Stem Cells International

Stem Cell and Tissue Engineering Applications in Orthopaedics and Musculoskeletal Medicine

Guest Editors: Wasim S. Khan, Umile Giuseppe Longo, Adetola Adesida, and Vincenzo Denaro

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Editorial

Stem Cell and Tissue Engineering Applications in Orthopaedics and Musculoskeletal Medicine

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Over the past two decades, there has been a tremendous interest in the application of tissue engineering protocols and stem cells, particularly, mesenchymal stem cells (MSCs) to develop cell-based strategies for regeneration, replacement, or repair of damaged tissues. The 1990s brought the advent of tissue engineering, where MSCs were differentiated in the presence of specific bioactive molecules towards bone, cartilage and other musculoskeletal tissues. The beginning of the 21st century saw the emergence of cell-based clinical therapies using MSCs at three different approaches. Firstly, tissue-engineering approaches in which MSCs are seeded into three-dimensional (3-D) scaffolds in order to generate functional tissues for replacement of defective tissues. Secondly, we see the use of MSC transplantation to replace defective host cells and thirdly, harnessing the properties of MSCs to act as cytokine/growth factor producers to stimulate repair or inhibit degenerative processes.

Reflecting the enormous interest of both our readership and authors in this promising field, we are pleased to present this special issue. This special issue presents a total of 21 paper including research papers and comprehensive reviews that aim to provide a better understanding of current basic research findings, clinical data, and the challenges encountered on the application of biologicals (cytokines/growth factors), tissue engineering, and stem cells in the treatment of musculoskeletal lesions affecting bone, cartilage, intervertebral discs, ligaments, menisci, muscle, and tendons.

The treatment of large bone defects remains a major challenge for orthopaedic reconstructive surgeons. Generation of bone grafts with osteoconductive, osteoinductive, and osteogenic properties via tissue engineering strategies may resolve this problem. W. Khan et al. review available literature on bone tissue engineering to understand its principle, current limitations, and clinical relevance in bone reconstructive surgery.

Hyaline cartilage is an avascular tissue covering the end of long bones in diarthrodial joints where it fosters near frictionless movement and load bearing. Current treatment options for damaged hyaline cartilage lead to the formation of fibrocartilage tissue that neither recapitulates the molecular phenotype nor the biomechanical properties of hyaline cartilage. While MSCs have the capacity to be differentiated into cartilage cells: chondrocytes, current methods of chondrogenic induction of MSCs result in fibrocartilage formation and expression of templates for osteogenesis. In this special issue, U. Longo et al. provide a comprehensive review of available literature on the role of gene therapy and MSCs to develop therapeutic strategies for hyaline cartilage regeneration.

Low back pain is one of the largest health problems in the Western world today affecting 8 in 10 of the adult population at some point in their lives and has an enormous impact on Western economies due to occupational incapacities and healthcare cost. While the causes of low-back pain are thought to be multifactorial, the degeneration of intervertebral discs (IVD) is frequently seen in almost all cases. U. Longo et al. provide two review papers on the prevention and management of IVD degeneration. One paper focused on the role of biologics: growth factors and

anticatabolic agents, to stimulate synthesis of extracellular matrix molecules that are central to IVD functionality and inhibition of the enzymes responsible for IVD degradation. The other paper provided a review of *in vitro* and *in vivo* evidence on the potential application of MSCs to treat IVD degeneration.

Ligaments possess the remarkable capacity to connect two or more bones, stabilize joints, and to sustain excessive mechanical loads. However, they have a limited reparative and regenerative capacity. Current treatment modalities, that comprise the use of autografts and allografts, suffer from a variety of issues that include donor site morbidity, pain, altered harvest biomechanics, and disease transmission. Hence there is a need for new treatments. Tissue engineering strategies have been advocated as a potential approach to resolve these problems. Calgor Yilgor et al. provide a review paper summarizing the application of biologics, cells (MSCs and fibroblast) scaffolds and bioreactors in ligament regeneration. While in a paper, W. Yates et al. focused their review of current literature on the relevance of ligament tissue engineering on anterior cruciate ligament reconstruction. S. Rathbone et al. report that most British orthopaedic surgeons will use tissue-engineered anterior cruciate ligament in clinical practice.

The meniscus is a vital tissue for normal healthy knee joint biomechanics. Meniscus injury is a major risk factor for the development of osteoarthritis. Unfortunately, the reparative capacity of the meniscus is limited to its vascularized region. In a research article, D. Ferris et al. used equine autologous bone marrow stromal mesenchymal stem cell and fibrin glue to demonstrate healing of lacerated equine menisci in a nude mice model. Their study may serve as a promising model system to study cell-based therapies for meniscus repair. In their paper, U. Longo et al. produced a review of available literature on current strategies of tissue engineering for the management of meniscal defects. In another paper, U. Longo et al. focused their review on proposed biological strategies directed to enhance meniscus repair in the avascular region.

Despite the fact that skeletal muscle has the capacity to repair and regenerate, muscle function is compromised due fibrotic scar tissue formation after injury. U. Longo et al. review current literature on the application of biologicals, such as platelet rich plasma and tissue engineering strategies comprising scaffolds and MSCs in the management of skeletal muscle injuries. While S. MacLean et al. discuss current literature on different stem cell types, gene therapy, and tissue engineering approaches that may be applicable to skeletal muscle regeneration following injury and muscular dystrophy.

Tendons are central to normal functionality of the musculoskeletal system where they connect muscle and bone to produce joint movement. Unfortunately, the reparative and regenerative capacities of tendons are limited because of their low cellularity. Tendon injuries are a common cause of morbidity in the population and account for a significant health care burden. Preclinical studies with a variety of cell types including MSCs, tendocytes, dermal fibroblast, have been evaluated for tendon repair or regeneration. While these studies show promise of cell-based options in treating tendon disorder, there have been little clinical applications. U. Young reviews the application of stem cells in tendon disorders. L. Gulotta et al. review current literature on the use of stem cells with or without growth factors to enhance tendon repair. S. Maclean et al. review stem cell-based tissue engineering strategies in the management of tendon injuries. In a clinical study, C. Pascual-Garrido et al. report a 5-year follow up clinical improvement in a small cohort of patients with chronic patellar tendinopathy after treatment with untreated bone marrow stromal stem cells. N. Maffulli et al. discuss current literature on tissue engineering approaches to treat rotator cuff tears. While U. Longo et al. review current literature on available biological and synthetic scaffolds for tendon tissue engineering.

The study of stem cells isolated from a variety of tissues often offers unique insights into similarities or differences in differentiation potential between stem cell populations. Schmitt and coauthors discuss current literature on different stem cells use in orthopaedic regenerative medicine. While in a research paper, Marmotti and colleagues report a novel method of isolating stem cells from umbilical cord for orthopaedic tissue engineering. Furthermore, they showed evidence that the stem cells had the capacity to undergo myogenesis, adipogenesis, chondrogenesis, and osteogenesis. E. Fossett and W. Khan discuss in a review how MSCs seeding density, age, and gender of MSCs donor affect the proliferative capacity of MSCs.

In summary, we are very excited to present to our readers this special collection of outstanding review and research articles and would like to take this opportunity to thank all our authors for their contributions and support to *Stem Cells International*.

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Research Article

Minced Umbilical Cord Fragments as a Source of Cells for Orthopaedic Tissue Engineering: An In Vitro Study

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A promising approach for musculoskeletal repair and regeneration is mesenchymal-stem-cell- (MSC-)based tissue engineering. The aim of the study was to apply a simple protocol based on mincing the umbilical cord (UC), without removing any blood vessels or using any enzymatic digestion, to rapidly obtain an adequate number of multipotent UC-MSCs. We obtained, at passage 1 (P1), a mean value of 4, 2×10^6 cells (SD 0,4) from each UC. At immunophenotypic characterization, cells were positive for CD73, CD90, CD105, CD44, CD29, and HLA-I and negative for CD34 and HLA-class II, with a subpopulation negative for both HLA-I and HLA-II. Newborn origin and multilineage potential toward bone, fat, cartilage, and muscle was demonstrated. Telomere length was similar to that of bone-marrow (BM) MSCs from young donors. The results suggest that simply collecting UC-MSCs at P1 from minced umbilical cord fragments allows to achieve a valuable population of cells suitable for orthopaedic tissue engineering.

1. Introduction

The repair and regeneration of bone, articular cartilage, and muscle are a major challenge in biomedical research. One of the most promising approaches is represented by mesenchymal stem-cell-based tissue engineering. Mesenchymal stem cells (MSCs) have been under constant investigation since the 1990s [1] for their excellent proliferation potential and their capability for differentiation into multiple lineages. Moreover, their immunosuppressive properties make them a suitable candidate for allogenic cell therapy. Allogenic cell-based approaches imply MSCs to be isolated from a donor, expanded, and cryopreserved in allogenic MSC banks, providing a readily available source for cell replacement therapy.

Bone marrow (BM) represents the most commonly used source of adult MSCs. BM-MSCs have been functionally defined as plastic-adherent, nonhaematopoietic, multipotential cells that support haematopoietic stem cells expansion in vitro and that are able to differentiate into cells of various connective tissues. Various cell-surface markers have been associated with a mesenchymal phenotype, as CD105, CD73, CD90, and HLA-ABC proteins, while lack expression of CD45, CD34, CD14, or CD11b, CD79 alpha or CD19 and HLA-DR were also considered characteristic of this cell population [2]. Previous studies have extensively shown their ability to differentiate into bone [3, 4], muscle [5], adipose tissue [6], cartilage [7], and tendon [8]. Nevertheless, several limitations as the painful procedure for BM collection, the limited number of BM-MSCs available for autogenous use,

and the concomitant reduction in allogeneic BM donations have raised an increasing interest in identifying alternative sources of MSCs.

Human umbilical cord (UC) has been recently suggested as a valid alternative tissue for MSCs [9]. The UC is a tissue of extraembryonic origin lying between the mother and the fetus, consisting of two arteries, one vein, intervessels connective tissue (the Wharton's jelly), and umbilical epithelium. The UC is normally discarded after birth. Therefore, UC collection does not require any invasive procedure nor implies major ethical concerns. MSCs have been isolated from all compartments of the umbilical cord tissue, namely, the umbilical vein endothelium and subendothelium and the Wharton's jelly. Within Wharton's jelly, MSCs have been isolated from three regions: the perivascular zone (UC perivascular cells), the intervascular zone, and the subamnion. MSCs can be also isolated from umbilical cord blood, but the limited amount of blood that can be collected and the technical difficulties of this procedure make umbilical cord blood less suitable than UC connective and perivascular tissues. Both Wharton's jelly-derived cells and umbilical vein perivascular cells (endothelium- and subendothelium-derived MSCs) have shown multilineage capability along with immunoregulatory properties [10, 11]. It has been shown that a single injection of MHC-mismatched unactivated human UC-MSCs did not induce a detectable immune response [12]; therefore, they can be tolerated in allogeneic transplantation [13]. These cells share with BM-MSCs several surface markers as CD73, CD90, and CD105 and did not express CD34 [14]. Moreover, UC-MSCs show low expression of HLA class I and no expression of HLA class II unless stimulated with IFN-γ [15, 16].

The aim of this study was to apply a simple protocol based on mincing the whole UC, without removing any blood vessels or using any enzymatic digestion, in order to obtain an adequate number of multipotent UC-MSCs at P1. This method did not imply selecting a single cell population from the different UC regions (Wharton's jelly, endothelium, and subendothelium) but allowed for accessing to a mixed population of MSCs from all UC areas. Multilineage potential of these cells, immunophenotype, origin, and telomere length were verified at P1. This study intends to identify a cell population suitable for tissue engineering applications in orthopaedics and musculoskeletal medicine with a simple method with minor cell manipulation, in order to establish a good manufacturing practice protocol for the isolation and expansion of multipotent UC-MSCs.

2. Materials and Methods

Approvals were obtained both from the Ethical Committee of MBC (Molecular Biotechnology Center), University of Torino, and from the Ethical Committee of Mauriziano Hospital, Torino (Italy).

2.1. UC Collection and Processing. After obtaining patient's own informed consent, 4 fresh UC samples of women with healthy pregnancies were retrieved at the end of gestation during caesarean deliveries from the Department of Obstetrics and Gynecology of Mauriziano Hospital (Torino, Italy).

The UC samples (mean length $29,5 \pm 4,8$ cm, range 25–35 cm, weight $30,5 \pm 5,3$ g, range 25–36 g) were collected in a phosphate-buffered saline (PBS) transfer medium containing 200 mg/100 mL ciprofloxacin (Bayer, Milan, Italy), 500 IU heparin (Pharmatex, Milan, Italy), and were immediately processed. After transferring under a sterile laminar flow cell culture hood, the cord length and weight were estimated and the UC was washed twice in PBS to remove the traces of contaminant red blood cells. The UC was firstly cut into 3 cm long segments, which were subsequently cut longitudinally and split open to expose the inner surface. The UC segments were transferred to a 60 cm^2 Petri dish (Corning, New York, NY, USA) containing 10 mL MSC expansion medium, consisting of Dulbecco's Modified Eagle Medium/F-12 (D-MEM) (Invitrogen, Carlsbad, CA, USA) enriched with 5% human platelet lysate obtained from healthy donors, 10% Fetal Bovine Serum (FBS), 1X penicillin/streptomycin, 1X sodium pyruvate, 1X nonessential amino acids (Invitrogen, Carlsbad, CA, USA), 500 IU heparin (Pharmatex, Milan, Italy). The UC segments were then manually minced into very small cuboidal fragments (4–7 mm length) using no. 15 sterile scalpels. The small UC fragments were then transferred and evenly distributed into 6-7 different 60 cm^2 Petri dishes (approximately 40–45 fragments/Petri dish) and incubated in the MSC expansion medium at 37°C in a humidified atmosphere with 5% CO_2 (day 0) (Figures 1(a), 1(b), and 1(c)). Fragments of UC were left undisturbed in culture and monitored for up to 2 weeks to allow identification of MSCs in the dishes.

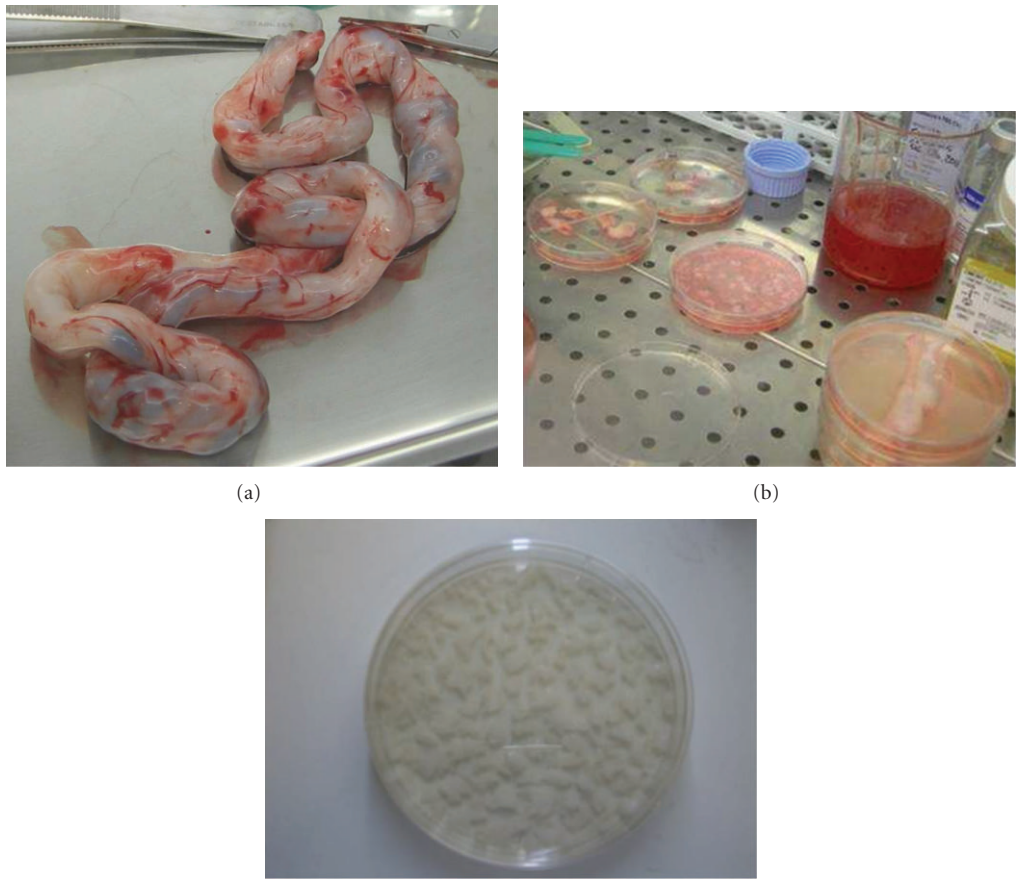
2.2. Culture of UC-MSCs. After 2 weeks from the initial seeding (day 14), the UC tissue was removed and adherent cells were allowed to expand for 2 additional weeks. Forty percent of the medium was changed every 3-4 days. After this time period (day 28), the adherent cells (P0) were trypsinized, centrifuged at 1200 rpm for 10 min, resuspended in MSC expansion medium, and replated for one consecutive expansion step at a density of 100–200 cells/cm^2, until full confluence was reached (P1). Cell confluence at P1 was reached after approximately 14 days (day 42).

At the end of P1 passage (day 42), the living cells were counted by trypan blue dye exclusion (Sigma-Aldrich, St. Louis, MO, USA).

UC-MSCs from three UC were used for immunophenotypic characterization, multilineage differentiation, and fluorescence in situ hybridization. UC-MSCs from one UC were used for telomere length analysis.

2.3. Immunophenotypic Characterization of UC-MSCs. Immunophenotyping of the expanded UC-MSCs was done using flow cytometry at the P1 passage of culture. $1,5 \times 10^6$ UC-MSCs were used for flow cytometry.

The following antibodies were used: CD90-Peridinin Chlorophyll Protein (PerCP)-cyanine dye Cy5.5, CD105-fluorescein isothiocyanate (FITC) (Biolegend, San Diego,



(a) (b)

(c)

Figure 1: Umbilical cord processing method (a, b, c).

CA), CD73-Allophycocyanin (APC), CD34-phycoerythrin (PE), HLA-DR-FITC, HLA-PerCP, HLA-ABC-PE, CD29-APC (BD Biosciences, San Jose, CA), CD44-Alexa Fluor (Cell Signaling Technology, Danvers, MA), PE-conjugated anti-mouse immunoglobulin G (IgG) (Southern Biotechnology Associates, Birmingham, Alabama, USA), isotypematched IgG-FITC, IgG-PE and IgG-PE-Cy5 control antibodies (Biolegend, San Diego, CA).

Analysis was performed on a FACScan (Becton Dickinson (BD), Buccinasco, Italy) for at least 10.000 events and using CellQuest software (BD).

2.4. UC-MSCs Differentiation. UC-MSCs at P1 from three UC samples were assessed for multilineage differentiation.

The adipogenic, osteogenic, chondrogenic, and myogenic differentiation ability of UC-MSCs was determined as briefly described, following previously published methods [1, 14].

For adipogenic differentiation, 5×10^5 UC-MSCs were cultured with EUROMED Adipogenic Differentiation Kit (EuroClone, Pavia, Italy) for 3 weeks. To evaluate the differentiation, cells were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and stained with 0.5% Oil Red O (Sigma-Aldrich, Milan, Italy) in methanol (Sigma-Aldrich, Milan, Italy) for 20 minutes at room temperature.

Osteogenic differentiation was assessed by culturing 5×10^5 UC-MSCs in EUROMED Osteogenic Differentiation Kit (EuroClone, Pavia, Italy). Medium was changed twice a week for 3 weeks. To evaluate the differentiation, cells were fixed with 4% paraformaldehyde for 20 minutes and stained with Alizarin Red, pH 4.1 (Lonza, Bergamo, Italy) for 20 minutes at room temperature. Cells were also studied with Alkaline Phosphatase stain (Alkaline Phosphatase Kit-based on naphthol AS-BI and fast red violet LB, Sigma-Aldrich). For immunofluorescence analysis, we also cultured cells directly on coverslips in the same conditions to identify the presence of osteocalcin (Abcam, Cambridge, UK) [17].

A pellet culture system was used for chondrogenesis (Figures 5(a) and 5(b)). Approximately 1×10^6 UC-MSCs were centrifuged in 15-mL conical polypropylene tube (Falcon BD Bioscience, Milan, Italy) at 150 g for 5 minutes and washed twice with DMEM. The pellets were cultured in EUROMED Chondrogenic Differentiation Kit (EuroClone, Pavia, Italy) supplemented with 10 ng/mL of Transforming Growth Factor $\beta 3$ (SeroLab, Lausanne, Switzerland). Medium was changed every 3 days for 28 days. Pellets were fixed in ethanol 80% overnight, and the paraffin-embedded sections were stained for glycosaminoglycans using Safranin O and for sulfated proteoglycans with Alcian blue (Sigma-Aldrich, Milan, Italy) an with Toluidine Blue [18].

Myogenic differentiation was performed using 5×10^5 UC-MSCs, plated in six-well culture plates (BD Falcon, Milan, Italy) on coverslips in DMEM with 10% knockout serum, 1% penicillin, 1% streptomycin, 0.1 mM β-mercaptoethanol, and 40 ng/mL fibroblast growth factor (FGF) [14]. The medium was changed every 3 days. After 21 days in culture, cells were immunostained with a monoclonal antibody against human myogenin (Abcam, Cambridge, UK).

The same osteogenic, chondrogenic, and adipogenic differentiation protocols were used on a population of BM-MSCs as a control.

2.5. Immunofluorescence Analysis. Immunofluorescence analysis of cells is briefly described as it follows.

The UC-MSCs grown on glass coverslips were rinsed briefly in phosphate-buffered saline (PBS 1X) and fixated with 4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature. Samples were washed three times with PBS 1X. To obtain permeabilization, samples were incubated for 1 min with PBS 1X containing 0.5% Triton X-100 and washed three times with PBS 1X. For blocking, cells were incubated with 1% BSA in PBS 1X for 1 hour to block unspecific binding of the antibodies; then samples were incubated with the primary diluted antibody (antiosteocalcin or antimyogenin, depending on the cell line) in 0.1% BSA in PBS 1X in a humidified chamber for 1 hr at room temperature (dilution 1 : 50). Then, the cells were washed three times in PBS 1X and subsequently incubated with the secondary antibody (anti-mouse conjugated with Alexa Fluor 488, Invitrogen, Milan, Italy) in 0.1% BSA for 1 hr at room temperature in dark (dilution 1 : 1000). The solution was washed three times with PBS, and, for counterstaining, cells were incubated with DAPI (4′,6-diamidino-2-phenylindole, DNA stain) for 7 min. Then, cells were rinsed with PBS 1X three times for 15 min; finally, the coverslip was mounted with a drop (5 μL) of mounting medium for observation.

2.6. Fluorescence In Situ Hybridization. The origin of UC-MSCs was performed using a fluorescence in situ hybridization (FISH) for 2 UC samples. This was carried out in the two cases of male newborns among the four UC specimens collected. 1×10^5 cells were used.

The probes used were X centromere Xp11.1-q11.1 (DXZ1) (green) and Y heterochromatin Yq12 (DYZ1) (red). The enumeration probe set contained chromosome specific DNA repeat sequences located at the centromere of chromosome X and in the heterochromatic block of chromosome Y (Cytocell Aquarius, Cambridge, UK).

Cells derived from P1 were fixed in Carnoy's fixative, according to the institutional protocol guidelines. FISH protocol was performed according to the manufacturer's instructions (Cytocell Aquarius, Cambridge, UK). Results were analyzed using fluorescence microscope (Olympus-BX41, magnification 100x, triple filter RED-GREEN-DAPI).

2.7. Telomere Length Analysis. Telomere length was evaluated on UC-MSCs at P1 from one UC, and results were compared to telomere length of bone marrow MSCs taken from bone marrow aspirate of 6 healthy adult volunteers (age 20–30).

Approximately, 4×10^6 cells were used for telomere length analysis. This analysis was performed on one UC only. Telomere length was determined using a Southern Blot analysis as previously described [19]. 2 μg of DNA were digested by mixing Hinf I (20 U) and Rsa I (20 U; Roche Diagnostic, Mannheim, Germany) and incubated at 37°C for 2 hours. Digested DNA fragments were separated by 0.8% agarose gel electrophoresis in 1X Tris-acetate-EDTA running buffer. Positively charged nylon membrane was used to transfer DNA separated by electrophoresis. After overnight transfer, the nylon membrane was exposed to ultraviolet light to fix DNA fragments. TeloTAGGG Telomere Length Assay Kit (Roche Diagnostic) was used for the hybridization phase. The membrane was submerged in a prehybridization solution for 2 hours at 62°C under gentle stirring and then incubated in the hybridization solution (2 μL of the digoxigenin (DIG-) labeled telomere-specific probe added to the prehybridization solution) for 3 hours at the same temperature. After hybridization, the membrane was washed twice at room temperature in stringent wash buffer I (2X SSC, 0,1% SDS) for 10 minutes and then twice at 37°C in stringent wash buffer II (0.2X SSC, 0,1% SDS) for 20 minutes; it was incubated with blocking solution 1X for 30 minutes at room temperature and then with a DIG-specific antibody covalently coupled to alkaline phosphatase (AP). Finally, CDP Star, a chemiluminescent substrate, was dropped onto the membrane to stimulate AP to produce light emission. This emission was detected by X-ray film (Lumi-Film Chemiluminescent Detection Film, Roche Diagnostic) and scanned for analysis. Analysis was performed using Quantity One (BioRad). For each telomere smear, peak telomere restriction fragments length and the point of maximum signal intensity defining the highest concentration of telomere repeats were calculated as the median value of telomere length of the cell population examined.

2.8. Statistical Methods. The Student's t-test was used to compare telomere lengths evaluated in UC-MSCS and in healthy people bone marrow. Differences were considered significant for $P < 0,05$. Statistical analysis was carried out with the statistical software package GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Morphologic and Immunophenotypic Characterization of UC-MSCs. In primary cultures, typical spindle-shaped adherent cells were observed migrating from the UC tissue fragments and initiating the colony formation approximately at day 14 after UC fragments seeding. After removing the UC fragments at day 14, cells at P0 took approximately 10-days period to gain 60% confluence (Figure 2), while full confluence was observed after 14 days. The UC-MSC clones (P0) were then collected at day 28 and replated for further expansion (P1). Confluence at P1 was observed after 14 days of culture (day 42).

At day 42, we obtained at P1 a mean value of $4,2 \times 10^6$ cells $\pm$ 0,4 from each UC. From the initial UC fragments

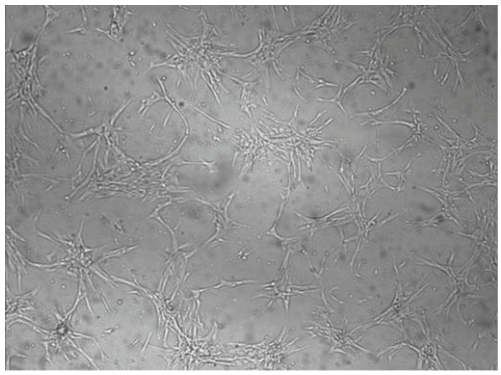


Figure 2: MSCs from the umbilical cord tissue during cell culture. Cell cultures (P0) 10 days after UC fragments removal (24 days of culture), magnification 10x. Cells show a fibroblast-like morphology.

seeding (day 0), we obtained at the end of P1 (day 42) $0{,}14 \times 10^6$ cells/g of UC seeded.

The phenotype of UC cells was analyzed by flow cytometry. Data from one representative experiment are reported in Figure 3. The majority of collected UC cells showed a positive expression of the main MSC markers CD73, CD90, and CD105, as well as of CD44 and CD29. Furthermore, they were negative for the typical haematopoietic marker CD34.

The data also demonstrated the presence of HLA-ABC proteins and the absence of HLA-DR. Additionally, we have visualized a notable presence (40%) of negative double cells for both HLA-ABC and HLA-DR proteins.

3.2. Differentiation of UC-MSCs. In the osteogenic-stimulated cultures, significant calcium deposition was observed with Alizarin Red staining inside the cluster of cells after 21 days, consistent with osteogenic commitment of UC-MSCs. UC-MSCs showed a pattern similar to bone-marrow MSCs after 21 days of cultures with the same medium, as it is shown in Figures 4(a) and 4(b).

Cells were also positive for alkaline phosphatase stain (Figure 4(c)).

Osteocalcin was found in cytoplasm at immunofluorescence in UC-MSCs after commitment toward osteogenic pathway (Figures 4(d) and 4(e)).

Chondrogenic commitment with the pellet culture system was observed at 28 days. At the histological evaluation, pellets of UC-MSCs from all three UCs exhibited positive staining for Alcian Blue and Safranin O (Figure 5) and Toluidine blue methods (data not shown). UC-MSCs showed roundish shape and a pattern similar to bone-marrow MSCs cultured in the same conditions, as it is shown in the Figure 5.

Myogenic commitment was observed in cell cultures (Figure 6) after 21 days. At immunofluorescence, cells from all three UCs were positive for antimyogenin antibody. Positivity was observed predominantly in the nuclei and lesser in the cytoplasm, according to the literature [20].

In the adipogenic-stimulated cultures (Figure 7), UC-MSCs showed lipid deposition and changes in the cellular morphology after 21 days. In all cultures from three UCs, intracellular lipid granules staining positive with Oil Red O were detected after 3 weeks. At variance with bone marrow derived adipocytes, that show larger vacuoles, in our culture UC-MSCs showed smaller lipid vacuoles, possibly related to brown fat commitment.

3.3. Fluorescence In Situ Hybridization of UC-MSCs. Cytogenetic analysis of UC-MSCs was performed in two cases of male newborns. The method showed that UC-MSCs were mainly XY (95% and 100%, resp.). This is consistent with a prevalent newborn origin of these cell populations.

3.4. Telomere Length Analysis. Telomere length of UC-MSCs from one UC was determined and compared to telomeres of BM-MSCs at P1 taken from 6 adult volunteers (age 20–30) (Figure 8).

No significant difference was observed between the two cell population. Median value of UC-MSC telomere was 9023 base pairs (9,023 kbp), while median value from all 6 donors was 9340 base pairs (range 7,872–9,867 kbp).

4. Discussion

In this study, we apply an easy, reliable, and repeatable method to isolate a mixed population of UC-MSCs from umbilical cord fragments. This protocol was based on simply mincing UCs directly in the MSC expansion medium with minimal mechanical manipulation. We did not remove any blood vessels, and we did not use any enzymatic digestion or any additional purification steps in order to avoid the possible selection of cellular subpopulations. With this feasible method, we collected an adequate number of UC-MSCs already at P1. At immunophenotypic characterization, cells at P1 were positive for the major MSC markers (CD73, CD90, CD105, CD44, and CD29) and negative for the typical haematopoietic marker CD34. Furthermore, we did not find HLA class II in all cells, and we have also observed the presence of a peculiar subpopulation of double negative (HLA-I and HLA-II) UC-MSCs. UC-MSCs obtained with this protocol seem to have a newborn origin and are capable to be committed towards multiple lineage as bone, fat, cartilage, and muscle. Telomere length was similar to that of BM-MSCs taken from young donors. Taken together, all these observations suggest that collecting UC-MSCs at P1 from minced umbilical cord fragments allows to achieve a valuable population of UC-cells that could be used for orthopaedic tissue engineering applications.

In orthopaedics, cell therapy is widely used to enhance tissue repair in different pathologic conditions involving long bone defects or osteochondral lesions. Different cell sources are proposed by tissue engineering, as autologous bone marrow aspirate or allogeneic cells (i.e., allogeneic BM-MSCs or allogeneic chondrocytes). These cells are usually loaded onto suitable scaffolds and directly transferred to the lesion site. This "one-stage" approach eliminates patient's own cell "in vitro" expansion and is considered less expensive than traditional autologous cell culture and implantation, especially in the field of cartilage repair [21].

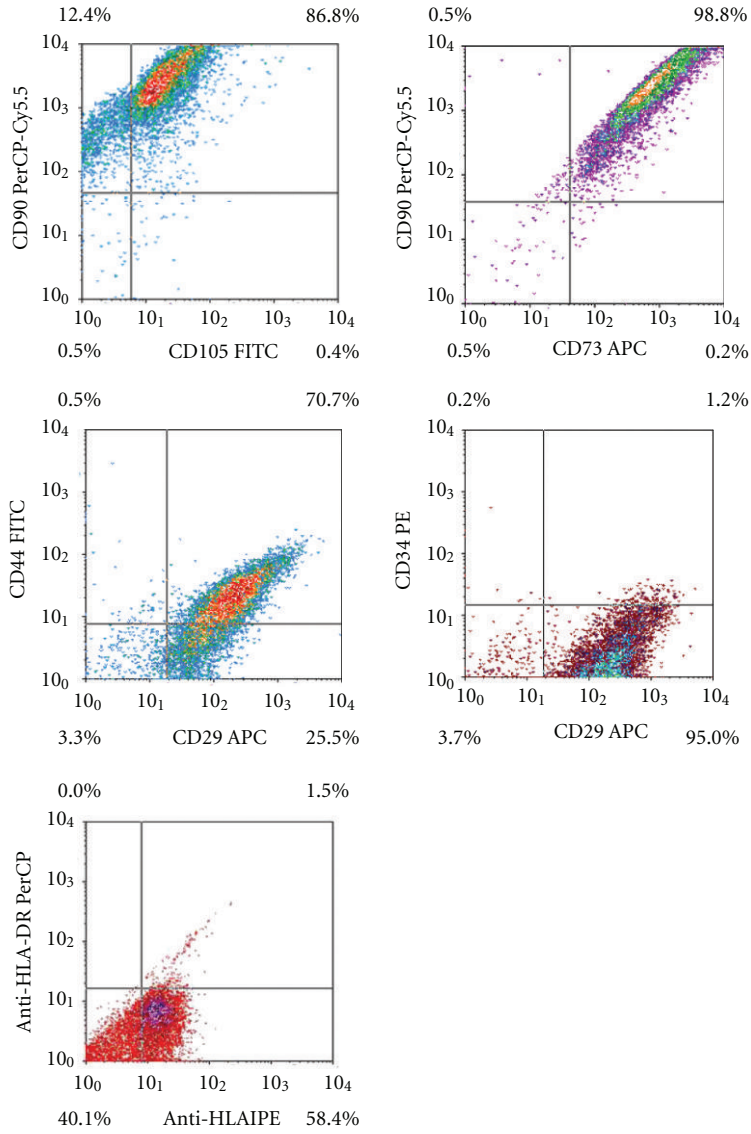


Figure 3: Flow cytometry analysis of surface-marker expression on umbilical cord mesenchymal cells, after one stage in culture. Flow cytometry reveals positivity for CD73, CD90, CD105, CD44, CD29 and a notable presence of negative double cells for both HLA-ABC and HLA-DR proteins.

Autologous bone marrow cells and bone marrow concentrate are regarded as the "gold standard" for bone and cartilage repair [22, 23]. Nevertheless, donor site morbidity is a drawback of this cell source. Additionally, proliferative and differentiation capacity of BM-MSCs are shown to decline with increasing patient's age [24]. Moreover, in bone marrow concentrate, a small number of MSCs are available, thus reducing the efficacy of the cell delivery.

Allogeneic bone marrow could be considered as a solution for these limitations [25], but reduced availability of this resource and decline in BM donations [26] make this solution impractical for large-scale clinical use in orthopaedics.

UC has been recently introduced as a potential alternative to BM in musculoskeletal tissue engineering. Many hypothetical advantages make UC an interesting source of cells. UC is readily available in great quantity, as it is usually discarded during both normal vaginal delivery or cesarean sections. Collecting this source of cells implies no invasive procedure and low costs. Being UC an extraembryonic tissue usually abandoned at the end of the delivery, few ethical problems and legal concerns are involved in this procedure, provided that a complete and informed written consent is obtained from the mothers. Recent studies have shown that MSCs can be obtained from all different compartments of UC, as the Wharton's jelly [10], the perivascular regions [27], and the subendothelium and endothelium areas of UC vessel [11]. All these works have also shown the multilineage potential of these cell populations. Greater expansion capability and long telomere sequences have been observed in UC-MSCs, suggesting a late onset of senescence of this cell population compared to BM-MSCs

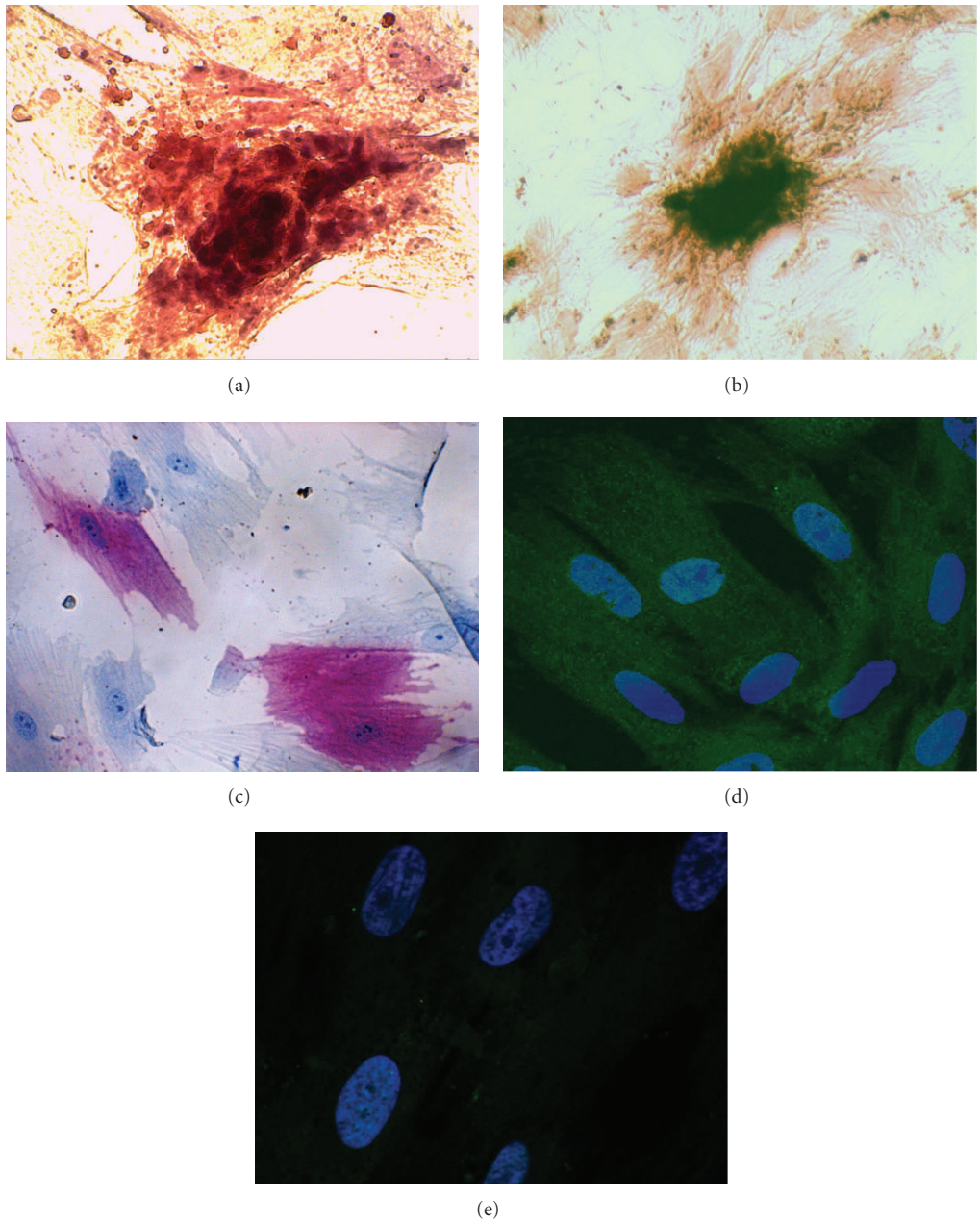
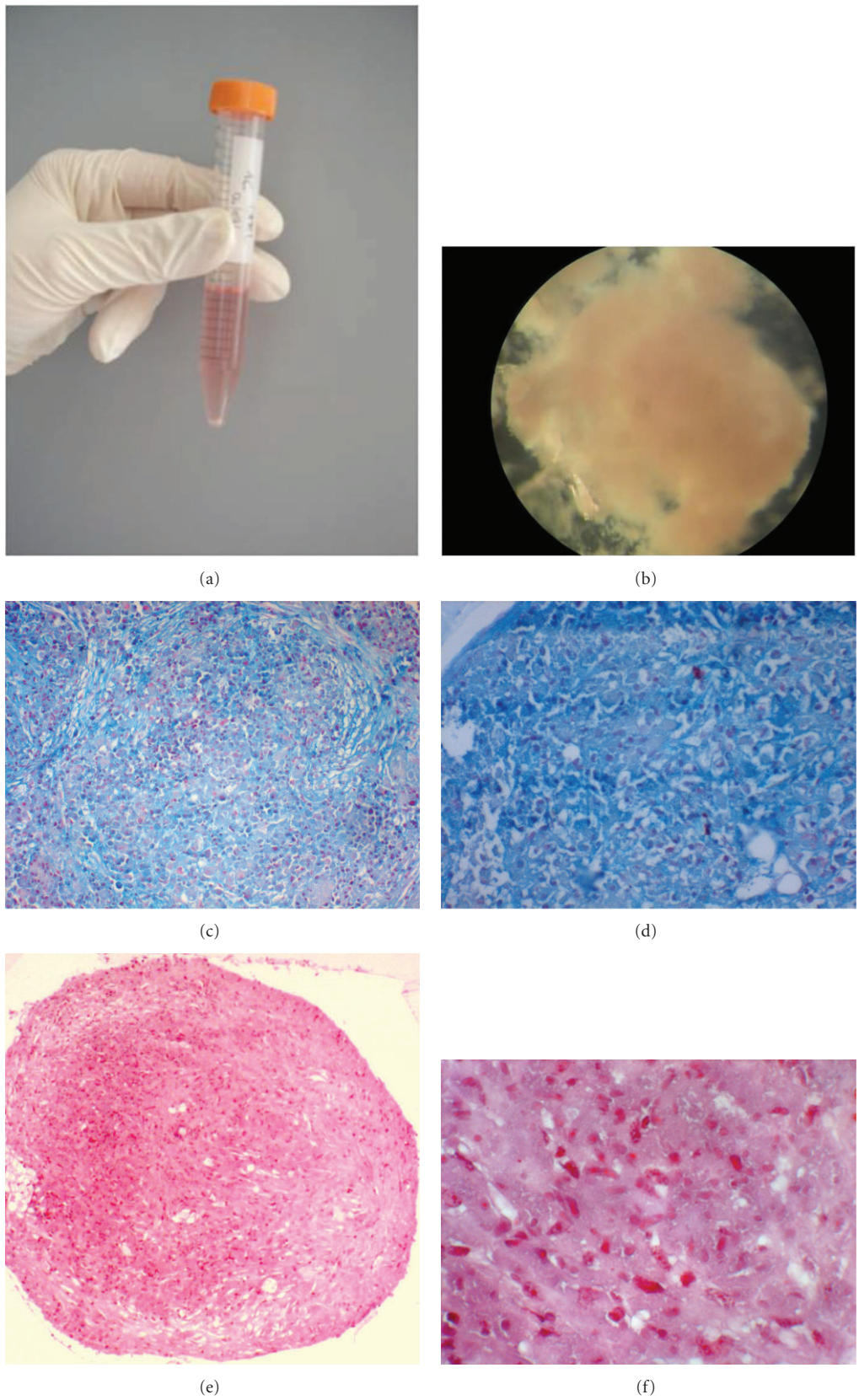


Figure 4: Osteogenic differentiation. Alizarin red staining revealing formation of calcium deposition in UC-MSCs (a) and in human BM-MSCs (b); (c) alcaline phosphatase cytoenzymatic staining of UC-MSCs; immunofluorescence for osteocalcin in UC-MSCs cultured in osteogenic medium; (d) osteocalcin stained with primary antibody against and with a secondary antibody anti-mouse (conjugated with Alexa Fluor 488) and merged with DAPI (blue, nuclei), (e) negative control with DAPI and secondary antibody only.

during in vitro expansion [28]. Immunosuppressive capacity of UC-MSCs and the absence of tumorigenic potential and cytogenetic abnormalities of this cells, when expanded in culture or implanted in vivo, have been extensively described in previous studies [26, 29]. Finally, the theoretical possibility to combine UC-MSCs from multiple donors increases the availability of this source of cells and might be beneficial when a great number of cells are required for a single procedure [30, 31]. All these observations confirm the attractive therapeutic potential of UC-MSCs.

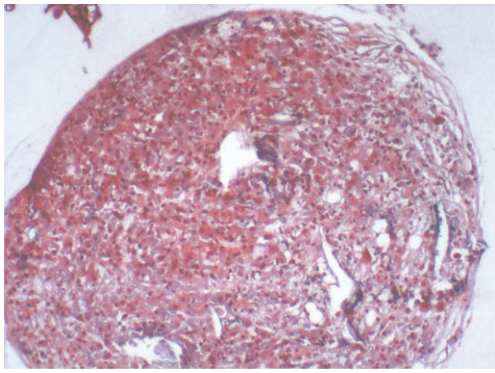
In this study, we have applied an easy and rapid method to collect an adequate number of UC-MSCs, by simply mincing the UCs and cultivating the small UC fragments and the migrating MSCs for a total of 6 weeks in a standard culture medium enriched with human platelet lysate and fetal bovine serum.

Previously reported methods have described the isolation of MSCs from UC through multiple steps in order to select a specific cell source. In 2003, Mitchell et al. introduced a technique to collect multipotent stem cells from Wharton



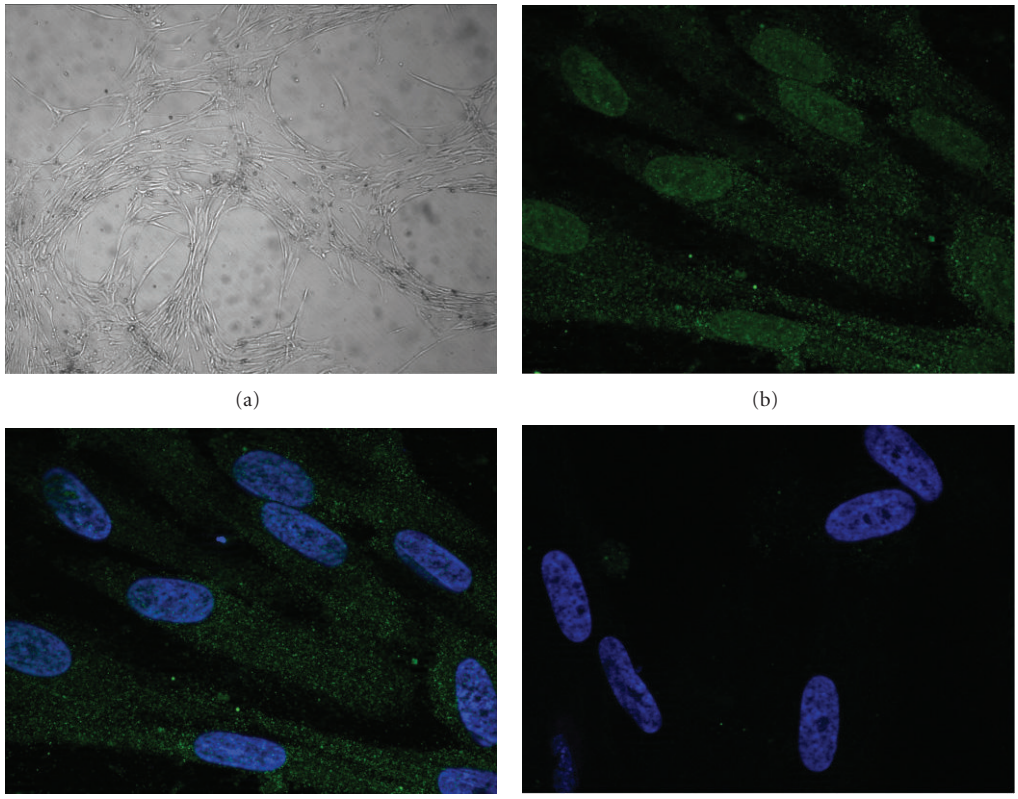
(a) (b) (c) (d) (e) (f)

Figure 5: Continued.



(g)

Figure 5: Chondrogenic differentiation. Cells growing in pellet culture system in chondrogenic medium (a, b). Histological section after chondrogenic commitment; UC-MSCs (c) and BM-MSCs (d) stained with Alcian Blue; UC-MSCs (e, f) and BM-MSCs (g) stained with Safranin O (f): higher magnification).

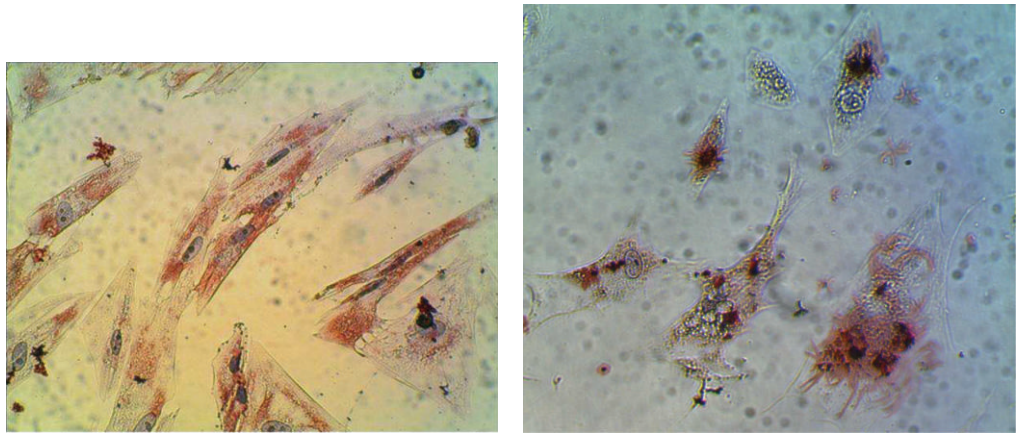


(a) (b)

(c) (d)

Figure 6: Myogenic differentiation. Phase contrast microscopy of cultures grown in myogenic medium for 21 days (a) at low magnification, immunofluorescence for myogenin in cells cultured in myogenic medium for 21 days; (b) myogenin stained with primary antibody against and with a secondary antibody anti-mouse (conjugated with Alexa Fluor 488); (c) merged with DAPI (blue, nuclei); (d) negative control with DAPI and secondary antibody only.

jelly by removing all vascular network [32]. In 2004, Wang et al. proposed to scrape off the Wharton jelly from the whole UC and to perform an enzymatic digestion with collagenase and trypsin [33]. Recently, Montanucci et al. [34] demonstrated a procedure involving multiple enzymatic digestion with hyaluronidase and human recombinant Liberase of UC samples and centrifugation of the digestion product to cleave the cells out of the original Wharton jelly matrix. Complex methods are also required to isolate human umbilical cord perivascular cells, implying tissue dissection in order to isolate UC vessel, collagenase digestion, centrifugation, and magnetic bead depletion protocol [27].



(a) (b)

Figure 7: Adipogenic differentiation. Oil Red O staining of adipogenic differentiation of UC-MSCs (a) with formation of smaller lipid vacuoles and of BM-MSCs (b) with formation of large vacuoles.

In all these studies, selected cell populations were obtained with detailed procedures that, albeit effective, implied non-negligible tissue manipulation. All these procedures may be considered too complicated for a clinically oriented large-scale cell therapy.

These observations encouraged us to apply a simple, practical, and economic method to rapidly obtain a mixed MSC population from minced umbilical cord, similarly to recent studies [26, 30].

In order to reduce the risk of external contamination, only UCs from cesarean sections were utilized. In our opinion, UCs from cesarean sections are more suitable for tissue engineering than UCs from vaginal delivery, due to the possibility to collect these samples in the clean environment of the operating room.

The method described in this study has some practical advantages. It allows to cut off time-consuming steps involving the use of enzymatic solution and the need for long incubating period. It involves a minimal tissue manipulation consisting in mechanically mincing the tissue. This principle does not impair the vitality of the tissue, as shown in previous work involving cartilage biopsies [35] and UC samples [30]. Moreover, a mixed MSC population is obtained with our method, in order to preserve the "mesenchymal properties" of all UC compartments.

With our protocol, an adequate number of cells was obtained to perform all studies from each UC without multiple expansion passages. This study was not designed to primarily obtain large number of cells, harvesting the maximum number of UC-MSCs from each cord as a clinical use should require. This method was aimed to extract a consistent number of cells with minimal manipulation for a preliminary in vitro pilot study. Indeed, the natural tendency of MSCs to attach to plastic dishes was the main element of our separation technique. For this reason, a great amount of umbilical cord was discarded after 15 days of incubation and not used to obtain more MSCs. The final number of cells, albeit not exceedingly high, was nevertheless sufficient for the whole design of the experiment including the cell characterization and the commitment toward osteoblastic or chondroblastic or myoblastic or adipoblastic line. In light of an in vivo application, these methods could be anyway suitable, because the umbilical cord is a virtually unlimited source of cells normally discarded after birth and the extraction efficiency can be therefore a secondary problem when the primary source of cells is widely available at no costs. We are nevertheless aware that processing the whole umbilical cord with different harvesting procedures and different method of mincing and expanding the cell population would theoretically lead to a greater number of cells available from each UC.

The MSCs collected showed a fibroblast-like morphology, when adherent to the plastic dishes. At the immunophenotypic characterization, cells exhibited a phenotype similar to that of BM-MSCs, being positive for the main MSC markers (CD73, CD90, CD105), for CD44, CD29, and HLA class I and negative for the haematopoietic marker CD34 and for HLA class II, in agreement with other reports [36]. Interestingly, a peculiar population of UC-MSCs negative both for HLA class I and HLA class II was found. This "double negative" cell population seems particularly suitable for an allogeneic use. Further studies will aim to properly separate these specific cells in order to apply them in future in vivo experiments. The mixed UC-MSC population obtained with this method was shown to have a newborn origin. This can partially explain the great plasticity of these cells.

Indeed, we obtained osteogenic, adipogenic, chondrogenic, and myogenic early commitment after culture with differentiation media. We are aware that we did not reached a morphology similar to differentiated tissues, but rather we observed a commitment toward a specific cell type or, in other words, a progression toward an osteoblastic or chondroblastic or myoblastic or adipoblastic line. Moreover, we still observed differences in the plasticity of these cells compared to BM-MSCs, that show a more advanced differentiation stage in similar culture conditions (see comparative Figures 4, 5, 6, and 7).

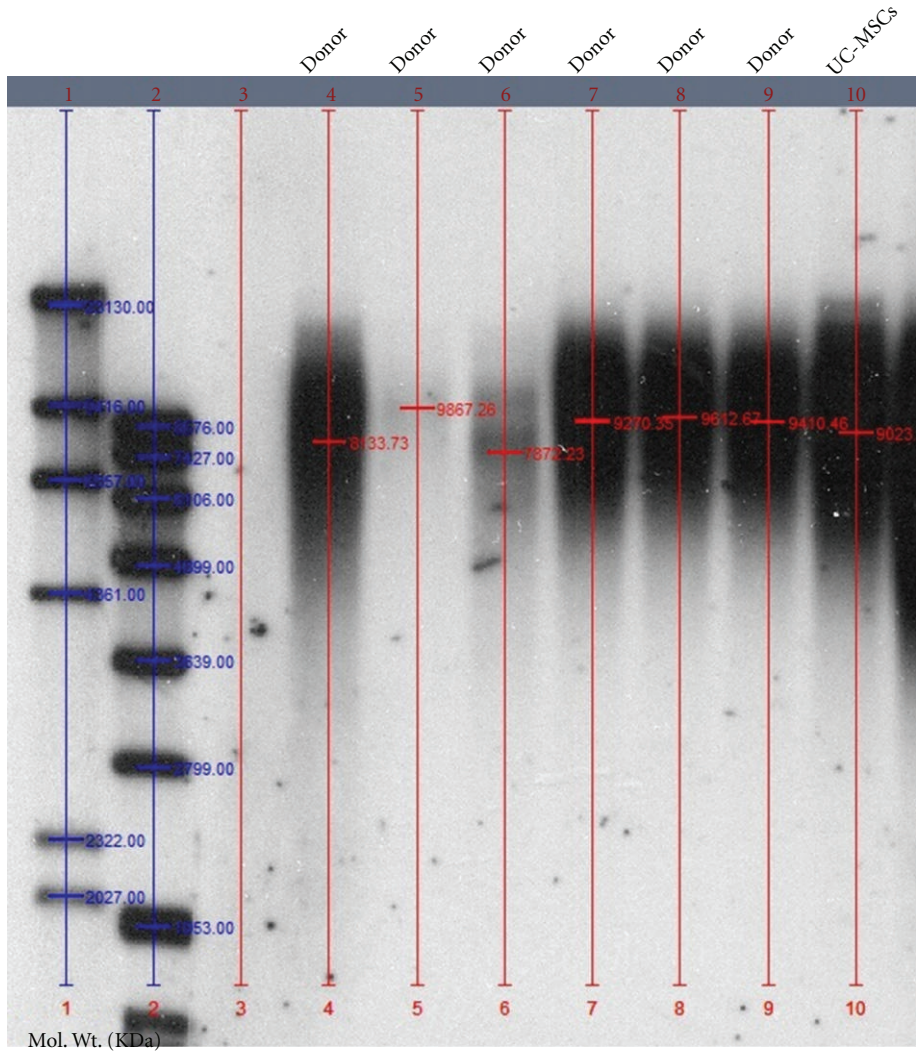


Figure 8: Telomere length of UC-MSCs compared to telomere length of BM-MSCs from 6 different donors (age 20–30) as assayed by Southern Blot analysis of DNA restriction fragments.

Published data on UC-MSC differentiation potential are still controversial; our results are in contrast with some previously reported observations showing low capacity of UC-MSC to differentiate towards bone, adipocytes, and chondrocytes [15, 26, 37]. We believe that the osteogenic, adipogenic, and chondrogenic commitment obtained in this study may be related to the specific composition of the medium used in this protocol and possibly could be further improved. These early commitment stages could be further enhanced by the influence of the in vivo microenvironment to complete the differentiation process.

For all these reasons, these cells could be considered a putative candidate for cell therapy in orthopaedic tissue engineering.

We have also assessed the length of the telomere in UC-MSCs collected with this method, as indicator of cell replication history and senescence, and we have compared the result with the telomere length of BM-MSCs obtained from healthy young donors. We observed analogous results in the two different cell populations. This is in agreement with the literature [28] and shows that the mixed UC-MSCs population at P1 obtained with this method share an equivalent proliferative potential with MSCs from BM aspirate of young donors. Nonetheless, it also suggests that the procedure of cell isolation described in our study does not induce substantial cell senescence in the UC-MSCs.

5. Conclusion

In conclusion, UC-MSCs can be obtained after a primary culture at P1 with this simple and rapid method. This mixed cell population of predominant newborn origin has shown signs of osteogenic, adipogenic, and chondrogenic commitment along with long telomere sequences suggestive for a high proliferative potential. Thus, UC-MSCs at P1 seem to have the potential to be good candidates for tissue engineering applications in orthopaedics. The concept of this study may indeed be considered as a future hypothetical

option for patients who might benefit from stem cells therapy. However, given these preliminary results, testing in vivo the regenerative potential of this cell population in an animal models, including large animals, will be the next logical step.

Conflict of Interests

The authors indicate no potential conflict of interests.

Acknowledgment

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Review Article

Mesenchymal Stem Cell for Prevention and Management of Intervertebral Disc Degeneration

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Intervertebral disc degeneration (IVD) is a frequent pathological condition. Conservative management often fails, and patients with IVD degeneration may require surgical intervention. Several treatment strategies have been proposed, although only surgical discectomy and arthrodesis have been proved to be predictably effective. The aim of biological strategies is to prevent and manage IVD degeneration, improve the function, the anabolic and reparative capabilities of the nucleus pulposus and annulus fibrosus cells, and inhibit matrix degradation. At present, clinical applications are still in their infancy. Further studies are required to clarify the role of mesenchymal stem cells and gene therapy for the prevention and treatment of IVD degeneration.

1. Introduction

The normal intervertebral disc (IVD) has a rich extracellular matrix (ECM), and three parts are anatomically distinguished: the central gelatinous nucleus pulposus (NP), which contains chondrocyte-like cells surrounded by a more fibrous annulus fibrosus (AF) containing fibroblast-like cells along with cartilaginous endplates (EP) connecting the adjacent vertebral bodies [1–6].

The IVD matrix consists of a well-organized framework of macromolecules which are able to attract and retain water [7]. The structural components of the IVD matrix are predominantly represented by collagen and proteoglycans [8]. Collagenous proteins are mostly present in the AF, and proteoglycans are the major components of the NP. In fact, collagenous proteins comprise 70% of the outer annulus dry weight, but only 20% are located in the central NP, while proteoglycans make up 50% of the NP [8].

Functionally, the collagen provides shape and tensile strength, while proteoglycans confer tissue viscoelasticity, stiffness, and resistance to compression, through their interaction with water.

A proper balance between matrix synthesis/apposition and degradation is responsible for the maintenance of the integrity of the IVD and hence the mechanical behaviour of the IVD itself. This is related to the activity of cytokines, growth factors, enzymes, and enzyme inhibitors that act in a paracrine or/and autocrine way [9–11].

With aging, morphological and molecular changes in the IVD can induce progressive degeneration and pathologic alteration of this particular tissue [12]. The morphological changes include dehydration and tears of the AF, NP, and EP [13]. The molecular changes consist of decreased cell viability and proteoglycans synthesis and reduced diffusion of nutrient and waste products. Accumulation of apoptosis debris, degraded matrix macromolecules, and an increased degrading enzymatic activity along with a modification of the collagen distribution also represent the degenerative changes that occur at a molecular level [8, 11, 14–16].

A variety of growth factors have anabolic function in IVD cell metabolism, resulting in accumulation and synthesis of matrix, while the opposite effect has been observed for cytokines, since they inhibit the synthesis of IVD matrix and promote the catabolic breakdown of its components [11, 17].

Although inflammatory mediators have been detected in IVD degeneration conditions, the actual pathologic role of these mediators is either unknown or not well defined. Nitric oxide (NO), interleukin-6 (IL-6), prostaglandin E2 (PGE2), TNF-alpha, fibronectin, and matrix metalloproteinases (MMPs) are few of the many identified mediators [12, 13, 18, 19].

IL-6, NO, and PGE2 seem to exert an inhibitory effect on proteoglycan synthesis. These factors are activated by interleukin-1 (IL-1), which also plays a role in the direct degradation of the proteoglycan matrix. This direct breakdown process by IL-1 occurs through a family of enzymatic mediators known as MMPs. In the cascade of inflammatory mediators, IL-1 likely plays a major role, although the nature of such role has not been defined yet.

To find new efficient treatment options, further investigations are required to identify the mediators that promote IVD degradation or matrix accumulation with the aim of targeting the homeostatic state of IVD-matrix metabolism by modulating the factors involved in synthesis and degradation towards the most favourable state to ensure their metabolic balance. Only recently, considerable attention has been placed on the use of stem cells as a clinically applicable therapeutic option for IVD degeneration.

The aim of this paper was to report the state of the art on the treatment of IVD degeneration (IVD-d), with mesenchymal stem cells (MSCs) based on the *in vitro* and *in vivo* experiences currently available.

2. IVD Degeneration

An active regulation and balance between anabolism and catabolism of the IVD cells is responsible for the maintenance of the physiological homeostasis in IVD tissue.

This mechanism is guaranteed through a complex and accurate coordination of the effect of a number of substances and molecules, including growth factors, enzymes, enzyme inhibitors, and cytokines, that act in a paracrine or/and autocrine way.

IVD degeneration commonly begins during the second decade of life, and it progresses towards severe conditions with ageing [17].

The lack of an adequate nutrient supply [8], along with the inappropriate mechanical load [20], may cause loss and alteration, as well as dysfunction, of cell viability and IVD properties.

IVD herniation, radiculopathy, myelopathy, spinal stenosis, instability, and low back pain represent the common medical conditions usually associated with symptomatic IVD-d, and they represent the most common among the conditions managed by spine surgeons, in addition to being the leading cause of disability in people younger than 45 years of age.

Various therapeutic strategies have been proposed for IVD-d in the last decade, with the most frequently used ones consisting of surgical interventions characterized by discectomy with vertebral fusion or, as an alternative, conservative management of IVD-d (e.g., lifestyle changes, physical therapy, pain medication, rehabilitation). Neither of such approaches deals with the loss of IVD tissue, and therefore new biological strategies appear challenging.

3. MSCs in IVD Degeneration (IVD-d)

Researchers and clinicians have focused their attention on tissue engineering and regenerative medicine to identify long-term tissue repairing options offering the possibility to restore IVD tissues [21].

Attention has been posed on stem cells as a potential source of cells to repopulate and regenerate the IVD. There is a large number of potential sources of MSCs, including adipose tissue, bone marrow, and other tissues [22, 23] as embryonic and fetal stem cells, which are pluripotent cells with a potential to differentiate into any body tissue. Regarding the embryonic stem cells, these cells are highly flexible since they are able to differentiate into any type of tissue, but both the procurement of human embryonic stem cells and research are limited, given the related ethical issues [24].

In terms of IVD regeneration, many studies have shown that IVD-d can be treated or repaired with the injection of MSCs, multipotent cells able to differentiate into tissues of mesenchymal origin including bone, cartilage, fat, muscle, and fibrous tissues depending on the biological environment. These cells can be obtained from multiple adult tissues, including bone marrow, trabecular bone, articular cartilage, muscle, and adipose tissue, which represent a variant of the adult stem cell.

Several authors have speculated that MSCs can also differentiate into the chondrocyte-like cells present in the NP, but, since the phenotype of NP cells has not been clearly characterized yet, confirmation of the differentiation from MSCs to NP cells has not been achieved [23, 25, 26], mainly because AF, NP, and EPs components have a different cartilage or bone composition and different kinds of matrix macromolecules and proteins.

While a variety of cell sources in the field of stem cell research have been proposed for clinical application [21], autologous adipose stem cells (ASCs) as a cell source in bone and cartilage repair have gained significant attention. In fact, adipose tissue is considered a suitable source of stem cells for clinical use, given the easiness of the procedure to retrieve adipose tissue, since this procedure is minimally invasive and can be performed in outpatient clinics and also due to the larger number of cells obtained [27]. Treatment or repair of IVD can occur with an injection of autologous adipose stem cells (ASCs), in presence or absence of growth factor prestimulation or matrix attachment [28].

4. Mesenchymal Stem Cells (MSCs)

4.1. In Vitro Evidences on MSCs. Based on the high incidence of symptomatic IVD-d caused by disc herniation, radiculopathy, myelopathy, spinal stenosis, instability, and low back pain, there is a great interest around the use of MSCs to repair degenerated IVD tissues. Since the IVD phenotype of NP cells has not been clearly defined yet, there is still a lack of knowledge about differentiation from MSCs to NP cells, complicated by the fact that AF, NP, and EPs components are differently composed of cartilage or bone and matrix.

Nevertheless, *in vitro* experiments have demonstrated that the application of growth factors and specific culture conditions can guide MSCs to differentiate into particular cell phenotypes [29].

In an *in vitro* study conducted with MSCs cultured in the presence of TGF-b, it was observed that the MSCs were able to express a phenotype similar to NP cells with the production of type II collagen and aggrecan [30]. Moreover, under specific culture conditions such as hypoxia, it was initially seen that hypoxia contributed to maintain an NP cell phenotype in *in vitro* cultures, and subsequently the combination of hypoxia and TGF-b1 exposure proved to advance differentiation of the MSCs into an NP-like phenotype. On the basis of this experience, researchers suggested that the hypoxic environment of the IVD could promote differentiation of the MSCs towards an NP-like phenotype *in vivo* [31, 32].

The effect of cocultures of MSCs with AF and NP cells obtained from human degenerative IVDs in 3-dimensional pellet cultures has also been investigated. The coculture determined an increased proliferation in both the MSC-AF and MSC-NP cultures, but a higher proteoglycan production was found only in the MSC-AF coculture. Based on the interaction between AF cells and MSCs, a direct implantation of the MSCs *in vivo* may be applied without any need to differentiate the MSCs before implantation [33].

In their research in the IVD implantation of MSCs, Sakai et al. [34–36] initially implanted MSCs into a normal IVD and subsequently repeated the same experiment in a IVD degeneration model in rabbit. Based on the results obtained in their initial study, they reported that autologous MSCs implanted into a rabbit IVD survive and proliferate, with differentiation into a NP-like phenotype. At 48 weeks since implantation, labelled MSCs (i.e., tagged with the gene for green fluorescent protein (GFP) with the GFP-labelled cells followed tracking their effects for a period of 48 weeks) expressed factor-a (HIF-a), glutamine transporter-1, and MMP-2, which represent hypoxia-inducible NP phenotypic-related markers [36]. Autologous MSCs injected into the IVD could reestablish a chondrocyte-like cell population able to produce proteoglycans and collagen II in a rabbit degenerating IVD. At MRI imaging, degenerating IVDs treated with MSCs showed improvement compared to controls both in IVD height and hydration [35].

To repair the matrix of degenerative NP tissue, transplanted cells must produce large quantities of collagen, proteoglycans (e.g., aggrecan), and other matrix proteins [37]. Generally, these matrix proteins are produced by cells located in the central regions of the IVD and chondrocytes, making them candidates for cell-based IVD repair. Also, progress recorded in stem cell research in the past decade provides an attractive scenario for the use of adult MSCs (ASCs) for NP tissue engineering [30, 38–46]. Thereafter, while a variety of cell sources in the field of stem cell research has been proposed for clinical application [21], ASCs as a cell source in bone and cartilage repair have gained significant attention. Particularly, application of ASCs in NP tissue regeneration has been the subject of a number of recent studies [27, 47–49]. One study reported that the differentiation of ASCs into a NP cell-like phenotype occurred in coculture of human ASCs and NP cells in a micro-mass-cultured mode. In other experiments, expression of proteoglycan and type I collagen in 3D-cultured sand rat ASCs could be significantly stimulated either through treatment of TGF-b or coculture of human IVD cells [27, 49].

Moreover, in coculture of NP cells and ASCs combined in micromasses, separated by a permeable membrane to allow the diffusion of soluble factors, an upregulation of the aggrecan and collagen II expression was detected [50]. Therefore, soluble factors released by NP cells may have a direct effect on promoting the differentiation of the ASC into the NP-type lineage [51].

Coculture of human NP and ASCs improves the quality of the tissue reconstructed *in vitro* both in terms of 3D cell organization and matrix production [47]. Conversely, cell expansion is essentially needed to obtain sufficient cell quantities for transplantation, and the common monolayer culture approach has determined a number of concerns including cell dedifferentiation, senescence, and genetic mutagenesis [40].

However, adipose tissue might be considered as an adequate source of stem cells for clinical use, based both on the ease of the procedure to retrieve adipose tissue and the cell quantities that can be obtained. In fact, on one hand, the collection of adipose tissue occurs through a minimally invasive procedure allowing for this to be straightforwardly performed in outpatient clinics, and, on the other hand, it allows for yields of adherent ASCs to reach up to 25,000/g of tissue. Therefore, although somewhat new in the stem cell research area, ASCs have gained intensive attention as a cell source in bone and cartilage repair [27] and the use of ASCs as seed cells in NP tissue engineering has been the subject of further investigations [52, 53].

The results of cocultured rabbit ASCs with NP tissues through a specifically designed device showed that ASCs responded to soluble mediators from NP tissues [53]. In experimental studies, NP or AF tissues were cocultured with alginate beads containing ASCs isolated from inguinal fad pads of NZW rabbits and following real-time RT-PCR analysis and it was observed that NP tissues could notably stimulate type II collagen and aggrecan genes expression in ASCs, whereas this was not the case for AF tissues [53].

Recently, confirmation of the feasibility of a stem cell therapy for IVD repair has been obtained by a parallel *in vitro* and *in vivo* study. In the *in vitro* portion of the study, in which bone-marrow-derived stem cells (BSCs) and IVD cells were cocultured, the results showed an improvement of ECM

production, while the *in vivo* study persistence of BSC for at least 24 weeks after implantation in rabbit IVD was shown [54].

4.2. In Vivo Evidences on MSCs. The future of treatment options for IVD degeneration should deal with evidences regarding the use of MSCs in animal studies.

The possibility of allogeneic MSC transplantation has also been suggested and should be considered [55], based on a number of potential advantages of allograft stem cells versus autograft stem cells.

It is theorized that the use of autograft MSCs may have the same genetic predisposition for degeneration as the native NP cells, while it should be possible to escape this genetic predisposition by using an allograft cell population. The use of allograft MSCs may in fact allow for the selection and maintenance of a cell population that is less likely to undergo degeneration. Also, in comparison to collection and culture of MSCs for each individual patient, allograft cells could be maintained for "off the shelf use" with a higher convenience and reduced costs [24].

Several *in vivo* animal experiments conducted with the implant of MSCs into the IVD have highlighted promising results in terms of long-term viability of the implanted cells and differentiation into a phenotype similar to the native NP cells with the production of type II collagen and aggrecan [24].

Crevensten et al. [56] were the first in 2004 to report the viability of allograft MSCs implanted in a rat IVD. They observed the proliferation of the stem cells as well as a trend towards increased IVD height after a 4-week follow-up period, which suggested an enhanced production of proteoglycans.

Subsequently, allograft MSCs were also transplanted into the New Zealand white rabbit: the cells survived, proliferated, and differentiated into native NP cells like phenotype. After grafting, the animals were followed for 6 months, and no immune reactions occurred towards these allograft cells. In the IVDs treated with MSCs, type II collagen production along with an increased proteoglycan concentration was detected [57].

The effectiveness of MSC transplantation has also been confirmed in large animal models (chondrodystrophoid breed canine with nucleotomy) closer to humans. In fact, in a canine model of IVD degeneration, MSC transplantation determined a positive outcome on preservation of immune privilege, possibly by differentiation of the transplanted MSCs into FasL expressing cells [38]. These data suggest that the NP region may tolerate MSCs from other areas of the body or even other individuals [58].

Other researchers have also investigated the use of allogenic MSCs for IVD regeneration, despite the concerns for immunologic rejection of the allograft tissue. Similarly to previous experiences showing *in vivo* viability of allograft NP tissue, the "immunologically privileged" environment of the avascular IVD space may also allow for long-term viability of allograft stem cells [59, 60].

The ability of allogenic MSCs to escape alloantigenic immune recognition and determine an immunomodulatory effect has been noted also in other studies [61, 62]. The ability to elude immune recognition may arise from the lack of major histocompatibility complex class II molecules on MSCs. Nevertheless, major histocompatibility complex class II expression has been observed as MSCs differentiate or are exposed to inflammatory cytokines [61, 62].

An immunomodulatory effect of MSCs on the local environment by producing anti-inflammatory cytokines such as TGF-b and IL-10 has also been reported [61, 63]. In addition to enhancing the probability for MSC graft survival, this immunomodulatory effect of MSCs may also play a role in IVD regeneration by modifying the environment from a proinflammatory, catabolic condition to a more anti-inflammatory, anabolic environment [4–6, 24, 64, 65].

Several studies have now confirmed the effectiveness of MSC transplantation, demonstrating that MSCs transplanted into degenerating IVDs *in vivo* can survive, proliferate, and differentiate into cells expressing the phenotype of NP cells with suppression of inflammatory genes [35].

There are a large number of different types of stem cells and a variety of possible sources of MSCs including bone marrow, adipose tissue, and other tissues that have received significant attention as potential sources of cells to repopulate and regenerate the IVD.

Bone marrow aspiration, which has been used by spinal surgeons in fusion procedures, is an excellent source of MSCs [24]. When bone-marrow-derived stem cells (BSCs) combined with a hyaluronic acid-derived scaffold were injected into pig IVD, IVDs presented a central NP-like region. This finding suggested that BSC are able to differentiate in IVD-similar cells and also to produce matrix proteins similar to normal IVD tissues [66]. Further confirmation comes from experiments performed in previously degenerated or degraded IVDs in rabbits, that showed how, following transplantation in these IVDs, BSCs were able to proliferate and differentiate into cells expressing and producing some of the main, although not all, extracellular components of the IVD [67].

Recently, other stem cells such as synovial MSCs have been studied as a potential source of cells to be used for IVD-d. In an induced IVD-d model in rabbits, allogenic synovial MSCs have been transplanted and evaluated up to 24 weeks postoperatively with imaging analyses such as magnetic resonance imaging, radiographs, and histological analysis. T2-weighted MR imaging revealed a higher signal intensity of the NP in the MSC group. Radiographs showed that the height of IVD in the MSC group remained higher compared to that in the degeneration group. Immunohistochemical analyses showed a higher expression of type II collagen around NP cells in the MSC group compared to that of the normal group. These results brought to the conclusion that synovial MSCs injected into the NP space promote synthesis of the remaining NP cells to type II collagen as well as inhibition of degrading enzymes and inflammatory cytokines expression, resulting in the structure of the intervertebral IVD being maintained [68].

Several authors have recently reported their successful results using different types of stem cells, but there is still a lack of published evidence regarding adipose-derived stem cells (ASCs) implantation for IVD degeneration management, and, only recently, experiments with ASC have confirmed this type of stem cells as another potential cell source for IVD repair and regeneration. In fact, in a goat ABC chondroitinase degeneration model [69], ASC can be beneficial for cell therapy of IVD disease because these cells are easily isolated compared to other MSCs such as BSCs [70, 71].

Despite the promising results deriving from animal studies, there is still a lack of studies performed in humans.

To our knowledge, the only trial conducted in humans, a nonrandomized, noncontrolled study evaluating autologous hematopoietic stem cells transplanted into IVD for the treatment of low back pain in 10 patients, did not report any significant improvement of the patients' condition [72].

Therefore, further research and randomized controlled studies are required for a better definition of the effectiveness of the administration of ASC, rather than BSC or other types of stem cells for the management of IVD degeneration.

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Review Article

Most British Surgeons Would Consider Using a Tissue-Engineered Anterior Cruciate Ligament: A Questionnaire Study

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Donor site morbidity, poor graft site integration, and incorrect mechanical performance are all common problems associated with autografts for anterior cruciate ligament (ACL) reconstructions. A tissue-engineered (TE) ligament has the potential to overcome these problems. We produced an online questionnaire relating to tissue engineering of the ACL to obtain input from practising clinicians who currently manage these injuries. 300 British orthopaedic surgeons specialising in knee surgery and soft tissue injury were invited to participate. 86% of surgeons would consider using a TE ACL if it were an option, provided that it showed biological and mechanical success, if it significantly improved the patient satisfaction (63%) or shortened surgical time (62%). 76% felt that using a TE ACL would be more appropriate than a patellar tendon, hamstring, or quadriceps autograft. Overall, most surgeons would be prepared to use a TE ACL if it were an improvement over the current techniques.

1. Introduction

Some of the most frequently ruptured ligaments occur in the knee joint, often through sporting activities such as skiing, football, and basketball. Ninety percent of knee ligament injuries involve the anterior cruciate ligament (ACL) and medial collateral ligament (MCL) [1]. The MCL can self-heal, but the ACL is not able to do so from a combination of poor vascularisation and its intra-articular location. Therefore, alternative methods, including regenerative medicine, have focused heavily upon the ACL with the aim of producing a fully functional tissue *in vitro*.

The current gold standard procedure to reconstruct a torn ACL is surgical autografting. This involves using part of the patients own patellar, hamstring, or quadriceps tendon to replace the torn ACL [2]. However, this causes donor site morbidity [3–6] and is associated with pain and a recovery period for this region [5, 6]. Generally, 75–90% of patients have good or excellent long-term success rates from the current reconstruction techniques regarding functional stability and symptomatic relief upon return to normal activities, but many patients experience unsatisfactory results, which could be attributed to graft failure [7]. Some of these patients continue to endure pain, suffer from loss of motion secondary to the procedure, and their instability is not corrected [7]. Others suffer from degenerative joint disease such as osteoarthritis or experience reinjury [6]. Alternatively, allografts can be used; in this instance, the donor tendon is taken from a cadaver, but the disadvantages associated with this include scarcity of suitable tissue, the risk of the recipient contracting a disease from the donor, or tissue rejection [8, 9]. Prosthetic replacements (synthetic grafts) have previously been used, but they are inadequate due to wear and degeneration [10]. Surgical reconstruction techniques have limitations and do not always provide completely satisfactory long-term results in a high proportion of patients, consequently affecting their quality of life [7, 11]. Because of this dilemma, regenerative medicine could be an option, whereby *in vitro* tissue engineering of ligaments could offer a solution to the problems associated with the current surgical methods [4, 6]. Tissue-engineered ligaments could provide better performance in the long run by improved biocompatibility, better integration into host tissue, and the ability to remodel their own extracellular matrix [12].

Tissue engineering is a method which combines knowledge from material science, engineering, molecular biology, and medicine [12]. The basic procedure normally involves using scaffolds to act as structural supports for cell growth and maturation *in vitro*, where a chemical or mechanical stimulus may also be applied to promote the formation of a functional tissue. This concept was originally developed to repair skin, cartilage, and bone, but is now being considered as a possible option to produce a functional ACL.

It is important to have input from some of the end users, namely, the orthopaedic surgeons, on developing tissue engineering strategies so that scientists working in this area can determine whether their research is heading in the correct direction. We wanted to ascertain whether the orthopaedic surgeons that routinely carry out ACL reconstruction would choose to use a-tissue engineered ACL for implantation if it were an option and to elucidate any reservations that they may have regarding this emerging technology. Therefore, we developed an online questionnaire to investigate the current clinical opinion on using tissue- engineered ACLs to treat their future patients.

2. Materials and Methods

An online questionnaire was designed to ascertain how satisfied knee surgeons were with the current autografting methods for ACL reconstructions and to gain their opinions towards using a tissue-engineered ACL for implantation as an alternative method to the current autografting procedure. We also wanted to determine whether the surgeons felt it would be advantageous to surgeon, patient, and the National Health Service (NHS) to use a tissue-engineered ACL. The following questions and responses were included as described in Table 1.

Many of the questions above required "Yes, No or Do not know" answers, while others were categorised or required the input of values. The questionnaire was peer reviewed and subsequently approved by the local research ethics committee (Project no. 09/H1204/64, approved 15/10/09). Between July and October 2010, 300 orthopaedic surgeons specialising in knee surgery and soft tissue injury in the UK were contacted by e-mail and invited to participate. From this e-mailed input request, 79 surgeons responded.

The sample of surgeons contacted for inclusion in this study were those who were members of the British Orthopaedic Association (BOA), who also belonged to one of two specialist societies, the British Association of Surgery of the Knee (BASK) and the British Orthopaedic Sports Trauma Association (BOSTA). The e-mail addresses were obtained from the BOA handbook of 2006, where the invitation and link to the survey was sent to 300 surgeons. Reminders (aimed at nonresponders) were sent at 8 days, 42 days, and again at 69 days after the first invitation to ask for their feedback. The results remained anonymous and were analysed using Microsoft Excel.

3. Results

The overall response rate in total was 26% (79/300): we did not receive an answer from 221 surgeons. Of those who did respond, 10% (8/79) did not perform ACL reconstruction and,therefore, could not provide feedback. Most of the surgeons who completed the survey had been an orthopaedic consultant for more than 10 years (62%, 44/71), with 15% (11/71) of the respondents having been a consultant for less than 5 years. Sixteen of 71 (23%) respondents had been an orthopaedic consultant for 5 to 10 years. From the 71 surgeons, 69% (49/71) had over 10 years of experience performing ACL reconstructions, with 23% (16/71) having 5–10 years of experience, and 8% (6/71) having less than 5 years of experience.

The average number of ACL reconstructive surgeries that were performed by the respondents every month varied from 1 to 15. The majority of respondents (77%, 55/71) performed between 2 and 8 ACL reconstructions a month.

Forty-eight surgeons (68%, 48/71) responded to question 5 regarding their opinion of the current success of using the patellar tendon as an ACL repair. More surgeons (a total of 66/71, 93%) completed question six regarding their opinion of the treatment success of the hamstring tendon for ACL repair, whereas fewer surgeons (13%, 9/71) responded to question 7 regarding their opinion on the use of the quadriceps tendon for ACL repair. Figure 1 shows the opinion of the respondents with regards to the success of each of these three currently used ACL treatment methods.

The majority of surgeons (86%, 41/48) found patellar tendon autografts to be very successful/successful for treating athletes (any age) and adults of 16–40 years in age. On average, 54% (26/48) of surgeons felt that the patellar tendon was very successful/successful in patients with a lower typical daily exercise regime who were 56 years or older. Surgeons felt that the patellar tendon was either satisfactory (32%, 15/48) or very unsuccessful/unsuccessful (13%, 6/48) for these older patients.

The hamstring tendon procedure was viewed by 88% (58/66) of surgeons to be very successful/successful for athletes (any age) and patients with "normal" activity up to the age of 40. 73% (48/66) of surgeons felt that this procedure was very successful/successful for patients with "normal" activity over the age of 56. Surgeons felt that for these patients (older than 56 years of age) the hamstring tendon procedure was either sufficient (23%, 15/66) or very unsuccessful/unsuccessful (3%, 2/66).

Fewer surgeons felt that the use of quadriceps tendon was very successful/successful for young athletes (42%, 4/9) and athletes over 26 years (66%, 6/9) in comparison to the patellar tendon and hamstring tendon. In these cases, 25–28% of surgeons felt that this technique was unsuccessful for all people (all ages and activity levels).

Of 71 surgeons, 67 (94%) were familiar with the term tissue engineering and its implications as a future therapy. 86% of the surgeons (61/71) felt that they would consider using a tissue-engineered ACL as a treatment provided that it had demonstrated biological and mechanical success *in vitro* and *in vivo*. 6% (5/79) surgeons would not consider this treatment option, and a further 8% (6/79) were unsure.

The time that the surgeons stated they would be prepared to wait for a tissue-engineered ACL to be prepared varied from 2 to 26 weeks. From the 64 who answered this question,

Table 1: Questionnaire used online for orthopaedic consultant feedback.

	Question
1	How many years have you been a consultant?
2	Do you perform anterior cruciate ligament (ACL) reconstructions?
3	Approximately how many ACL reconstructions do you perform each month?
4	How long have you been performing ACL reconstructions?
5	How successful do you rate patellar tendon ACL reconstructions? (e.g., the patient being able to return to physical activities without experiencing another injury to the reconstructed ACL)
6	How successful do you rate hamstring ACL reconstructions? (e.g., the patient being able to return to physical activities without experiencing another injury to the reconstructed ACL)
7	How successful do you rate quadriceps ACL reconstructions? (e.g., the patient being able to return to physical activities without experiencing another injury to the reconstructed ACL)
8	Are you familiar with tissue engineering as future clinic therapy? (Definition of tissue engineering: To grow autologous tissue *in vitro* in order to replace damaged body parts.)
9	If tissue engineering an ACL for the patient were an option (either through the NHS or privately), would you consider using a newly developed tissue-engineered ACL? (If it had shown mechanical and biological success *in vitro and in vivo*)
10	If an autologous tissue were tissue-engineered in the laboratory, what time limit would you see as acceptable from the moment the patients cells were harvested to the moment the engineered ACL was ready for implantation?
11	If you were to hypothetically use a tissue-engineered ACL, would you be concerned about the successful integration of the engineered ACL into the bone?
12	An engineered ACL could be an exact match to the native ACL. Do you feel that this would be more appropriate for implantation than a hamstring, quadriceps, or patellar tendon (which are only similar in tissue type to the ACL and not an exact match)?
13	Approximately how long on average does your current treatment strategy for ACL replacement take (a) regarding operation length (b) regarding full recovery time with no pain
14	Do you think it is likely that some patients would prefer to wait to receive a tissue-engineered ACL from their own cells, rather than receiving the current surgical ACL reconstruction using their own patellar tendon/hamstring tendon/quadriceps tendon?
15	With respect to tissue engineering (a) Do you believe that using a tissue-engineered ligament would produce less scarring? Donor site scarring (e.g. patellar tendon, hamstring, quadricep), implant site scarring, skin scarring as a result from donor tissue harvest? (b) Do you believe that using a tissue-engineered ligament would take less surgical time? (c) By how much would surgical time need to be reduced for you to consider it to be a significant improvement? (d) Do you believe that using a tissue-engineered ligament would give patients a shorter full recovery time? (e) By how much would recovery time need to be reduced for you to consider it to be a significant improvement? (f) Do you believe that using a tissue-engineered ligament would give recovering patients reduced pain or recurring injuries?
16	Currently ACL reconstructions cost £2,061 (NHS) and £3,500–£5,000 (privately). If a tissue-engineered construct cost more than your current procedure (for instance, up to twice the amount) but significantly improved the patient's satisfaction (resolution of instability/mobility/strength), would you consider using this technique?
17	Any other personal suggestions? For example, what do you see as an advantage/disadvantage regarding using tissue-engineered constructs? Do you see a need to improve current surgical techniques?

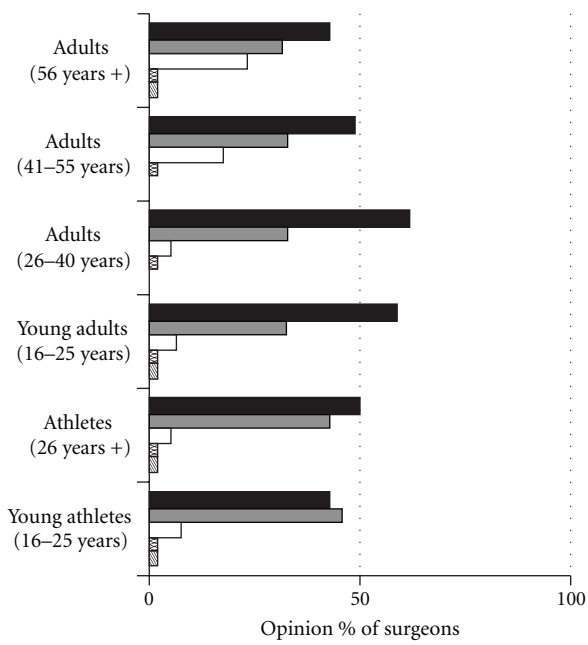
the majority (59/64) would wait up to 12 weeks. Of those 59 surgeons, 8% (5/59) felt that they would be prepared to wait 4 weeks, 44% (26/59) 6 weeks, 12% (7/59) 8 weeks, and 36% (21/59) said they would wait 12 weeks for such an implant to be ready for clinical use.

The surgical opinion of concern regarding the successful integration of a tissue-engineered ligament into the native bone was as follows: very concerned (25%, 18/71), concerned (42%, 30/71), slightly concerned (15%, 11/71), not particularly concerned (15%, 11/71), and not at all concerned (1%, 1/71).

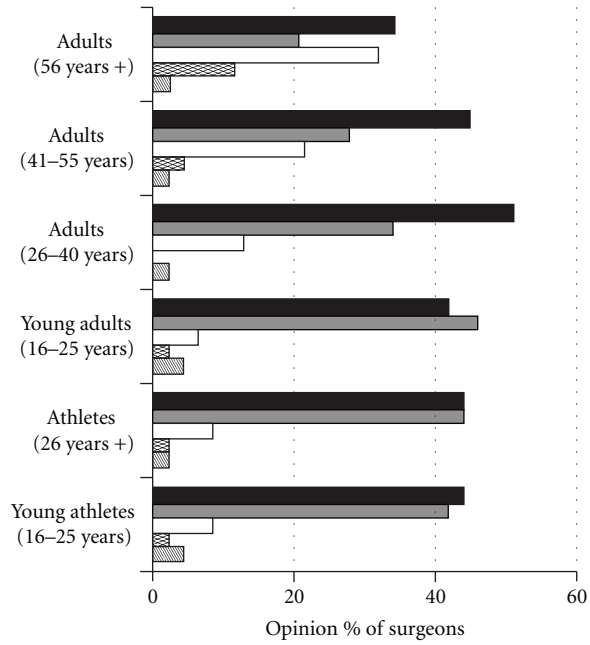
75% (53/71) of the participating surgeons felt that it was more appropriate to use a tissue-engineered ACL which had the capability to be an exact match to the native ACL than the currently used tendon autografts. 10% (7/71) of surgeons felt that it was not more appropriate, and 14% (10/71) of surgeons did not know.

The length of time taken for the surgeons to operate using their current tendon autograft technique varied from approximately 60 to 90 minutes per operation. The surgeons stated that recovery time for these currently used autograft techniques for ACL treatment varied from 1 to 18 months, with 10% (7/71) surgeons stating 2 months, 43% (31/71) surgeons stating 6 months, and 13% (9/71) surgeons stating 9 months are needed for full recovery time after surgery. The minority (34%) suggested that a longer period was necessary.

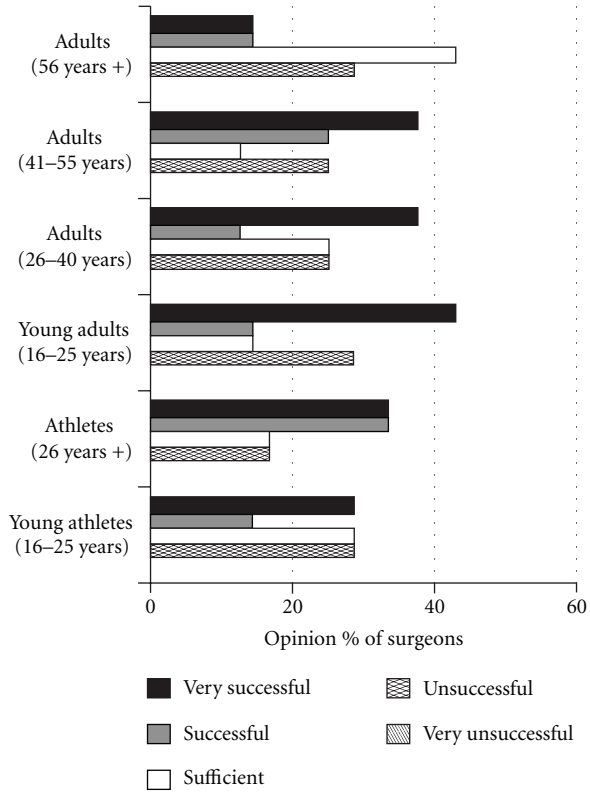
The surgeons felt that 60% (43/71) patients would prefer to wait for a tissue-engineered ACL to be prepared for their ACL repair instead of using current techniques. Another 28% (20/71) of surgeons were unsure of what the patients



(a) Hamstring tendon autograft success



(b) Patellar tendon autograft success



(c) Quadricep tendon autograft success

Figure 1: Graphs demonstrating current surgical opinion on the success rate of the use of (a) hamstring tendon, (b) patellar tendon, and (c) quadriceps tendon for the repair of anterior cruciate ligament injuries.

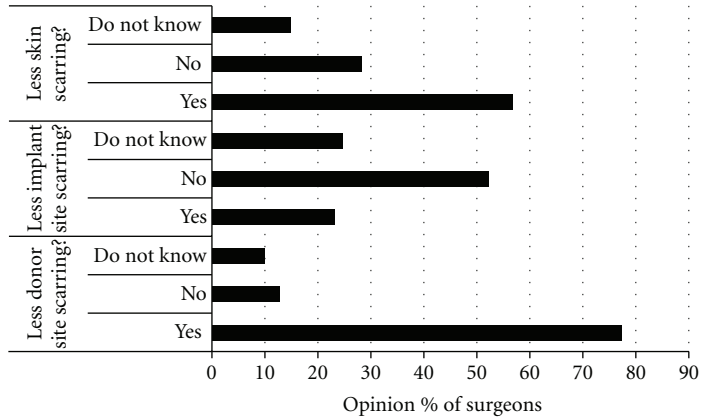


Figure 2: Graph to demonstrate the % of surgeons who believe that using a tissue-engineered ligament would produce less scarring in relation to location in the body.

response would be, and 11% (8/71) felt that the patient would probably not prefer to wait for a tissue-engineered ligament to be produced for them.

Figure 2 shows the opinions of the surgeons regarding whether scarring will or will not be reduced as a result of using tissue-engineering as a new therapy for ACL reconstruction. 77% (55/71) of the surgeons felt there would be less scarring at the donor site as a result of using tissue-engineered implants. 62% (44/71) of surgeons believed that using a tissue-engineered ACL would reduce operative time, 24% (17/71) felt that time would not be reduced in surgery, and 14% (10/71) stated that they were unsure if surgery time would be reduced. The surgeons felt that anywhere between 5 and 30 minutes reduction on surgery time would be of significant improvement to them, with 39% (28/71) of surgeons stating that 10 to 15 minutes would make a difference to them and 21% (15/71) stating that 20 minutes would be beneficial.

When surgeons were asked whether they believed that using a tissue-engineered ligament would give patients a shorter full recovery time, 23% (16/71) said yes, 47% said no (33/71), and 30% (21/71) were unsure. When questioned further to discover how much the patient recovery time needed to be reduced for them to consider it to be a significant improvement, the surgeons replies (from a total of 47 who responded to this question) varied between 4 and 26 weeks. The majority (45/47) indicated there needed to be a reduction of up to 12 weeks. 33% (15/45) of surgeons felt that a 4-week reduction in recovery time would be significant, 38% (17/45) felt 6 weeks would, and 29% (13/45) felt that 12 weeks would be of significance.

38% (27/71) surgeons believed that using a tissue-engineered ligament would give recovering patients reduced pain or recurring injuries, with 35% (25/71) of surgeons being unsure whether this was true. 27% (19/71) surgeons felt that a tissue-engineered ACL would not give these added benefits.

When surgeons were asked whether they would consider using a tissue-engineered construct that costs more than their current procedure (for instance, up to twice the amount) but significantly improved the patients satisfaction (resolution of instability/mobility/strength) 63% (45/71) stated yes, 10% (7/71) said no, and 27% (19/71) were unsure.

There were a variety of comments left by the surgeons when prompted. A recurring theme that surgeons were concerned about with regards to the use of a tissue-engineered ACL was of fixation. For example, how will the new device be fixed into the bone and how successfully will it integrate? Another recurring theme was the importance of reducing donor site morbidity, which appeared to be very appealing to the surgeons. Other comments included the need for initial mechanical integrity of the construct to be immediately load bearing. Many surgeons also felt that the engineered ACL should come prepared with whip stitches ready for implantation. Individual comments include the following: "Would be very useful in revision ACL reconstruction," "The whole issue is stability. If the tissue is as good and not significantly more expensive I would use it," "The key will be getting the graft to incorporate and pick up a blood supply. The graft will not have a nerve supply and postoperative rehab will be unchanged as I see it while patients restore proprioception. Engineered grafts may be of use in revision reconstructions and in multiligament knee injuries where autograft harvesting results in significant donor site morbidity and takes time."

4. Discussion

Tissue engineering has been of increasing interest as a new and emerging therapeutic strategy. These techniques have been applied clinically with varying success for skin and cartilage. Researchers are beginning to investigate new approaches to engineer replacements for the ACL [13, 14]. It is important to include a dialogue with the end users of these products during their development to ensure the correct criteria are being addressed.

As such, this survey was targeted at surgeons who performed ACL reconstruction to gain their views on how satisfied they are with the current methods for ACL reconstruction, whether there is a need for an alternative method,

and how they would feel about using a tissue-engineered ACL if it were an option. As with most surveys, there were two potential sources for bias. The first was an incomplete sampling frame because not all specialist surgeons in the UK could be contacted (unavailable/invalid addresses), and the second was represented by nonresponders. However, the data obtained are the first, to our knowledge, to give an indication of current clinical opinion of the potential of tissue engineering for the ACL. This feedback is essential to tissue engineering scientists to ensure that the correct product goal posts are set with respect to functionality and culture growth rate *in vitro*. An assessment of the potential use of this new and emerging strategy from the perspective of the end users of a tissue-engineered product is important to be incorporated from the early stages of product development.

Our data demonstrated that most of the participants had performed surgery for more than 10 years, performing from 1 to 15 reconstructions per month, and therefore regarded as experienced, making their opinions valuable to us. Generally the results implied that patellar tendon and hamstring grafts were very successful for all age groups (including athletes,) regarding their return to physical activities with no recurring injuries, whereas quadriceps grafts were very successful in patients aged <56 years, with a very high percentage rating them as "unsuccessful" compared to the patellar tendon or hamstring. The patellar tendon is known to be a very stiff, strong tissue, if not stiffer than the ACL, and is also associated with a short healing period [15], making it suitable choice for reconstructions, as confirmed by the high success rate indicated by the responses. Currently, the most commonly used grafts are patellar tendon and hamstring, but the quadriceps tendon is also becoming a popular graft, producing fewer donor site problems than harvesting of the patellar tendon, with excellent mechanical characteristics [15]. From the data obtained, the percentage of respondents who found a quadriceps graft to be very successful was much lower than for patellar tendon and hamstring, with a relatively large proportion suggesting that it was unsuccessful.

Although the current surgical procedures are successful in a high proportion of patients, surgeons would consider using alternative methods if they were an improvement over existing techniques. A high proportion would be prepared to use a tissue-engineered ACL if it were shown to have biological and mechanical success (*in vivo* and *in vitro*) and significantly improved patients' satisfaction, but there were some concerns about its successful integration into bone. It was also recognised that there would be a reduction in scarring when tissue harvesting was not required (no donor site), which would be another advantage to the patient. In particular, it may be worthwhile from a tissue engineer point of view to develop tissue-engineered ACL constructs for the older patient (56 years plus), as surgeons have less confidence in ACL reconstruction in this age group [16].

If the procedure time could be reduced using a tissue-engineered ACL, it could be of benefit to both patient and the NHS, reducing anaesthetic time and surgery costs, where a 10–30 min reduction would be needed to consider it an improvement over existing methods. Currently, the surgeons feel that tissue engineering an ACL will not significantly reduce recovery time. As such, it is important for scientists to design appropriate experiments to effectively demonstrate that the products being developed reduce recovery time. This should be translated into the appropriate animal model—for example, a reduction of an 18-month recovery time by 3 months (96% of surgeons stated that 12 weeks reduction in recovery time would be significant to them) would produce a 20% recovery time reduction. If a mouse ligament model was to normally take 10 weeks to fully recover, the tissue-engineered counterpart should demonstrate full recovery by at least 8 weeks. Scarring did not appear to be an issue for the surgeons.

Surgery time to reconstruct the ACL was typically stated to last between 60 and 90 minutes. A significant reduction of this surgery time to the surgeons would be 20 minutes. Again, this translates to an approximate reduction of surgery time of 25%. These convincing statistics need to be incorporated and considered into the preclinical animal models used by tissue engineers when testing their products. The need for ways to reduce surgery time was highlighted further when the respondents were also asked for their comments which included comments such the need for preprepared whip stitches and easy insertion techniques.

One important factor to consider is how long the *in vitro* culture can be in order to obtain the functional ligament. This survey identifies a clinical need for a stable, usable construct by 4–6 weeks. Approximately two-thirds of the surgeons surveyed were concerned about the method of integration of the tissue-engineered ACL into the host bone. These factors should be fully considered when designing such a construct, and efforts to demonstrate this efficacy should be included. Cost was not as much as a concern to the clinicians than the actual performance of the replacement ACL. The stability and potential reduction of full recovery time of the replacement ACL was far more important than the initial expense of the tissue engineered ACL. Recent reviews indicate the need for improving ligament repair [17, 18] as such, this current study indicates that clinicians may be open to improving current methods using tissue-engineering strategies.

5. Conclusions

The majority of British surgeons were familiar with tissue engineering as a concept and are very open to its potential use. Current treatments for patients over 56 years of age have a greater need for improvement than for younger patients. Greater patient awareness is needed with regards to tissue engineering as a potential therapy for an increase in potential acceptance of new strategies by these end users. Surgeons clearly detailed a need to have a fully load-bearing construct for implantation. Therefore, it is important for tissue engineers to ensure that they have reliable mechanical integrity data of their developing constructs. Currently, surgeons are not convinced that tissue-engineered ligaments could shorten the recovery time of the ACL in comparison to the current methods used. Again, it is important that the data gathered by the tissue engineer researchers effectively

demonstrate this potential. Improvements to currently used procedures need to reduce surgery time by at least 20 minutes and reduce the patient time to full recovery by a minimum of 12 weeks before surgeons feel that there is a benefit. If these criteria are met, it was not deemed as a problem if the operation costs increased up to double the current costs. Lastly, there is a need to have these tissue-engineered constructs ready for use after a minimum of 4–6 weeks *in vitro* culture. Any potential longer *in vitro* culture time becomes unattractive for the surgeons.

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Review Article

Application of Stem Cells in Orthopedics

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Stem cell research plays an important role in orthopedic regenerative medicine today. Current literature provides us with promising results from animal research in the fields of bone, tendon, and cartilage repair. While early clinical results are already published for bone and cartilage repair, the data about tendon repair is limited to animal studies. The success of these techniques remains inconsistent in all three mentioned areas. This may be due to different application techniques varying from simple mesenchymal stem cell injection up to complex tissue engineering. However, the ideal carrier for the stem cells still remains controversial. This paper aims to provide a better understanding of current basic research and clinical data concerning stem cell research in bone, tendon, and cartilage repair. Furthermore, a focus is set on different stem cell application techniques in tendon reconstruction, cartilage repair, and filling of bone defects.

1. Introduction

Today great hope is set on regenerative medicine in all medical fields. Leland Kaiser introduced the term "Regenerative medicine" in 1992. He forecasted that "a new branch of medicine will develop that attempts to change the course of chronic diseases and in many instances will regenerate tired and failing organ systems" [1]. Since then, scientists all over the world try to develop cell-based approaches to regenerate damaged tissues, or even substitute whole organs [2].

Of course, regenerative medicine has developed to be of interest in orthopedics. There, great hope was set on regenerative medicine to develop alternative therapies for cartilage damage, arthritis, large bone defects, or atrophic tendon ruptures during the last decade. These are all indications, which are treatable only insufficiently with conventional implants and surgical procedures [3–10]. Therefore, they frequently result in decreased function of the musculoskeletal system or even loss of patients' mobility. In the worst case, the mentioned diseases even result in a loss of autonomy for the patient. In consequence, this implies immense costs for the health care systems all over the world.

In this review, we focus on application of stem cells in regenerative medicine for orthopedic indications. We present current approaches in stem cell-based therapy in orthopedics and review recent successes in basic science and clinical application of regenerative medicine approaches within the field.

2. Stem Cells

Stem cells are of particular interest in regenerative medicine. They inhere several unique characteristics that distinguish them from other cell types. Stem cells represent unspecialized cells, which have the ability to differentiate into different adult cell types. Here, it is important to distinguish embryonic stem cells, which are truly pluripotent from multipotent adult stem cells. Embryonic stem cells (ESCs) are only found in early developmental stages of the organism. They represent the only cell type, which has the ability to renew itself indefinitely and is truly pluripotent. As a unique precursor cell, it can differentiate into cells of all three germ layers [2]. In contrast, a variety of multipotent adult stem cells exists in assumedly all tissues of the organism. They are

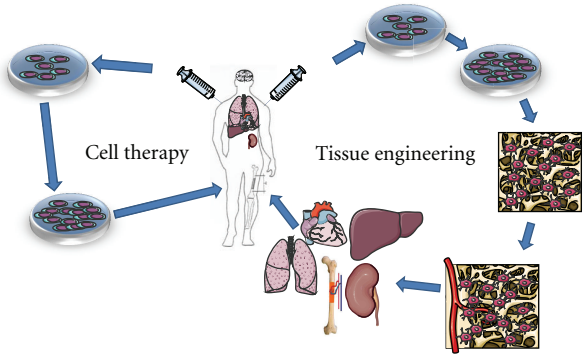

Figure 1: The two strategies of stem cell application in regenerative medicine. Stem cells are either isolated from the patient (autologous transplantation) or from other donors (allogenous transplantation). The cells are expanded *in vitro* and either applied directly to the patient to substitute lost cells ("cell therapy"), or seeded into 3 dimensional scaffolds ("Tissue engineering") and differentiated into the demanded cell type. The composed artificial tissue construct is subsequently implanted into patients' tissue defect.

responsible for maintaining the integrity of the tissue they reside in. Usually, these adult stem cells show limited differentiation potential to tissues of one germ layer [2].

The use of human ESCs as a resource for cell therapeutic approaches is currently an intensively researched field [11–13]. From a legal and ethical point of view, research involving human embryonic cells is highly controversial and many countries are reviewing their legislation. Besides the ethical concerns, the use of embryonic stem cells is problematic, as the application of allogenic pluripotent cells inheres a distinct oncogenic potential that currently forbids the application in patients.

The work of Takahashi and Yamanaka in 2006 has opened new perspectives in regenerative medicine. His group was the first to demonstrate successful dedifferentiation of somatic cells into a pluripotent ESC-like status by transfection with four embryonic transcription factors [14]. The so-called induced pluripotent stem cells (iPS cells) provide the possibility of autologous therapy with pluripotent and easily accessible cells in the future. Beside the great potential this technique undoubtedly represents, it bears some essential safety problems which are currently far from being solved. As ESCs, these cells inhere a high oncogenic potential which currently forbids application in patients. If they are injected in an undifferentiated state, they cause teratomas, and mice generated from iPS cells show high rates of tumors. This oncogenicity may be due to the transcription factors used for dedifferentiation which are known to be oncogenes, due to the insufficient epigenetic remodeling or due to the oncogenic retroviruses used for transfection [15].

The use of adult stem cells raises less ethical concerns and has proved to be much safer than pluripotent stem cells. In addition, these cells have further advantages compared to ESCs, for example, a use for autologous cell therapies, using patients' own cells to reduce possible immune responses, is easier to realize. Nonetheless, the limited differentiation potential of adult stem cells narrows their applicability. Typically, adult stem cells can differentiate into the cell types of the tissue in which they reside. Mesenchymal stem cells have been found to be the most promising candidates, as they show good differentiation potential towards cartilage, tendon and bone cells. They can be isolated from a number of mesenchymal tissues as for example bone marrow, fat, synovial membrane, periosteum, and others [16]. Interestingly, these mesenchymal stem cells have been found to differ regarding their differentiation potential dependent on their tissue source [17].

As ethical and safety concerns currently forbid application of iPS cells and ESCs in patients [2, 18], we will focus on adult mesenchymal stem cells within the rest of the paper.

3. Application of Mesenchymal Stem Cells in Regenerative Medicine

Regenerative medicine mainly includes two different strategies of cell-based therapy. In the first approach, cells are applied to substitute damaged cells within a tissue to reconstitute its integrity and function. During this procedure called "cell therapy" a cell suspension is simply injected into the damaged tissue or into the blood circulation. The second approach called "tissue engineering" is more complex. Here, cells are combined with a three dimensional matrix to compose a tissue-like construct to substitute lost parts of the tissue, or even whole organs (Figure 1) [2].

One of the most successful examples in "cell therapy" is the transplantation of hematopoietic stem cells. This procedure has now been practiced for decades to treat serious hematological diseases. For transplantation of bone marrow, hematopoietic stem cells are injected into the blood circulation of the recipient. Interestingly, they find their way to the bone marrow by a phenomenon termed "homing." Chemokines were found to play a key role in homing of hematopoietic stem cells [19–21].

Several experiments have proven the ability of homing to injured tissue for several types of stem cells. In animal models of hepatic intoxication, partial hepatectomy, myocardial infarction, nephropathy, cerebral ischemia, lung injury, lung fibrosis, and local irradiation, stem cells enriched in injured tissue and partially differentiated into tissue-specific cell types after systemic injection [22–38]. Cell therapy with systemically injected mesenchymal stem cells was also performed in humans, showing beneficial effects in graft-versus-host disease or osteogenesis imperfecta [39, 40].

However, cell therapy alone is not sufficient to regenerate large tissue defects or even replace whole organs. Therefore, the approach of "tissue engineering" is the more promising strategy. In the process, tissue-specific cells are seeded on a scaffold imitating the architecture of the tissue-specific extracellular matrix. In the last decade, basic science has made great advantages in tissue engineering research, resulting in *in vitro* composition of multiple different functional tissue constructs [41]. Nonetheless, tissue engineering therapy has barely reached the patient [42]. The reason for the modest entering of tissue engineering methods into the clinic is the yet unsolved problem of vascularization [43]. Thus, an

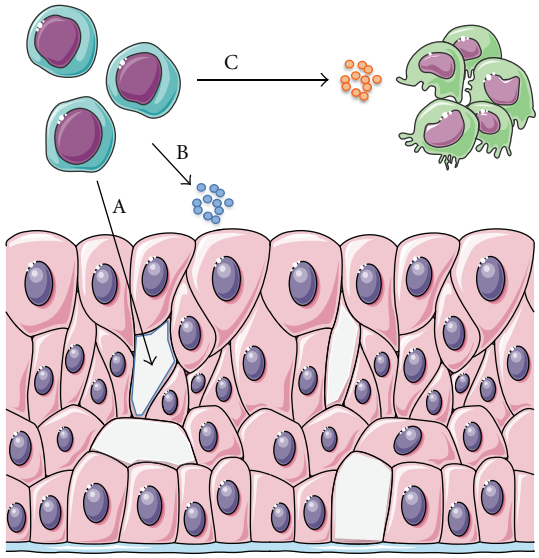

Figure 2: Stem cells participate in tissue regeneration in different ways. They directly differentiate into tissue-specific cells and thus substitute damaged or lost cells (A). They indirectly influence tissue regeneration by secretion of soluble factors. Here they promote vascularization, cell proliferation, differentiation within the tissue (B) and modulate inflammatory processes (C).

intact vascular network is a prerequisite to realize tissue constructs of more than 400 μm in diameter [44]. In the last decade many scientists in the field of tissue engineering have focused on solving the problem of vascularization. However, all efforts proving applicability for tissue engineering of large solid tissues or even whole organs in humans have failed so far (for paper see [43]). Nonetheless, tissue engineering was already successfully used in patients to substitute either hollow organs with limited wall diameter (trachea, bladder) or avascular tissues as cartilage [45–47]. In these cases, the diffusion trajectory is sufficient to maintain cell survival.

4. Participation of Mesenchymal Stem Cells in Tissue Regeneration

Mesenchymal stem cells have the ability to migrate chemotactically to tissues showing inflammation and injury in the organism [48]. Besides their unique ability to differentiate into different cell types, mesenchymal stem cells were found to secrete a variety of cytokines, showing anti-inflammatory activity and create an anabolic microenvironment [17]. Furthermore, direct cell-cell contact immunomodulation has also been shown. Thus, they participate in regeneration of injured tissues in different ways. On one hand, they directly differentiate into tissue-specific cells and thus substitute damaged or lost cells. On the other hand, they indirectly influence tissue regeneration by secretion of soluble factors. Thirdly, they are able to modulate the inflammatory response. Thus, they can promote vascularization, cell proliferation, differentiation and modulate an inflammatory process (Figure 2).

Indeed, there is evidence for all mentioned activities of MSCs in tissue regeneration from *in vitro* and *in vivo* experiments. The differentiation potential of MSCs was extensively studied *in vitro*. The cells were found to inhere the potential of multilineage differentiation towards possibly all kinds of mesenchymal cells such as cartilage, bone, tendon, and fat cells, and fibroblasts [69]. Excitingly, further studies revealed that differentiation capacity of MSCs seems not to be restricted to cells belonging to the mesenchymal lineage. They were shown to be able to differentiate towards cells from other germinal layers, as for example, neurons, glia cells, cardiomyocytes, endothelial cells and hepatocytes [70–73]. *In vivo* experiments and first clinical applications confirmed the ability of MSCs to engraft within a variety of injured tissues and differentiate into tissue-specific cells and thus substitute lost cellular function [17, 74].

In many studies, beneficial effects appeared without any detectable engraftment of the applied mesenchymal stem cells to the damaged tissue, however. Moreover, MSC protein extracts and conditioned medium from MSC cultures showed similar improvement of organ function in liver disorders or heart ischemia [75, 76]. Further investigation of MSCs revealed that they release paracrine factors for example IGF-1, HGF, VEGF, IGF-2, bFGF, or pre-microRNAs which protect's host cells, promote cell proliferation and enhance angiogenesis [77, 78]. These positive effects could partially be confirmed *in vivo*, where MSCs activated expression of some of the mentioned factors in the myocardium and promoted angiogenesis [79]. Furthermore, MSCs secrete paracrine factors which enhance lung function by regulating endothelial and epithelial permeability, decreasing inflammation, enhancing tissue repair, and inhibiting bacterial growth in acute lung injury and acute respiratory distress syndrome [80]. Beneficial effects of paracrine MSC signaling could also be confirmed in healing of cutaneous wounds [81]. The recently identified potential of paracrine MSC signaling on damaged tissue even caused some authors call MSCs an "injury drug store" [82].

Besides their mentioned differentiation potential and their ability to promote tissue regeneration by secretion of soluble factors, MSCs inhere extraordinary immunological properties. There is increasing evidence that the cells themselves are relatively nonimmunogenic and they can be readily transplanted between different individuals without initiating an immune response [83]. Furthermore, they proved to inhere anti-inflammatory and immunosuppressive capability *in vitro* and *in vivo*, where they can modulate immune responses on different targets. They inhibit maturation of immune cells, like helper T, cytotoxic T, dendritic, and B cells. Additionally, cells express a number of cytokines that can suppress inflammation, as for example TGF-beta1, NO, prostaglandin-E2, HLA-G, hepatocyte growth factor, and IL-10 [17]. The revealed anti-inflammatory effects of MSCs have opened a broad field of possible applications in transplantation and immune disorders. After confirming the anti-inflammatory effects in several animal models, first promising clinical applications have succeeded. In these applications, MSCs showed beneficial effects on graft versus host disease after hematopoietic stem cell transplantation and on Crohn's disease [84]. However, first results are promising and the *in vivo* application seems to be rather safe, as no serious

side effects have been reported. Nonetheless, randomized trials have to follow to confirm these first results.

5. Application Techniques in Orthopedics

To differentiate between favorable application strategies the aim of treatment is one important factor. As mentioned before, MSCs have the potential to rebuild injured tissue but also to secrete growth factors for enhancing tissue regeneration. Depending on the underlying pathology, the treatment strategies differ considerably. In one patient a large tissue defect has to be filled by means of tissue engineering, whereas in another the substantial defect is bridged with residual tissue of low quality and only an improvement of healing environment is indicated.

Besides the direct injection in the surrounding tissue, biomaterials are frequently used as carriers for drugs, bioactive molecules and cells. These materials have to fulfill some fundamental requirements. At first they have to be immune-compatible and nontoxic, whereas the degradation process must neither release toxic substances nor tissue-toxic concentrations of degradation products. For a later replacement with regenerated tissue, bio-degradable materials are important. The degradation velocity must be balanced as too fast and too slow are both detrimental. Beside these qualities, matrices formed from biomaterials must have distinct properties with regard to the desired kind of tissue. The prerequisite of mechanical strength, bioactivity, and kinetics of degradation and drug/cell release significantly varies between different repair tissues. Besides the used biomaterials themselves, the 3-dimensional structures of scaffolds have great influence on cell growth and differentiation. Scaffolds must be highly porous with interconnected pores of a diameter of at least 100 μm to allow ingrowth of cells and vessels [85]. Pore sizes between 100 and 400 μm are ideal.

Despite the tissue engineering of bone, for which various inorganic materials, such as hydroxyapatite, calcium phosphate, calcium carbonate, or glasses was tested, mainly organic biomaterials have been investigated for scaffold formation. These are either naturally derived, for example, collagen, fibrin, agarose, alginate, gelatin, silk or hyaluronic acid, or produced synthetically. Synthetically produced organic biomaterials are mainly polyhydroxyacids such as polyglycolides or polylactides. To control kinetics of degradation, recent studies were performed employing hydroxyl acid copolymers. Thus, it has been tried to adapt kinetics of degradation to those of tissue regeneration.

As these synthetic polymers often lack bioactivity, their surface was modified to alter cell adhesion, migration, differentiation, and proliferation in recent studies. Thus, they were coated or copolymerized with bioactive materials or functional groups were attached to the polymer chain before scaffold fabrication [86–88]. Apart from surface modifications with bioactive materials, scaffolds were coated directly with cytokines to control proliferation and differentiation of seeded cells [89]. Other authors describe the coating of scaffolds with genetic vectors to perform transfection of cells with different growth factors [90]. Biomaterials for tissue engineering can also carry drugs that prevent microbial colonization or control ingrowth of scaffolds into the surrounding tissue [91, 92].

5.1. Tendon Repair. Considering physiological properties of tendon tissue, an application technique via scaffolds with native extracellular matrix and the capability of cell seeding and adhesion would be ideal [93]. Based on this hypothesis, most of the current studies used scaffold application techniques. The few studies which favored direct application techniques injected the suspension of MSC into bone tunnels or on the bone surface before tendon refixation to improve tendon-to-bone healing [94, 95].

Scaffold application techniques for tendons can be divided into gel suspensions, 3D scaffolds of solid tissue, and hybrid techniques. Gel suspensions offer a perfect 3D filling of the defect, but the reduced stability in comparison to stable matrices may result in loss of gel at the repair site due to erosion. In a rabbit Achilles tendon model, Chong et al. [96] used a mixture of fibrin sealant and bone marrow-derived mesenchymal stem cells. The fibrin sealant was injected into the tendon and the repair site was additionally covered with the agent. Fibrin incorporates the advantages of a clinical use over years including FDA approval, bone marrow-derived mesenchymal stem cells remain viable in fibrin and published data indicate that fibrin itself has no effect on tendon healing [97]. In this study no differences between fibrin and fibrin with MSC could be shown histologically. In the early healing phase (3 weeks), significantly improved biomechanical properties were documented but not in subsequent time periods (6 and 12 weeks). In a rat rotator cuff model, Gulotta et al. [98] also used MSC in a fibrin sealant and placed it between tendon and bone before refixation of the tendon. In this acute tendon repair model they did not find any significant histological or biomechanical differences after 2 or 4 weeks, respectively. Noteworthy, the same group recently succeeded in enhancing tendon healing in the same rotator cuff model, applying transfected MSCs using the embryonic transcription factor MT1-MMP and the tendon transcription factor scleraxis [99, 100]. With a collagen gel, Awad et al. [101] presented a further gel-based application technique. They fixed a collagen gel with different concentrations of MSC to suture material and filled a defect in the rabbits' patellar tendon. After 12 and 26 weeks, significantly higher maximum stresses and moduli were documented compared to natural repair tissues. However, an adverse event was observed as there had been an increased number of intratendinous ossifications (28%). In comparison to the intact tendon only 25% of the ultimate load was reached with MSC. Regarding all groups, cell concentration had no significant influence on the outcome. This study group improved its application technique and presented a hybrid technique (MSC in a gel-collagen sponge composite) [102]. In the rabbit patellar tendon model, the biomechanical properties and cellular alignment were significantly improved in the MSC group after 12 weeks. A different matrix is presented by Omae et al. in *in vitro* and *in vivo* studies [103, 104]. Xenotendon slices with a thickness of 50 μm were decellularized and seeded with bone marrow stromal cells. The first results of the bundled construct in

a patellar tendon rat model showed a survival of the stromal cells in all layers. *In vivo* results with MSC have not been published yet but the approach is promising.

In conclusion, the application of MSC in tendon repair shows promising but inhomogenous results in animal models. Current *in vivo* data favor the culture of MSC into a tissue-engineered construct, with the advantage of primary stability and allowing the cells to produce their own extracellular matrix. But there is no consensus about the ideal carrier construct. Clinical data are not yet available for MSC application in tendon repair.

5.2. Cartilage. Besides autograft transplantation and autologous chondrocyte transplantation, current therapeutic concepts of cartilage defects include the recruitment of MSC. Drilling, abrasion, or microfracturing of the subchondral bone aims at the recruitment of MSC from the subchondral bone to stimulate the formation of cartilage repair tissue. In experimental and clinical studies of these standard techniques, a nonhyaline cartilage with high proportions of fibrous elements and inferior functionality has been found [105].

For autologous cartilage repair various two- and three-dimensional constructs are available. Most of the matrices consist of natural polysaccharides and proteins, such as alginate and collagen. Furthermore, synthetic polymers are also available for example, polyethylene glycol (PEG) or polylactic acid (PLA). Successful outcome of a stem cell-based cartilage tissue engineering also depends on the design of extracellular matrix for a proper differentiation of MSCs into chondrocytes [106]. The most important property, namely, mechanical stability, to provide appropriate cell-matrix interactions to stimulate tissue growth and capability of functional tissue growth. The ideal matrix has sufficient strength to protect the cells from axial load and shear forces, is highly adhesive to remain stable in the repair site and possesses enough porosity to allow nutrient and differentiation factors to diffuse through it. Currently, a large number of *in vitro* studies focus on the optimal three-dimensional matrix.

Increasingly innovative matrices are tested in *in vivo* animal models. For example, Shafiee et al. [107] performed MSC-based cartilage repair in a rabbit model with full-thickness cartilage defects. They used poly(vinyl alcohol)/polycaprolactone (PVA/PCL) nanofibers as matrix which showed a support of MSC proliferation and chondrogenic differentiation *in vitro*. The animals treated with MSC showed an improved healing of the defects compared with the untreated control. Tay et al. [108] used alginate-embedded MSC for the repair of focal cartilage defects in a rabbit model. They compared the macroscopic and histological results of MSC versus autologous chondrocyte transplantation 6 months postoperatively. MSCs had a similar effectiveness as chondrocyte transplantation, MSC even showed a significantly better macroscopic score. Both treatments resulted in superior tissue regeneration compared with untreated control defects. These promising results from the laboratory resulted in the first clinical studies about cartilage repair with support of MSC. The earliest data are case series of Wakitani et al. [109, 110]. They performed a bone marrow aspiration from the iliac crest and the MSC were expanded in culture. Four weeks later, the MSC were implanted using a collagen gel and the defect was additionally covered with a periosteal flap. The authors describe satisfying clinical and macroscopic results, but the small number of patients, the retrospective study design and the missing control has to be taken into consideration. Nejadnik et al. [111] performed a matched pair analysis of 36 patients in each group who underwent autologous cartilage transplantation or implantation of MSC. The postoperative followup after 24 months showed no significant difference of different functional knee scores between the groups.

In the treatment of osteochondral lesions, the group of Buda et al. [66, 112] published clinical results of lesions in the femur condyle and the talus. In the talus group, MSC were taken from the iliac crest and incubated with a hyaluronic acid membrane ($n = 25$) or collagen powder ($n = 23$) before implantation in the defect in a single step procedure. 48 patients were examined clinically and radiologically after an average of 29 months postoperatively. The clinical scores revealed a significant improvement compared to postoperative scores whereas in the MRI and histology of second-look arthroscopies none showed complete hyaline cartilage. In the 20 patients with MSC therapy of the femur condyle satisfactory clinical results (IKDC 90.4 points) were also reported after an average of 29 months postoperatively. The MRI showed a satisfactory integration of the graft in 80% of the patients. Instead of direct defect coverage, some groups describe a simple intra-articular injection of MSC [113], with the intention of the ability of homing of the MSC. Centeno et al. report about an injection in a patient with early osteoarthritis of the knee. In the MRI followup after 6 months, they revealed an increased cartilage volume compared to point of time before injection.

In summary, all applications for clinical use are based on very small case series. The MSC application technique was adopted from the clinical experience of autologous chondrocyte transplantation (fibrin, collagen gel, periosteal flap). Before a clinical use can be recommended, basic research to optimize application techniques, cell preparation, and concentration are essential [114]. With improved knowledge from basic studies further evaluation of the clinical potential of MSC application has to be performed in larger randomized controlled trials.

5.3. Bone. In bone, the main focus of regenerative medicine approaches lies on atrophic non union and replacement of lost bone tissue. Large bone defects are usually caused by trauma, infection, or tumors, as atrophic nonunion are usually caused by insufficient blood supply, interposition of soft tissue or consequence after infection. Current treatment strategies include autologous bone grafts from the iliac crest, which is actually the gold standard—and as salvage procedures—autologous fibula graft transfer and allogenic bone graft transplantation. However, all mentioned techniques show limitations, as bone supply is limited, autologous bone harvesting is accompanied with high rates of morbidity and allogenic transplantation inheres the risk of transmission of diseases or rejection [115, 116].

Table 1: Clinical applications of mesenchymal stem cells in bone regeneration.

Author	Diagnosis	Application	*n* patients	Results
		Treatment of nonunions		
Connolly et al. 1991 [49]	Atrophic pseudarthrosis	Percutaneous autologous bone marrow injection	20	Healing capacity comparable to autologous cancellous bone grafting
Garg et al. 1993 [50]	Nonunion in long bones	Percutaneous autologous bone marrow injection	20	17 out of 20 cases united in 5 months
Kettunen et al. 2002 [51]	Tibially delayed or non-union	Percutaneous autologous bone marrow injection	41	Appeared to be as effective as open techniques
Hernigou et al. 2005 [52]	Atrophic pseudarthrosis	Percutaneous autologous bone marrow injection	60	Application is effective and safe Positive correlation between number of progenitor cells and callus volume
Goel et al. 2005 [53]	Tibial non-union	Percutaneous autologous bone marrow injection	20	15 out of 20 patients showed bone union
		Treatment of osteonecrosis		
Hernigou and Beaujean 2002 [54]	Osteonecrosis femoral head	Injection of autologous bone marrow concentrate	116 (189 hips)	Very good results in early stages Injection of greater number of progenitor cells transplanted had better outcomes
Gangji et al. 2004 [55]	Osteonecrosis femoral head	Injection of autologous bone marrow concentrate	13 (18 hips)	Significant reduction of pain, progression and improvement of function
Hernigou et al. 2009 [56]	Osteonecrosis femoral head	Injection of autologous bone marrow concentrate	342 (534 hips)	High amount of progenitor cells as predictor for successful outcome
		Enhancing spinal fusions		
Neen et al. 2006 [57]	Spinal fusions	Autologous bone marrow aspirate on hydroxyapatite-collagen I-composite	50	Similar healing capacity as autologous cancellous bone grafting in posterolateral fusion Inferior results in interbody fusions
Gan et al. 2008 [58]	Spinal fusions	Bone marrow concentrate on tricalciumphosphate	41	After 34.5 months 95.1% cases showed good spinal fusion
		Filling bone cysts		
Wright et al. 2008 [59]	Simple bone cysts	Intralesional injection of autologous bone marrow aspirate	77	Inferior results compared to injection of methylprednisolone
Park et al. 2008 [60]	Simple bone cysts	Implantation of autologous bone marrow aspirate implanted in combination with either nonvital allogenic bone graft or injected with bone powder	20 (23 cysts)	Injection of bone marrow-bone powder mix is effective alternative to open treatment
Zamzam et al. 2009 [61]	Simple bone cysts	Percutaneous autologous bone marrow injection	28	Application is a safe and effective treatment
		Filling of bone defects		
Salama and Weissman 1978 [62]	Different bone defects	Xenograft with bone marrow aspirate	28	Results have been "most satisfactory"
Jäger et al. 2009 [63]	volumetric bone deficiencies	local autologous bone marrow/mesenchymal stem cell injection	10	May be a promising alternative to autogenous bone grafting
Marcacci et al. 2007 [64]	Large bone diaphysis defect	autologous MSCs were expanded in vitro and seeded on hydroxyapatite scaffolds	4	Followup up to 7 years after surgery, good integration of implant, no secondary fractures
		Various applications		
Hendrich et al. 2009 [65]	various bone healing disturbances	Bone marrow concentrate	101	Autogenous bone marrow concentrate application is safe
Giannini et al. 2009 [66]	Osteochondral talus defects	arthroscopic-assisted injection of autologous bone marrow aspirate	48	Functional improvement
Dallari et al. 2007 [67]	High tibial osteotomy	Lyophilized bone chips with platelets-enriched plasma with bone marrow aspirate	33	Lyophilized bone chips with platelets-enriched plasma with or without bone marrow aspirate enhance healing
Kitoh et al. 2009 [68]	femoral and tibial lengthenings	Application of MSC expanded *in vitro* with PRP	28 (51 osteotomies)	No enhancement of bone healing by MSC/PRP

In the last two decades, regenerative medicine approaches have been extensively studied to improve bone healing, or even generate functional bone tissue to substitute lost bone. Many *in vitro* studies were performed to investigate applicability of different stem cell types for bone regeneration. Here, promising capacity for differentiating towards bone cells, enhancing bone healing and vascularization could be proven for embryonic stem cells and different adult mesenchymal stem cells. However, due to the ethical and safety concerns mentioned above, only adult stem cells are presently taken into consideration for therapeutic applications [63]. Here, mesenchymal stem cells presently seem to be the most promising candidates for bone regeneration, due to their excellent osteogenic differentiation capacity [69].

In vitro trials found out that MSC strongly promote angiogenesis by paracrine factors after mechanical stimulation, as occurring during fracture healing [117], which makes MSC more interesting for bone regeneration. This paracrine enhancement of angiogenesis in bone regeneration could also be confirmed in animal models *in vivo* [118].

The capacity of mesenchymal stem cells for homing to injured tissues known from other fields was also demonstrated for fractures. Here, mesenchymal stem cells showed migration towards the fracture site after systemic application in a mouse model. The study further revealed that the cells enriched there and participated in fracture healing by paracrine induction of tissue healing, reduction of systemic and local inflammation and differentiating into bone cells [74]. However, the majority of the stem cells were trapped in the lungs after systemic application, thus making local application more practicable for bone regeneration [119].

Different groups achieved to compose small bone-like tissue constructs *in vitro*, by composing MSC with a variety of different biomaterials. Implanted into animals, several of these constructs survived *in vivo* [120]. However, researchers did not succeed in composing vital bone pieces in larger volumes, or even whole bones. This is due to the diffusion tract being larger than 200 μm. Beyond 200 μm, diffusion is not sufficient for providing cells with oxygen and nutrients. Therefore, functional vascularization is a prerequisite for survival of such solid tissues. Up to now, the problem of vascularization in tissue engineering is not yet solved, inhibiting the translation of tissue engineering methods into the clinic [43].

Nonetheless, regenerative medicine for bone healing has reached the patient in form of cell therapy approaches to treat localized bone defects or systemic diseases of the skeleton [39]. Here, autologous bone marrow or autologous mesenchymal stem cells was successfully implanted in a number of patients to enhance fracture/osteotomy healing, fill bone defects, treat pseudarthrosis, bone cysts, osteonecrosis, or enhance spinal fusion. Relevant clinical applications are summarized in Table 1.

6. Conclusions

Current data provides a number of interesting approaches to treat musculoskeletal pathologies with the support of mesenchymal stem cells. But considering the limited, partially only preclinical data we believe that a standardized clinical application will take at least an additional 5 to 10 years. In order to realize the full therapeutic potential of stem cells, a number of open questions has to be to be answered. Besides the necessity of establishing further data about native stem cell function and pathways, basic research in the understanding of native tendon, bone, and cartilage regeneration also has to be continued. Especially signal pathways have to be understood because single-MSC application might be insufficient or only partially sufficient without the adequate signal for inducing tissue regeneration. The regenerated tissue also has to provide the appropriate 3-dimensional structure including production of extracellular matrix and biomechanical behavior according to native tissue. Therefore, tissue engineering will play an important role in the next years. In the near future, an interdisciplinary approach with biologists, bioengineers, and clinicians will be essential to achieve the clinical application of mesenchymal stem cells.

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Review Article

Stem Cells and Gene Therapy for Cartilage Repair

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Cartilage defects represent a common problem in orthopaedic practice. Predisposing factors include traumas, inflammatory conditions, and biomechanics alterations. Conservative management of cartilage defects often fails, and patients with this lesions may need surgical intervention. Several treatment strategies have been proposed, although only surgery has been proved to be predictably effective. Usually, in focal cartilage defects without a stable fibrocartilaginous repair tissue formed, surgeons try to promote a natural fibrocartilaginous response by using marrow stimulating techniques, such as microfracture, abrasion arthroplasty, and Pridie drilling, with the aim of reducing swelling and pain and improving joint function of the patients. These procedures have demonstrated to be clinically useful and are usually considered as first-line treatment for focal cartilage defects. However, fibrocartilage presents inferior mechanical and biochemical properties compared to normal hyaline articular cartilage, characterized by poor organization, significant amounts of collagen type I, and an increased susceptibility to injury, which ultimately leads to premature osteoarthritis (OA). Therefore, the aim of future therapeutic strategies for articular cartilage regeneration is to obtain a hyaline-like cartilage repair tissue by transplantation of tissues or cells. Further studies are required to clarify the role of gene therapy and mesenchimal stem cells for management of cartilage lesions.

1. Introduction

Hyaline articular cartilage is a highly specialized tissue. The function of cartilage is to protect the bones of diarthrodial joints from friction, forces associated with load bearing and impact [1, 2]. The peculiar problem of this tissue is its durability. Once articular cartilage is injured or degenerated, it has very limited capacities for self-repair and regeneration. In partial thickness lesions, in whom the defect is completely contained within the articular cartilage, there is no involvement of the vasculature. Consequently, chondroprogenitor cells from marrow or blood cannot reach the damaged region to repair the lesion or contribute to the healing of the tissue. The most considerable consequence of cartilage avascularity is that articular chondrocytes are not able to migrate towards the lesion and to produce reparative matrix to fill the defect. As such, the defect is not repaired and remains permanently [1, 2].

Full thickness cartilage lesions result in the damage of the chondral layer and subchondral bone plate. The rupture of blood vessels promotes the formation of the hematoma at the injury site. In this condition, the repair response is promoted and the defect is filled with fibrocartilaginous tissue within weeks [1, 2].

Usually, in focal cartilage defects without a stable fibrocartilaginous repair tissue formed, surgeons try to promote a natural fibrocartilaginous response by using marrow stimulating techniques, such as microfracture, abrasion arthroplasty, and Pridie drilling with the aim of reducing swelling and pain and improving joint function of the patients. These procedures have demonstrated to be clinically useful and are

usually considered as first-line treatment for focal cartilage defects [3–5].

However, fibrocartilage presents inferior mechanical and biochemical properties compared to normal hyaline articular cartilage, characterized by poor organization, significant amounts of collagen type I, and an increased susceptibility to injury, which ultimately leads to premature osteoarthritis (OA).

Therefore, as outlined in the modern literature on the subject, the aim of future therapeutic strategies for articular cartilage regeneration is to obtain a hyaline-like cartilage repair tissue by transplantation of tissues or cells [2, 3, 6–8].

Tissue transplantation procedures such as periosteum, perichondrium, or osteochondral grafts have shown positive results for a limited number of patients, especially in the short term, but long-term clinical results are uncertain, with tissue availability for transplant that seems to be the major limitation, especially in large cartilage defects [2, 3, 6–8]. The autologous chondrocyte transplantation (ACT) procedure has been performed since 1987 in combination with a periosteal cover to treat chondral or osteochondral lesions of the knee, reporting good clinical results [9–11].

Recently, several authors improved this procedure embedding chondrocytes in a three-dimensional matrix before transplantation into cartilage defects [4, 12, 13].

Good results have also been obtained especially regarding clinical symptoms, such as pain relief and joint motion, but none of the current treatment options has proved the capacity to reproduce the biochemical properties of articular hyaline cartilage [3, 10, 14].

Moreover, in the last years, tissue engineering approaches have been investigated with the aim to produce cartilage grafts *in vitro* to facilitate regeneration of articular cartilage *in vivo*. While promising *in vitro* data have been obtained compared to current cartilage repair options, various problems remain unresolved for a successful repair associated with the formation of hyaline cartilage *in vivo* [2, 7, 15, 16].

2. Gene Therapy

The gene transfer to articular tissues was firstly described and performed by Evans et al., as a method to treat patient with rheumatoid arthritis [17, 18]. Initial successful experiments in several animal models using retroviral-mediated gene delivery promoted subsequent clinical trials to evaluate the safety and feasibility of using gene therapy for rheumatoid arthritis [17, 18]. The study was performed on 9 patients without any complications; all the nine participants tolerated the treatment and, in addition, in all the treated joints, intra-articular gene transfer and expression was observed [17, 18]. The relative success of these studies suggests that this new treatment option can be used in major articular disorders for which only unsatisfactory treatment options are currently available.

Nowadays research and recent results indicate that the design of a successful genetic treatment for cartilage repair and restoration includes a refined strategy of gene delivery that takes into account the complexities of treating this particular tissue.

For the purpose of cartilage repair, potentially useful complementary DNAs (cDNAs) include members of the transforming growth factor- (TGF-) β superfamily, including TGF-βs 1, 2, and 3, a number of bone morphogenetic proteins (BMPs), insulin-like growth factor- (IGF-) 1, fibroblast growth factors (FGFs), and epidermal growth factor (EGF).

Alternatively, to support production and maintenance of the proper hyaline cartilage matrix, delivery, and expression of cDNAs encoding specific extracellular matrix (ECM) components such as collagen type II, tenascin, or cartilage oligomeric matrix protein (COMP) may also be used [19].

Another class of biologics that may be useful in cartilage repair is represented by transcription factors that promote chondrogenesis or the maintenance of the chondrocyte phenotype. SOX9 and related transcription factors (i.e., LSOX5) and SOX6 have been identified as essential for chondrocyte differentiation and cartilage formation [20].

Signal transduction molecules, such as SMADs, are also known to be important regulators of chondrogenesis [21]. However, since these molecules function completely in the intracellular environment, gene transfer may represent the only way to harness these factors for repair, as they cannot be delivered in soluble form.

Other secreted proteins, such as indian hedgehog (IHH) or sonic hedgehog (SHH), play key roles in regulating chondrocyte hypertrophy [22] and could be beneficial for modulating the chondrocytic phenotype of grafted cells.

Prevention or treatment of cartilage loss may also require the inhibition of the activity of certain proinflammatory cytokines, such as interleukin- (IL-) 1 and tumor necrosis factor- (TNF-) α, as these are important mediators of cartilage matrix degradation and apoptosis after trauma and disease. Therefore, anti-inflammatory or immunmodulatory mediators, such as interleukin-1 receptor antagonist (IL-1Ra), soluble receptors for TNF (sTNFR) or IL-1 (sIL-1R), IL-4 or IL-10, inhibitors of matrix metalloproteinases, and others, may be administered to effectively reduce loss of repair cells and matrix [23].

Inhibitors of apoptosis or senescence, such as Bcl-2, Bcl-XL, hTERT, i(NOS) and others, may also be beneficially employed to maintain cell populations which are capable of favourable repair responses at the injury site [24, 25]. Different candidate cDNAs may also be administered in combination, especially when favouring complementary therapeutic responses. For example, the combined administration of an anabolic growth factor (e.g., IGF-1) together with an inhibitor of the catabolic action of inflammatory cytokines (i.e., IL-1Ra) has the potential both to control the matrix degradation and to allow partial restoration of the damaged cartilage matrix [26, 27].

There are two general modes of intra-articular gene delivery, a direct *in vivo* and an indirect *ex vivo* approach. The direct *in vivo* approach involves the application of the vector directly into the joint space, whereas the *ex vivo* approach involves the genetic modification of cells outside the body, followed by retransplantation of the modified cells into the body.

The choice of which gene transfer method as to be used depends on several considerations, including the gene to

be delivered, and the vector used. In general, *in vivo* and *ex vivo* delivery can be performed using adenovirus, herpes simplex virus, adenoassociated virus vectors, lentivirus, and nonviral vectors. Due to their inability to infect nondividing cells, retroviral vectors are more appropriate for *ex vivo* use. While *ex vivo* transfer methods are generally more invasive, expensive, and technically wearisome, they finally allow control of the transduced cells and safety testing prior to transplantation. *In vivo* approaches are simpler, cheaper, and less invasive, but these methods require the introduction of viruses directly into the body, which limits safety testing [28].

Towards the treatment of damaged articular cartilage, the three primary candidate cell types to target genetic modification are synovial lining cells, chondrocytes, and mesenchymal stem cells.

Direct intra-articular injection of a recombinant vector [29–31] represents the most straightforward strategy for gene delivery to diseased joints. Cartilage and synovium are the two primary tissues to be considered for this application.

Within articular cartilage, chondrocytes are present at a low density and are located at varying depths within the dense matrix. Due to this situation, it has not been possible to achieve an efficient genetic modification of chondrocytes *in situ* [32–35]. Conversely, gene delivery within the synovium tissue has resulted much more feasible since it is usually characterized by a thin lining of cells that covers all internal surfaces of the joint except that of cartilage. Also, because of its relatively large surface area, the synovium represents the predominant site of vector interaction. Both the implant of modified cells and direct intra-articular injection of vector promote the synthesis and release of therapeutic proteins into the joint space, which then bathe all available tissues, including cartilage.

Substantial progress has been made in defining the parameters that are critical for effective gene transfer to synovium and prolonged intra-articular expression by using different types of vectors in *ex vivo* and *in vivo* approaches. Through research conducted in the field of rheumatoid arthritis, the effectiveness of synovial gene transfer of various transgenes has been well documented [23]. *Ex vivo* gene delivery to joints has been taken into phase I clinical trial and shown to be feasible and safe in humans with rheumatoid arthritis [17, 36]. Data relevant to direct intra-articular gene delivery are beginning to emerge, although to date most of the work in this field has been focused towards the study and treatment of rheumatoid arthritis, mainly because of the potential of this approach in treating OA [37], and also to expand repair methods of focal cartilage defects [28, 38–40].

For example, encouraging results have been reported for adenovirally delivered IGF-1 or IL-1Ra using animal models for OA and localized cartilage injury [32, 41].

Through both direct and *ex vivo* gene transfer to synovium, it is possible to obtain biologically considerable levels of transgene expression while for delivery of certain growth factors, this approach is not compatible. In fact, it was observed that adenoviral mediated delivery of TGF-β1 or BMP-2 to the synovial lining determined osteophytes, cartilage degeneration, joint fibrosis, and significant swelling [42–45]. In the perspective of cartilage repair, these results suggest that synovial gene transfer may be more appropriate for the delivery of chondroprotective agents rather than strong anabolic transgenes with pleiotropic effects of their products. It has been shown that this property is common to many anti-inflammatory cytokines.

For the gene-based delivery of certain intracellular proteins or growth factors, it appears that a strategy based on increased localization of the transgenes with the gene products contained in the lesion of the cartilage may be more practical. To achieve this goal, the most direct approach may be represented by implantation into a defect of a three-dimensional matrix preloaded with a gene delivery vehicle, allowing infiltrating cells to acquire the vector and secrete the stimulating transgene products locally [37, 46].

In order to increase the healing of ligaments and bones, cartilage implants, activated genetically, have been designed [47–52]. For example, it has been seen that hydrated collagen-glycosaminoglycan matrices containing adenoviral vectors stimulate localized reporter gene expression *in vivo* for at least 21 days, after implantation into osteochondral defects localized in rabbit knees [50].

However, it is not known yet if this type of approach can promote an adequate biological response for repair due to the limited cell supply commonly present at the site of the cartilage lesion. To increase the graft cellularity, while preserving the feasibility of the procedure within one operative setting, autologous cells which are intraoperatively readily available, such as cells from bone marrow aspirates, could be mixed together with the genetically activated matrix. This genetically enhanced approach for tissue engineering would allow both the reduction of costs and execution time, while avoiding a significant effort for the *ex vivo* culture of cells [49, 50]. Nevertheless, the lack of control over gene transfer following implantation represents a limitation for their use.

Through the use of genetically modified chondrocytes, attempts have been made to further improve the quality of repaired tissue. Although chondrocytes have shown a certain resistance to transfection with plasmid DNA, it has been observed that some lipid-based formulations increase the efficiency of DNA uptake [53]. However, viral-based vectors are capable of producing far higher levels of transgene expression with enhanced persistence. It was found that transfection of monolayer-expanded chondrocytes with viral vectors such as Moloney Murine Leukemia Virus (MLV), lentivirus, adenovirus, and AAV occurs promptly. It has also been shown that adenoviral-mediated delivery of various transgenes, such as TGF-β1, BMP-2, IGF-1, or BMP-7, stimulates the production of a cartilage-specific matrix rich in proteoglycans and collagen type II and reduces tendency towards dedifferentiation [54–58].

It has been seen that following transfer of cDNA encoding matrix molecules, such as the collagen type II minigene, an increased extracellular matrix production occurs in human fetal chondrocytes [37].

Collagen type II expression of chondrocytes in three-dimensional culture *in vitro* has shown to be increased

following transduction with the transcription factor SOX-9 [59, 60], whereas overexpression of the transcription factor Runx-2 (Cbfa-1) promotes chondrocyte maturation and determines a hypertrophic phenotype, expressing high levels of collagen types II and X, alkaline phosphatase, and osteogenic marker genes [61, 62].

Since it has been found that chondrocyte biology can be positively influenced by genetic modification, attention of research has focused on their efficient delivery to cartilage defects. The delivery of genetically modified chondrocytes in suspension has represented the first approach. Several studies demonstrated that after engraftment onto cartilage explants *in vitro,* genetically modified chondrocytes have the ability of expressing transgene products at functional levels [63].

Compared to transplanted control cells, in these systems, genetic modification with IGF-1 [64], FGF-2 [65], or SOX9 [66] resulted in a considerable resurfacing and thicker tissue containing increased levels of proteoglycans and collagen type II [53]. Moreover, adenoviral-mediated IL-1Ra gene transfer to chondrocytes led to resistance to IL-1-induced proteoglycan degradation after engraftment [67].

Genetically modified chondrocytes have also been used as an alternative to delivery in suspension with the aim of enhancing tissue engineering procedures. This approach requires the transduction/transfection in monolayer cells subsequently seeded into a matrix for further transplantation into chondral or osteochondral lesions. Several transgenes including TGF β1, BMP-2, -4, -7, IGF-1, SOX9, among others have shown promising results in these three-dimensional culture systems due to their ability to maintain and stimulate the chondrogenic phenotype *in vitro* [16, 28, 40].

Initial studies highlighted that following genetic modifications with adenoviral, AAV, retroviral, or plasmid vectors, chondrocytes had the ability to efficiently express reporter genes in chondral and osteochondral lesions, and that when the genetically modified chondrocytes were seeded in three-dimensional matrices, transgene expression was extended over several weeks [68–71].

The results of efficacy studies demonstrating the effects of genetically modified chondrocytes in cartilage defects *in vivo* have just started to be reported.

In an *ex vivo* approach, adenovirally transduced chondrocytes expressing BMP-7 [54], integrated in a matrix of autogenous fibrin, were implanted into full thickness articular cartilage lesions in horses [54]. An enhanced tissue volume with increased production of a proteoglycan and collagen type II rich matrix was detected 4 weeks after surgery in the BMP-7-treated lesions, compared to control lesions treated with unrelated marker genes.

After 8 months, the mechanical features of the treated lesions as well as the levels of collagen type II and proteoglycan were however similar compared to the controls. This finding was attributed to some extent to the reduction of the number of allografted chondrocytes that persisted after 8 months in the lesions [54]. Nevertheless, these findings remain encouraging since they suggest that genetically modified chondrocytes can be used to increase a cartilage repair process in a large animal model.

3. Mesenchymal Stem Cells

Until recently, scientists have mainly focused on research involving two types of stem cells from humans and animals: nonembryonic "somatic" or "adult" stem cells and embryonic stem cells.

Embryonic stem cells are present in the blastocyst while adult stem cells are found in adult tissues. The normal turnover of organs that have a high intrinsic regenerative ability which include blood, skin, and intestinal epithelium is maintained by adult stem cells. Adult stem cells are generally unipotent or multipotent and they can be found in adults as well as adolescents and children.

Adult pluripotent stem cells are normally found in small numbers since they are very rare. However, they are present in several tissues including umbilical cord blood. The adult stem cells studied most extensively to date are the multipotent stem cells which are commonly referred to by their tissue origin (i.e., hematopoietic stem cells that differentiate into platelets erythrocytes, white blood cells, etc.) and the bone marrow stromal cells (also known as MSCs) [72, 73], which have the capacity to differentiate into connective tissue cells.

MSCs have the potential to differentiate into cells of connective tissue lineages [74] including bone [75–77], cartilage [77–79], ligament [80–82], muscle [78], fat [78, 83], and IVD [81, 82, 84]. It has been detected that these cells are also capable of differentiation along myogenic and neurogenic lineages, although these are not the common pathways used to prove multipotentiality of isolated MSCs.

Originally, adult MSCs were isolated from bone marrow by Pittenger et al. in 1999 [74], who demonstrated the potential for multilineage differentiation of these cells. Subsequently, a number of studies allowed to demonstrate the presence of stem cells in various adult tissues, including synovial fluid, articular cartilage, synovial membrane, periosteum, dermis, muscle, and adipose tissue.

To date, research has allowed for MSC-like progenitor cells isolation from trabecular bone, periosteum, synovium, skeletal muscle, adipose tissue, deciduous teeth [78, 80], and bone marrow [85].

Since no definitive markers of MSCs are available, a range of cell surface markers are normally used. These include immunopositivity for STRO-1, CD73, CD105, CD106, CD145, and CD166, associated with negative immunoreactivity for CD11b, CD31, CD34, CD45, and CD117.

Compared to the previous methods based on either density-gradient centrifugation or even simple plastic adherence, these markers allow to identify a more homogeneous population of cells.

Due to general heterogeneity of bone marrow cell populations, variable results can be obtained; however, MSCs have commonly shown the ability to differentiate along the adipogenic, chondrogenic, and osteogenic pathways. Research conducted by several authors suggests that MSCs are capable of differentiation to chondrocytes, osteoblasts, and nucleus pulposus (NP) cells of the IVD [84, 86–88]. However, since no definitive markers of NP cells are available,

a number of chondrocyte markers, with which they share a large phenotypic similarity, are typically used.

After Pittenger et al. [74] demonstrated the chondrogenic potential of MSCs, a number of approaches promoting MSC chondrogenesis [60] such as agarose [89] and alginate [90] gels have been described and more recently a range of tissue engineering biomaterials which allow or promote chondrogenesis have also been reported.

One of the most commonly used growth factors is TGF-b [74, 91], which has shown to promote chondrogenesis in addition to inhibiting adipogenic and osteogenic differentiation [92, 93].

Growth factors of the BMP family, principally BMP-7, and IGF-1 have also demonstrated the ability to promote chondrogenesis of MSCs and it has also been suggested that expansion of monolayer MSCs in medium containing FGF-2 induces chondrogenesis following transfer to a 3D culture environment [94–97].

However, with the *in vitro* differentiation approaches, the complexity of the signaling pathways involved in chondrogenesis represents one of the major problems, compared to the simplicity of culture systems.

Several studies have demonstrated the importance of cell-cell contact for MSC differentiation to either NP cells or chondrocytes [73] and pellet cultures mimic the mesenchymal compression that occurs during embryogenesis.

Similarly it is known that differentiation and matrix formation are induced by anabolic growth factors that exert their activity through a number of pathways, primarily the Smad and MAPKinase pathways [92, 96, 98].

The routine assessment of successful chondrogenesis is performed by the induction of SOX-9, which subsequently promotes the production of type II collagen as well as the enhanced expression of the PG aggrecan [99, 100]. Based on the similarities in the phenotype of NP cells of the IVD and articular chondrocytes [101], these markers are also used routinely to identify NP-like cells since no validated and highly specific NP marker genes are available. However, in standard *in vitro* culture systems MSC differentiation has shown to be unstable and it commonly leads to the expression of hypertrophic markers such as alkaline phosphatase and type X collagen [91, 102].

In terms of clinical application, the likelihood that chondrogenic differentiation may cause hypertrophy represents a problem since healthy surface and mid zone chondrocytes and NP cells do not express alkaline phosphatase nor type X collagen [103, 104].

This was demonstrated by Pelttari et al. [105] in pellet cultures comparing MSCs and chondrocytes, who reported that following implantation into SCID mice, the MSCs showed high levels of alkaline phosphatase and type X collagen expression which induced vascular invasion and calcification, while chondrocytes produced a cartilaginous matrix.

Improved differentiation or terminal differentiation inhibition may be induced with a number of growth factors. For example, it has been observed that the addition of PTHrP to TGF-b3-stimulated MSCs in poly-glycolic acid scaffolds also inhibits the expression of type X collagen of these cells and suppresses their terminal differentiation [106]. Also, the combination of TGF-b3 with BMP-2 has shown improved chondrogenic differentiation of MSCs compared to either growth factor alone or the combination of TGF-b3 with either BMP-4 or BMP-6 [107].

4. Conclusions

Hyaline articular cartilage is a highly specialized tissue. The peculiar problem of this tissue is that once articular cartilage is injured or degenerated, it has very limited capacities for self-repair and regeneration.

Usually, in focal cartilage defects without a stable fibrocartilaginous repair tissue formed, surgeons try to promote a natural fibrocartilaginous response by using marrow stimulating techniques, such as microfracture, abrasion arthroplasty, and Pridie drilling [108–111].

However, fibrocartilage presents inferior mechanical and biochemical properties compared to normal hyaline articular cartilage, characterized by poor organization, significant amounts of collagen type I, and an increased susceptibility to injury, which ultimately leads to premature OA [112–114].

The implementation of gene transfer techniques may allow to overcome the limitations of the current treatments for articular cartilage lesions. It has been shown that various approaches could be appropriate for an efficient transfer of exogenous cDNAs to cartilage lesions *in vivo* and for achieving sustained expression of the related gene products.

Initial efficacy studies have proven that gene-transfer techniques represent potent tools able to promote a significant biological response *in vivo*. However, the safety of gene transfer approaches for cartilage repair is also of particular importance because cartilage injuries are not life-threatening. Therefore the application of this technology for clinical use is strongly dependent on the use of safe and efficient delivery systems vectors and transgenes.

Although a number of animal models for OA and other types of arthritis are available, none of them allow to predict the equivalent disease in humans and most them are linked with problems. Further studies are required to establish the role of stem cells and gene therapy for cartilage repair.

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Review Article

Optimising Human Mesenchymal Stem Cell Numbers for Clinical Application: A Literature Review

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Adult mesenchymal stem cells (MSCs) are being investigated further for their use in stem cell therapies. However, as they are found in very low numbers in adult tissue, expansion *in vitro* is required to produce desired MSC numbers for clinical application. The need for effective cell-based therapies is increasing due to a rise in the ageing population, increasing the prevalence of musculoskeletal disorders. This review investigates how factors, age and gender of donor, as well as seeding density can affect MSC expansion. Age and gender of donor have received mixed results from studies, whereas seeding density studies have produced consistent results for numerous MSC sources, favouring lower seeding densities. Further research is required to reduce the risk of infection, loss of cell characterisation in cell culture, and making cell-based therapies more cost effective through creating rapid expansion of MSCs regardless of patient factors.

1. Introduction

Stem cells are an undifferentiated population, capable of endless self-renewal and differentiation down one or more lineages to produce specialised cell types [1]. Their ability to produce many cell types *in vitro* is one of the characteristics that has highlighted their importance for use in cell-based therapies. The earliest stem cell in the human body, the fertilised egg, is totipotent and has the capacity to differentiate into all cell types of the human body, as well as tissues to support the embryo. As the fertilised egg develops into cells of the human embryo, differentiation capacity down lineages become more limited [2].

Adult stem cells, also known as somatic stem cells, are located in many tissues of the human body and are required to restore normal function via repair and regeneration of tissues *in vivo*, for example, satellite cells in muscle tissue. They exist in a quiescent state until activated by mediators of injury or disease. Adult stem cells make up a small percentage of cells in a tissue and are surrounded by mature cells that have reached the end of the differentiation process and do not have the capacity to proliferate or differentiate [3]. The use of adult stem cells in clinical application is being investigated further due to restrictions and ethical issues surrounding the use of embryonic stem cells. Adult mesenchymal stems cells (MSCs) are suitable for use in clinical application as they have been found to adhere well to plastic, proliferate and differentiate well *in vitro*, and have suitable properties for transplantation: low immunogenic and high immunosuppressive properties, due to a low or absent HLA-2 marker on their cell surface [4, 5]. They have currently been shown to have the potential to enhance treatment of cardiovascular [6], neurological [7], and musculoskeletal disorders [8] by differentiating into cardiomyocytes and vascular cells, neuron and glial-like cells, and chondrocytes, respectively.

An early study by Friedenstein et al. reported that MSCs isolated from bone marrow, were found to be similar to fibroblasts [4, 9, 10]. These cells were clonogenic, adhered to plastic in culture, and replicated extensively *in vitro*. It has been demonstrated that these cells are multipotent, differentiating into osteoblasts, chondrocytes and adipocytes, when transplanted back *in vivo* [11, 12]. As well as the isolation of MSCs from bone marrow, other sources including adipose [13], skeletal muscle [14], synovium [15] and synovial fat

pad [16] have also been reported to contain MSCs capable of multilineage differentiation.

MSCs have shown great capabilities for use in clinical application; however, as they are found in very low numbers in adult tissue, expansion *in vitro* is required to reach the desired numbers before their use in clinical application. We have yet to develop a clear understanding of how to optimise MSC expansion efficiently, as many papers have reported discrepancies in results such as how age of donor, gender of donor, and seeding density can affect cell proliferation rate. Factors affecting optimisation of MSCs can be grouped into patient factors such as age and gender, as well as cell culturing factors such as seeding density used. This review article will look at how age, gender and seeding density affects the proliferation of adult mesenchymal stem cells.

2. Donor Age and MSC Proliferation

Mixed results have been published in existing literature about how age of donor affects mesenchymal stem cell expansion rate. Many have reported an inversely proportional relationship between proliferation rate and age; however, others have reported no relationship. Most literature has used BMSCs when looking at the effects of age, with a couple of papers using infrapatellar fat- pad-derived stem cells.

Khan et al. investigated the proliferation rate of synovial fat pad MSCs in two groups of patients with a mean age of 57 and 86, finding no significant difference in cell proliferation between both groups at five time points (days 2, 4, 6, 8, 10) [17]. This trend has also been supported by many papers investigating the effect of age using bone marrow-derived mesenchymal stem cells (BMSCs). Phinney et al. found up to 12-fold differences between patients when investigating growth properties of BMSCs from 17 healthy patients aged 19–45 years old; however, this difference showed no correlation with age of donor ($P < 0.05$) [18]. Both Suva et al. and Scharstuhl et al. extracted BMSCs from the neck and shaft of femur, respectively, at the time of hip arthroplasty [19, 20]. Suva et al. reported variable results for time required to reach the first passage, exponential cell growth, doubling time, and maximal cell amplification, but again none of these variations were found to be due to age-related differences of donors. Similarly with a sample size of 98, Scharstuhl et al. also reported that proliferative capacity is maintained with ageing after correlating proliferation with age.

On the other hand, Baxter et al. reported a severely reduced proliferative capacity with slower growth rate in a group of 59–75 years old patients compared to 0–18 years old [21]. This was supported by other studies that found that doubling time was almost 2-fold longer in older patients compared to younger [22, 23]. Culturing BMSCs over 4 months from "young" (7–18 years old), "adult" (19–40 years old), and "aged" (>40 years old) patients extracted from the posterior iliac crest, Stolzing et al. found differences between proliferation rate from week 5 in culture, describing that the proliferation rate of the "aged" BMSCs began to decrease and the growth curve started to plateau [24]. On the other hand, BMSCs from "adult" patients continued to increase in proliferation rate throughout the whole 4 months in culture. The "young" group of BMSCs were only investigated over 10 population doublings, where they also displayed a pattern of increasing proliferation rate with time. These findings were also supported by Dexheimer et al., who found a significant age-related decline in proliferation rate in BMSCs from older compared to younger patients [25]. Clonal expandability also decreased with increasing age with cells from an 80-year-old patient producing half the number of clones of that of BMSCs from a 20-year-old.

Interestingly, whilst investigating the effect of age on MSC proliferation from synovial fat pad tissue, one study explored this relationship at eight different seeding densities: 50, 250, 500, 1000, 2500, 5000, 750, and 10000 cells/cm^2 [26]. Extremely varied results were found, with five seeding densities (50, 250, 500, 5000, 7500 cells/cm^2) showing that there was an age-related decline in population doublings, whereas 10,000 cells/cm^2 showed an age-related increase in population doublings and two densities (2500 and 1000 cells/cm^2), showed no correlation with age. Varied results even between the same set of cells at different seeding densities shows that properties of MSCs and how they are altered are not properly understood. It also shows that proliferation can be affected by many factors, in which more research needs to go into providing knowledge about how MSCs are affected by a range of factors.

It is not known whether ageing of MSCs is due to factors inside the cells or factors in the surrounding tissue. It has been found that reduced synthesis of proteoglycans and glycosaminoglycans in the surrounding tissue results in a reduced proliferation and viability of MSCs *in vivo* [27]. Also, the production of advanced glycosylated end products (AGEs) inhibit proliferation of MSCs by activating apoptosis and reactive oxygen species (ROS) production [28]. In comparison, Zhou et al. suggested that intrinsic factors may be responsible for age-related changes in BMSCs [22]. He found that there was a 4-fold increase in the number of MSCs that were positive for senescence-associated β-galactosidase (SA- β-gal) and increased expression of p53 and its pathways genes (p21 and BAX) that may be responsible for mediating reduced proliferation potential. It has also been suggested that the expression of p16INK4a, an inhibitor of CDK4 and CDK6 which promote proliferation, increases with age [29]. MSCs from older patients were also found to be more apoptotic as physiological effects of ageing on MSCs induce senescence [22].

The effect of ageing on the properties of MSC remains controversial with inconsistent results, even when using MSCs from the same source type and patient. It is important to investigate how age affects the proliferation rate of mesenchymal stem cells to provide knowledge as to whether stem cell therapies can be achieved via autologous repair for older patients. This is a question which is becoming of great importance as with an ageing population, the prevalence of musculoskeletal disorders is increasing. Some studies have also shown that a higher proliferation rate is related to stronger osteogenic, chondrogenic, and adipogenic differentiation potential, crucial for repair of damaged or diseased musculoskeletal tissue [25]. The variable results could be due to

studies having a restricted age range due to the patient cohort available, studies having different age ranges for "young" and "old" categories, or not having a large enough sample size for young patients as quite a few studies use BMSCs extracted during hip arthroplasties, which on average occur more frequently in the older population. The variable results between studies call for more work on this subject to be undertaken to get a clear idea of how age of donor affects MSC proliferation rate. In particular, different sources of MSCs should be investigated and perhaps, if ageing is found to affect MSC expansion, then one source may be less affected and more suited to cell based therapies for the older population.

3. Gender and MSC Proliferation

There is little literature on the effects of gender on MSC proliferation potential, as the majority of papers group results from male and female patients together. MSC culturing techniques, such as duration and concentration of collagenase, and best harvesting site for maximal yield vary between males and females [30, 31]. Faustini et al. using adipose-derived stem cells (ADSCs) discovered that ADSC underwent most effective digestion when adipose tissue was incubated with 0.2% collagenase for 1 hour for males, whereas overnight digestion was more effective for tissue from females [31]. Females having a significantly higher yield of MSCs than males were also reported, with females having on average 2.05 ± 1.46 log-million cells compared to 1.44 ± 1.62 log-million cells from males. This, along with other knowledge that stem cells have estrogen and androgen receptors, suggests that there can be gender differences in regards to MSC proliferation. The higher levels of estrogens in females and androgens in males are thought to be responsible for the differences in male and female MSCs [30]. Most studies suggest that androgens have an inhibitory effect on the function of stem cells [32], whereas estrogens have an excitatory role controlling levels of cytokines and growth factor production in MSCs [33]. Estrogens have also been found to upregulate the expression of receptors on embryonic stem cells (ERa and ERb), increasing the production of 17b- estradiol which activates MAPK and cyclin-dependant kinases (cdk), which are intermediates in the cell cycle [34].

Despite suggestions and findings in some studies, Dexheimer et al. found no gender differences in MSC frequency and expansion of nonclonal MSC populations from bone marrow [25]. As gender could be a factor of influence for reaching desired cell numbers, research investigating the effect of gender on the properties of MSCs needs to be undertaken. This will help to make extraction and expansion of MSCs more efficient for all patients undergoing cell-based therapies as it can be known to extract more tissue from males than women, if females do have a higher yield as Faustini et al. reported. Also, more work should investigate the preferred isolation and culturing techniques for MSCs from male and female patients to ensure that the maximum yield of MSCs is extracted from the tissue source as possible.

4. Seeding Density and MSC Proliferation

Although limited, the literature suggests that seeding density does have an effect on cell proliferation rate. Both et al. found that BMSCs seeded at lower densities had a faster proliferation than higher densities, with MSCs at 100 cells/cm^2 reaching their target of 200 million cells, 4.1 days faster than cells that were seeded at a higher density (5000 cells/cm^2) [35]. Further decrease in seeding density below 100 cell/cm^2, also showed a further increase in proliferation rate. A similar relationship was found by Lode et al., when investigating the effect of seeding density on three-dimensional scaffolds [36]. They found that the highest seeding density of 1×10^6 cell/cm^2 resulted in a minimal increase in cell number compared to the lowest seeding density, which had a large increase in cell number. Extremely low densities (0.5–12 cells/cm^2) showed the size of single-cell-derived colonies that represent cell number and hence proliferation rate, to be inversely proportional to seeding density [37]. Another study looking at four different seeding densities for BMSCs also had results that were consistent with the previous findings. After 10 days in culture, BMSCs seeded at 2500, 250, 25, and 2.5 cells/cm^2 had mean results of 2.72 ± 0.48, 4.8 ± 0.42, 6.7 ± 0.53, and 7.6 ± 0.97 population doublings, showing that cells at a lower density have a faster proliferation rate than those of a higher density. Significant gain in population doublings was seen between 2500 and 250, as well as 250 and 25 ($P < 0.05$). On the other hand, although more population doublings occurred at lower densities, there was no significant difference between using 25 and 2.5 cell/cm^2. They also showed that seeding density did not affect cell characterisation as all cells had the same cell surface marker characterisation profiles [38]. This trend has been shown to occur in MSCs from various tissue locations not only bone marrow, confirming that seeding density does have a significant effect on proliferation rate. A study was carried out by Mochizuki et al. exploring the optimum seeding density for bone marrow-, synovium-, periosteum-, adipose-, and skeletal muscle-derived mesenchymal stem cells. Measuring cell growth and colony formation using crystal violet staining, they found that all MSCs had a greater colony size at lower seeding densities compared to higher; for example, MSCs derived from synovial tissue showed smaller colony size or cells became indistinct after 14 days when seeded at 10^5 and 10^6 cells/60 cm^2, compared to seeding densities of 10^3 or 10^4 cells/60 cm^2. Results showed that all sources had an optimum seeding density of 10^3 or 10^4 cells/60 cm^{2}or $10^3/10^4$ cells per cm^2 for BMSCs. In addition similar trends were seen when cells were seeded at 50 cells/cm^2 producing a higher cumulative number of cells than at 5,000 cells/cm^2. They also suggested that seeding density and total cell doubling times can affect senescence of cells. Synovial fat-pad derived MSCs observed results that were consistent with those from BMSCs [39].

The lower growth rate of cells seeded at higher densities could be due to contact inhibition. Higher growth rates at lower densities have been explained by the presence of small and agranular cells, also referred to as recycling stem cells, in the lag phase, which gave rise to large cells during the log

phase of exponential growth [37]. The log phase has been found to last for a longer duration in cells seeded at lower densities, and hence more population doublings occur due to a longer exponential growth phase [40]. Higher growth potential at lower seeding densities may also be due to more availability of nutrients per cell.

Finding the optimum seeding density for maximal expansion is useful in both laboratory investigations as well as potential clinical applications as the cell culturing procedure can be less time consuming, decreasing the risk of cell culture contamination, infection or loss of characteristics in cell culture, in addition to making the process more cost effective. These studies show that rapid expansion to reach a sufficient number of cells for clinical applications can be achieved by using lower seeding densities. As numerous papers have found that MSCs from a range of sources all have a faster proliferation rate/population doublings at lower seeding densities, if consistent relationships are found between age of donor and proliferation rate or gender and proliferation rate, then perhaps seeding density can be used to compensate for and be used to speed up expansion of MSCs for clinical application.

This review has looked at how some factors such as age and gender of donor as well as seeding density can affect MSC expansion for clinical application. Whilst studies disagree on the effects of ageing on MSCs, more work should be carried out to explore the effect of age on numerous MSC sources in the hope that a consistent relationship can be found. Identification of relationships will enable cell culturing techniques to be adapted for expansion of MSCs from particular age groups of patients, without increased risk of infection in cell culture by minimising time spent in culture. Gender is also a factor that should be investigated fully, as exposure to different concentrations of hormones may alter properties of MSCs. Research of how age and gender affects MSC proliferation should make sure that apart from the factor being investigated patients are all matched by gender, age, social factors, medical history, and chronic illness to ensure that all results are not due to potential confounders. In comparison, seeding density has shown consistent results over numerous studies as well as for numerous MSC sources. As the relationship between seeding density and proliferation rate of MSCs has been found, once age and gender relationships are also investigated, seeding density could perhaps be used to compensate for the effect of patient factors to result in rapid expansion of MSCs.

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Review Article

Stem Cell Applications in Tendon Disorders: A Clinical Perspective

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Tendon injuries are a common cause of morbidity and a significant health burden on society. Tendons are structural tissues connecting muscle to bone and are prone to tearing and tendinopathy, an overuse or degenerative condition that is characterized by failed healing and cellular depletion. Current treatments, for tendon tear are conservative, surgical repair or surgical scaffold reconstruction. Tendinopathy is treated by exercises, injection therapies, shock wave treatments or surgical tendon debridement. However, tendons usually heal with fibrosis and scar tissue, which has suboptimal tensile strength and is prone to reinjury, resulting in lifestyle changes with activity restriction. Preclinical studies show that cell therapies have the potential to regenerate rather than repair tendon tissue, a process termed tenogenesis. A number of different cell lines, with varying degrees of differentiation, have being evaluated including stem cells, tendon derived cells and dermal fibroblasts. Even though cellular therapies offer some potential in treating tendon disorders, there have been few published clinical trials to determine the ideal cell source, the number of cells to administer, or the optimal bioscaffold for clinical use.

1. Tendon Pathophysiology

Tendons are hypocellular, collagenous connective tissues, which are integral to the function of the musculoskeletal system. Tendons connect bone to muscle and are essential for transmitting forces to produce joint movement; hence, tendon injury is a major cause of population morbidity. For example, in the USA there are more than fifty thousand rotator cuff tendon repairs performed annually [1]. Healthy tendon has great tensile strength due to the high proportion of type I collagen (>90% of total collagen) which is arranged in a hierarchical structure [2]. After injury, the thinner type III collagen (usually <1%) is found and has the property of rapidly forming crosslinks to stabilize the injury [3, 4]. Tendon tissues are poorly vascularized and predominantly utilize anaerobic energy systems resulting in poor healing potential after acute or overuse injury [5, 6]. Mesenchymal stem cells have been identified within tendons, but currently no candidate gene transcription factor, promoting differentiation towards a tendon lineage, has been isolated. Tendon progenitors and tenoblasts are immature, proliferative cells and are the precursors to the terminally differentiated tenocytes, which lay down collagen within the extracellular matrix (ECM). Tendon specific growth factors and cell markers are at this point unknown.

Following acute tendon injury, five overlapping healing phases have been identified, in a process that lasts up to 10 weeks in healthy tendons. However, the resulting tendon is thickened, fibrotic, and less resistant to tensile stress than the preinjury state. Surgical repair or scaffold reconstruction is considered if there is poor quality tissue present, or in situations where the normal healing processes cannot occur, such as unstable apposition of the free ends. In clinical situations where surgical repair is technically difficult, or has too many complications, including a significant re-injury rate, then scaffold reconstruction is the preferred surgical treatment. There are three sources for reconstructive graft, namely, autologous tendon (e.g., patella, hamstring, or palmaris longus), tendon allografts, or synthetic acellular engineered scaffolds. These reconstructive procedures have

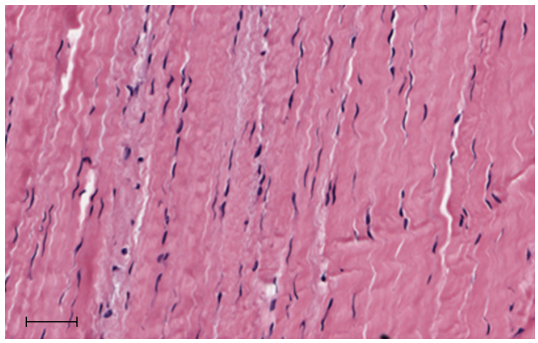

Figure 1: Normal Tendon. Note the relative paucity of cells.

recognized complications including donor site morbidity in autografts, potential immune rejection and infection transmission in allografts, and possible delayed implant failure in synthetic grafts. Hence, in injury resulting in tendon discontinuity, there is a clinical need for improved tissue-engineered scaffolds [7–9].

Tendons are also prone to overuse pathology which is associated with tenocyte depletion, microscopic collagen breakdown, and failed healing [10, 11]. This results in pain and altered function and contributes to tearing at lower strain thresholds. This process is defined as tendinopathy, which includes both tendinosis and tendinitis [12]. Tendinopathy demonstrates heterogeneous histological features with the presence of nontendon cell lines, such as fibroblasts, myofibroblasts, adipose, chondroid, and osteoid cells. There is an increase in ground substance with type III collagen (up to 30%), absence of inflammation, and marked reduction in the number of healthy tenocytes [10, 13]. Most tendons in the body can be affected, but the more disabling tendinopathies relate to the major joints such as the rotator cuff of the shoulder, the gluteal tendons of the hip ("greater trochanteric bursitis"), the common extensor tendons of the elbow ("tennis elbow"), and the Achilles tendon of the ankle. Initial treatment of tendinopathy is always conservative and is usually prolonged. There is reasonable evidence that exercise rehabilitation is beneficial, but limited evidence of efficacy for any of the other nonoperative treatments including platelet rich plasma injections which are purported to introduce autologous growth factors [14–16]. Surgical tendon debridement is sometimes undertaken for refractory cases, but this is expensive, disabling and the success is only modest [14]. Therefore, improved therapies for tendinopathy are required (Figures 1 and 2).

2. Cell Therapies

Preclinical studies have shown the potential for cellular therapies to increase tenocyte numbers and regenerate rather than repair tendon tissue. In the cellular treatment of tendon disorders, a small number of phase 1 and 2 clinical trials are being currently undertaken or have been completed. These trials have assessed the safety and efficacy of differing cell lines with varying degrees of cell potency, to treat tendinopathy.

2.1. Mesenchymal Stem Cells. Mesenchymal stem cells have the properties of proliferation and differentiation into mesenchymal tissue progenitors and are characterized by specific cell surface markers, adhesion molecules, growth factors, and ECM molecules [17]. MSCs can be isolated from a variety of tissues including; bone marrow, adipose tissue, the ACL, and tendon tissue [18–20].

MSCs can regenerate connective tissues, but there is increasing evidence that the mechanism of action may not be due to direct engraftment or differentiation [21]. MSCs secrete a variety of soluble autocrine and paracrine growth factors, which recruit MSCs, promote cell survival, and enhance the proliferation of endogenous connective tissue cells. These growth factors stimulate mitosis in tissue progenitors, induce angiogenesis, and reduce apoptosis [21–23].

MSCs are immune privileged which are thought to be due to their lack of MHC-II expression, disruption of T cell rejection mechanisms and secretion of anti-inflammatory mediators such as prostaglandins and interleukin-10 [24]. The use of allogeneic MSCs permits more efficient harvesting and expansion, but has the disadvantage of potential transmission of viral or prion vectors. Allogeneic MSCs can be used "off the shelf" in emergency situations, as they are always available (cryopreserved) and not rejected by host immune mechanisms. However, once differentiated, the evidence regarding persisting immune-privileged properties is inconclusive. MHC-II antigens can still be detected intracellularly by western blotting, even though they are not expressed on the cell surface [25]. Toma showed that a limited number of human MSCs persisted after differentiation into cardiomyocytes, after engraftment in a murine heart [26]. However, in contrast to Toma's findings, Huang demonstrated that in vitro differentiation of rat autologous and allogeneic MSCs, into myogenic lineages, reduced MHC I and increased MHC-II expression [27]. After 5 weeks only, autologous cells were present.

In preclinical animal models, both tendon laceration/defects and collagenase-induced tendinopathy are commonly studied. Chong et al. showed that intratendinous allogeneic MSCs, implanted in lacerated and sutured rabbit Achilles tendons, improved (accelerated) the histological and biomechanical parameters in the early stages of tendon healing [28]. In collagen gel scaffolds seeded in vitro and then implanted in rabbit tendons, ectopic calcification (due to osteogenesis) was found in up to 28% of cases, irrespective of the cell seeding density [29]. In a follow-up study, the authors noted that alkaline phosphatase activity was elevated around the sutures but only when the cells were in a 3D construct and not when in a monolayer [30]. The authors concluded that the osteoblastic proliferation was due to in vitro factors. Butler et al. advocated lower seeding density, with end posts rather than sutures and augmentation of the gel with type I collagen sponge and his group produced bioscaffolds with improved repair stiffness and improved force to failure [31]. No ectopic calcification was produced with this technique. Ouyang et al. demonstrated that PLGA scaffolds, seeded with allogeneic MSCs, repaired 1 cm defects in rabbit Achilles tendons with improved tensile stiffness

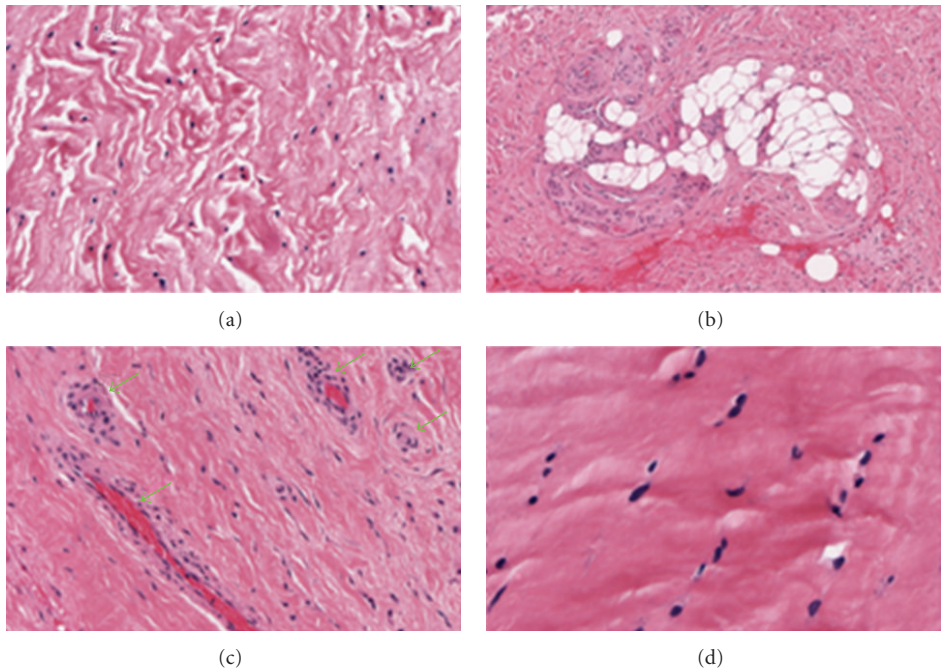

Figure 2: Tendinopathy in Rotator Cuff Tendons (a) Fibre disruption (b) Adipose tissue deposition (c) Vascular hyperplasia (d) Rounding of nuclei (Courtesy of University of Western Australia).

compared to acellular scaffolds. However, the tendons with tissue engineered bioscaffolds only had 62% of the tensile stiffness compared to surgically repaired control tendons at 12 weeks [32].

In rabbit bone-patellar-bone ACL autografts, Soon et al. showed that autologous bone marrow (bm) MSCs improved osteointegration of the bone anchors compared to controls. However, Young's modules and graft stiffness were reduced [33]. Synovium-derived stem cells have also been shown to improve osteointegration in ACL tendon-bone healing [34, 35]. Ouyang et al. fabricated a bone marrow stromal cell sheet which was assembled on a poly l-lactide (PLLA) scaffold and produced an engineered ligament which was largely type I collagen [36]. The MSC that incorporated PLLA scaffold was stronger and more functional compared to acellular controls.

Current ACL reconstructive practice generally utilizes tendon auto or allografts that undergo a prolonged remodeling and revascularization process. Wei et al. transfected bmMSCs with an adenoviral vector expressing TGF-β1/VEGF165, which were then implanted into rabbit tendon ACL scaffolds [37]. The treated tendons demonstrated accelerated remodeling, angiogenesis, and improved mechanical properties compared to controls.

In an in vivo collagenase-induced tendinopathy study, Lacitignola et al. demonstrated that both autologous bmMSCs and bone marrow mononuclear cells (bmMNCs) could be injected intratendinously into equine tendons, and both produced effective tendon regeneration [38]. Similarly, Crovace et al. demonstrated that intralesional MSCs regenerated more type I collagen than control tendons, which had more type III collagen [39]. No calcification or ectopic tissue has been reported by serial ultrasound or at autopsy on these or a number of similar equine tendinopathy studies [30, 40].

MSCs are now used as a therapeutic intervention in the equine thoroughbred industry to treat flexor digitorum superficialis (FDS) tendinopathy. This injury is often career-ending and has of a recurrence rate of 56% with conventional treatments [41]. Pacini et al. treated 11 horses with FDS tendinopathy, with targeted intralesional injection of MSCs and 9 horses recovered [42]. Allogeneic equine adipose MSCs have been successfully used to treat 14 out of 16 horses with FDS tendinopathy without complication [43]. In a controlled trial Smith et al. demonstrated that intratendinous injection of 10 million autologous bmMSCs resulted in statistically significant improvements in tendon cross-sectional area, cellularity, crimp pattern, and DNA content compared to controls [44]. Currently, over 1800 thoroughbred horses have received therapeutic autologous MSCs for tendinopathy, and the recurrence rate is 27% (http://www.vetcell.com/), whereas the quoted recurrence rate with conventional, noncell-based treatment is 56% ($P < 0.05$). There have been no reported cases of ectopic tissue production detected on serial ultrasounds. Twelve horses have now undergone postmortems (17 tendons), which have revealed good healing with minimal inflammatory cells, with crimped organized collagen fibers and no ectopic or neoplastic tissues [45].

The author of this review paper is currently undertaking a phase 1 trial in the use of allogeneic mesenchymal stromal cells in the treatment of human chronic (refractory) Achilles tendinopathy (Figures 3 and 4).

2.2. Embryonic Stem Cells. Embryonic stem cells (ESCs) are pluripotent cells with greater plasticity and proliferative

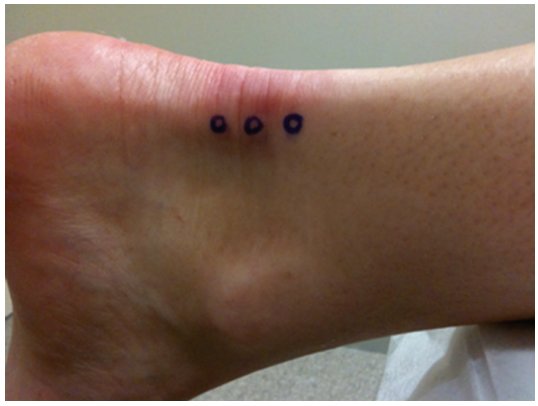

Figure 3: Injection sites for Achilles tendon.

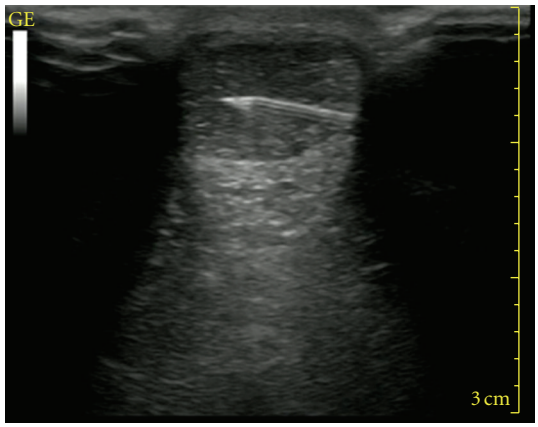

Figure 4: Ultrasonographically guided intratendinous Achilles injection.

capacity compared to adult MSCs. This means that they can provide an unlimited supply of MSCs and connective tissue progenitors. MSCs do improve tendon architecture, but have not induced the degree of tendon regeneration that is seen in injured fetal tendon [46]. A disadvantage of ESCs is their capacity to form teratomas. Chen et al. were the first to show that tendon regeneration could be achieved by in vitro differentiation into MSCs and then tenocytes [47]. These researchers used a xenograft model with human ESCs, which were differentiated into MSCs, then seeded in a fibrin scaffold before being implanted into a rat patellar tendon model. The hESC-MSCs had much better structural and mechanical properties than did the controls. The hESC-MSCs remained viable at the tendon wound site for at least four weeks and secreted human fetal tendon-specific matrix components and differentiation factors, which then activated the endogenous regeneration process in tendons. No ectopic tissue or teratomas were reported in this study, but the authors state that calcification was noted (unreported) in some of their other cases of patellar tendon fibrin ESC scaffolds. The authors concluded that improved differentiation techniques were required for ESCs for use in bioscaffolds for tendon repair. In a blinded placebo-controlled randomized trial of ESCs in equine collagenase-induced tendinopathy, intratendon injections of undifferentiated ESCs were shown to improve tissue architecture, tendon size, and tendon linear fiber pattern [48]. The eight horses were followed up with ultrasound and MRI scans, and no calcification or teratoma production was noted.

Induced pluripotent stem cells (iPS cells) are formed by reprogramming a nonpluripotent somatic cell, such as dermal fibroblasts using transfection of stem cell genes such as, c-myc, sox-2, oct-4, and klf-4. This avoids the ethical issues relating to embryonic stem cells but currently there are no clinical trials using iPS cells or ESCs in tendon research.

2.3. Tendon-Derived Cells. Until recently, little was known about the characteristics of tendon cells and their precursors. In 2007, Bi et al. isolated a rare cell population from an ECM niche and demonstrated clonogenicity, self-renewal, and multipotent differentiation capacity [20]. The cells in the population showed heterogeneity in these properties and so were referred to as tendon stem/progenitor cells (TSPCs). These cells reside in a niche, which includes biglycan (Bgn) and fibromodulin (Fmod), which in turn controls the fate of TSPCs by modulating BMP activity. Lower levels of Bgn and Fmod in the ECM are associated with osteogenesis, which can be found in tendinopathy [20]. No tendon-specific marker was identified in TSPCs, but compared to bone marrow MSCs, they highly expressed the tendon-related factors Scx, COMP, and Tenascin-C. When injected into mice, TSPCs were more likely to form tendon than bmMSCs, which preferentially formed bone. Tempfer et al. biopsied human rotator cuff tendons and isolated cells expressing both scleraxis and CD133, which is a marker of endothelial and hematopoietic stem cells [49]. The authors suggested that these were perivascular tendon cells in a vascular tendon niche, which had been activated for tendon repair. Further characterization of these cells is required to establish stem cell characteristics. Currently, there are no published trials on the use of tendon-derived stem/progenitor cells (TSPCs) in tendon engineering.

Autologous tenocytes can be harvested and expanded, prior to reimplantation. In 2002, Cao et al. seeded PLGA scaffolds with autologous tenocytes and successfully repaired hen flexor digitorum profundus tendon defects [50]. The cell-seeded scaffolds had aligned collagen, which had 83% of normal tendon strength, whereas the unseeded scaffolds only had 9% of normal strength. Similarly, autologous tenocytes were seeded on both porcine small intestine submucosa and type I/III collage bioscaffold (ACI-Maix), to repair rabbit rotator cuff models [51]. In a randomized controlled trial of rabbit collagenase tendinopathy model, Chen et al. showed that autologous tenocytes (either from tendon or epitendineum tissue) improved tendon remodeling, histological outcomes, collagen content, and tensile strength [52]. The autologous tenocytes that improved type I collagen expression, did not affect type III collagen and secreted protein rich in cysteine (SPARC) expression. In a phase I/II clinical trial of expanded autologous tenocytes, in 25 subjects with lateral epicondylitis [53], demonstrated improved grip strength, reduced pain, and a reduced Quick

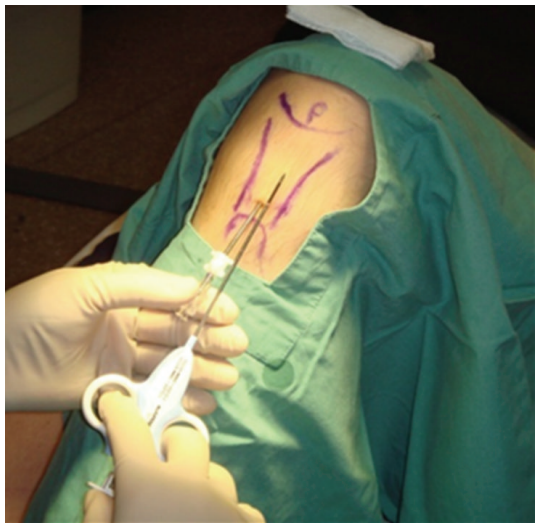

Figure 5: Trucut biopsy of patella tendon for tenocyte harvesting (Courtesy of M Zheng).

DASH Score over a six-month followup (*in press*). No ectopic tissue, tumors, infection, or other complications were reported. Autologous tenocyte implantation (ATI) is currently available in Australia, and over 100 procedures have been undertaken with no reported complication (*personal communication*). Tendon harvesting is usually performed with a Trucut needle, and the donor site is typically the patellar tendon, and ultrasound guidance is recommended. In athletes who play weigh-bearing sports, a miniopen biopsy of the palmaris longus tendon of the wrist is the preferred tendon donor site. These biopsies do require technical skill and are mildly invasive. Currently, in the Netherlands, a registered double-blind randomized controlled trial is being undertaken to assess the efficacy of ATI in 90 subjects with Achilles tendinopathy (Figure 5).

2.4. Dermal Fibroblasts. Dermal fibroblasts (DFbs) have been used in tissue engineering due to their abundant supply, ease of harvesting, and reprogrammability. They have multidifferentiation potential and have been shown to develop into brain, glia, muscle, and adipose lineages [17]. In vitro experiments have shown promise in tendon engineering [54, 55]. A concern regarding the use of DFbs in tendon engineering is the production of scar tissue. In a controlled trial, Deng et al. showed the importance of applying tatic mechanical strain on PGA constructs seeded with dermal fibroblasts (DFbs). After 14 weeks, histology revealed longitudinally arranged collagen with spindle-shaped cells in the strain group, compared to disordered fibrous tissue with randomly aligned collagen and reduced strength to failure in the controls (no strain) [56]. The researchers also compared the histology to PGA scaffolds seeded with tenocytes and reported no difference between the cell source [54]. When static tension is applied to DFbs in bioreactors, the cells produce type I and type III collagen, but other similarities to tenocytes cannot be confirmed, as there are no tenocyte-specific markers.

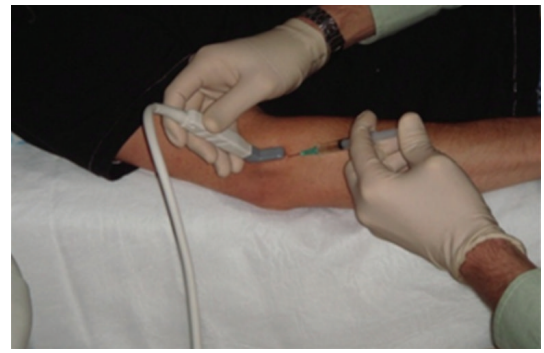

Figure 6: Injection of lateral epicondyle under ultrasound guidance.

Connell et al. demonstrated that DFbs could be expanded, stretched, and induced to lay down collagen in a similar fashion to tenocytes [57]. In a prospective study on twelve subjects with refractory lateral epicondylitis ("tennis elbow"), these researchers implanted 10×10^6 DFbs with precision-guided ultrasound intratendinous injection. Over the 6-month follow-up, there was improvement in disability scores and ultrasound tendon parameters ($P > 0.05$) in all but one subject. However, the collagen-producing fibroblasts were administered with platelet-rich plasma, which has been reported to improve healing in clinical trials [58, 59]. In a randomized trial of 60 cases of patellar tendinopathy, comparing ultrasound guided intratendinous injection of dermal fibroblasts to plasma controls, a faster response to treatment and significantly greater reduction in pain and improved function was noted in the treatment group [60]. One patient in the treatment group experienced tendon rupture, and subsequent biopsy showed relatively normal tendon tissue with type I collagen and tenocytes with normal morphology, and no ectopic tissue was noted.

Currently there are no current registered trials in the use of dermal fibroblasts in tendon-ligament engineering (Figure 6).

3. Gene Therapy

The therapeutic plasticity of stem cells means that specific transcription factors can be introduced which leads to reprogramming and phenotype transition [61]. A master transcription factor for the tendon lineages is yet to be discovered. Scleraxis (Scx) is the most studied potential marker of neotendon formation discovered to date [18]. However, other candidate genes include SIS1, SIX2, EYA1, EYA2, THBS4, and TNMD [62, 63], showed that MSC differentiation into neotendon was mediated by smad8 expression, which the authors felt was inhibitory of the normal osteogenesis pathway induced by bone morphogenic protein 2 (BMP2) [63].

Stem cells can also be gene modified to secrete growth factors which have autocrine and paracrine effects and which can lead to mesenchymal stem cell (MSC) recruitment, MSC differentiation into tenocyte lineages, and collagen synthesis. However, it appears that no specific tenogenic

growth factor has yet been identified. A number of proteins have been shown to induce neotendon formation including fibroblast growth factor (FGF)-2, transforming growth factor (TGF)-β, insulin-like growth factor (IGF)-1, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and members of the BMP superfamily (such as the growth and differentiation factors—GDFs) [40, 64–67]. The local administration of VEGF improves tendon revascularization, but not graft mechanics [34, 68]. TGF-β1 promotes improved strength in Achilles tendon regeneration by regulating collagen I and III synthesis, cross-link formation, and matrix remodeling [69].

As most growth factors have a restricted biological half-life, slow release preparations or transient secretion by MSCs is required during healing and regeneration processes. A number of animal studies have confirmed that transitory expression of growth factors including TGF-β1, GDF5, and IgF-1 produce some improvement in tendon histology, biomechanics, or healing rate [69–71]. However, most gene delivery methods require viral vectors with associated potential risks including immune rejection, uncontrolled transgene expression, and insertional mutagenesis [24]. At present, there are no registered clinical trials using gene-modified cell therapies in tendon disorder.

4. Bioscaffolds

Currently, autologous tendon or tendon allografts are the preferred scaffolds of choice for tendon defects requiring surgical reconstruction or augmentation. Because tendons have similar histological and physical properties to ligaments (which connect bones to bones), there is considerable overlap and interchange in scaffolding technology between these two structures. In clinical practice, tendon auto or allografts are the preferred donor tissue of choice for ligament repair, primarily because tendons are larger and more easily accessible and can be sacrificed with less morbidity than ligament donor sites.

Clinical examples requiring consideration of surgical scaffolding include

(i) an elderly patient with a painful chronic degenerate massive rotator cuff tendon tear, who has poor quality tissue (further retracted by unopposed muscular contraction) with a high chance of failure of primary surgical repair.

(ii) a young professional athlete, with a high-grade anterior cruciate ligament (ACL) injury, who may only experience mild painless knee instability when pivoting at speed, but the injury is effectively career-ending.

Potentially synthetic scaffolds offer clinical and cost benefits over current grafting techniques, due to accelerated healing with no harvesting morbidity, resulting in shorter hospitalization and rehabilitation periods. Tissue-engineered scaffold materials suitable for cell seeding ("bioscaffolds") are classified as natural or synthetic. Natural bioscaffolds include collagens, small intestine submucosa, and silk fibers, whereas most of the synthetic bioscaffolds have been derived from poly-l-lactic acid (PLA) and poly-lactic-coglycolic acid (PLGA) [72, 73]. Important factors for bioscaffold design include biocompatibility, biodegradation rates, mechanical properties, porosity for cell infiltration, nutrient transmission, and the biologic role of the ECM [18]. Type I collagen gels have been the most studied type of bioscaffold. The seeding density affects mechanical stability and the cellular alignment and reorganization of the matrix [74]. Collagen gels have been enhanced by in vitro seeding and collagen hybridization with PLA or cross-linking with dicatechol nordihydroguaiaretic acid (NDGA) [75]. At present, no tenocyte-collagen scaffold constructs have been able to achieve similar mechanical properties to native tendon [74].

An ACL scaffold must biomechanically match the native ligament, and the graft must allow for osseous attachment with current surgical techniques. Cartmell and Dunn produced a potential ACL scaffold in vitro by decellularizing a patellar tendon allograft to reduce antigenicity and then seeded the graft with fibroblasts [76]. These modified allografts have the potential to be developed into mechanically functional delivery vehicles for cells, gene therapy vectors, or other biological agents. Silk is emerging as a promising material for connective tissue scaffolds. Sahoo and colleagues developed a biohybrid scaffold system by coating bioactive basic (b) FGF-releasing ultrafine PLGA fibers over mechanically robust slowly degrading degummed knitted microfibrous silk scaffolds [77]. The bFGF stimulated MSC proliferation and tenogenic differentiation, and the resulting collagen production contributed to the enhanced mechanical properties of the tendon analogue.

Currently, there are no registered clinical trials using cell-seeded scaffolds to repair tendons (or ligaments).

5. Mechanostimulation

In clinical practice, exercise rehabilitation is a reasonably efficacious intervention for the treatment of tendinopathy; however, the exact exercise prescription (frequency, amplitude, and intensity) and type of exercise (eccentric, concentric or stretching) are still not fully established [78]. Mechanical loading of tendons is known to produce a trophic cellular response with stem cell proliferation and differentiation into tendon progenitors, with a resulting increase in deposition of extracellular matrix [20, 79, 80]. There is an associated increased scleraxis upregulation and secretion of cellular cytokines, including TGF-β and IGF-I [18, 81, 82].

In vitro, the type and axis of loading of bioscaffolds affect the cellular response. Compression loading leads to the formation of more cartilaginous tissue, whereas shear stress produces increased matrix metalloproteinases (MMP-1 and 3) in rabbit tendon fibroblasts, which results in matrix disruption [83, 84]. Repetitive loading, at higher construct strains, results in production of PGE_2 and BMP2, leading to differentiation into nontendon lineages [85, 86]. Zhang and Wang demonstrated that in vitro uniaxial loading of rabbit tendons at 0.5 Hz for 12 hours upregulated tenogenesis

and type I collagen production at 4% strain, but increased adipogenesis and osteogenesis at 8% strain.

Repetitive uniaxial mechanical stretching of seeded bioscaffolds increases ECM production and fibrillar alignment in a number of cell lines, including cultured tendon fibroblasts, isolated tendon fascicles, dermal fibroblasts, and MSCs. Chen et al. found that poorer outcomes resulted when stress was applied in the first three days after cell seeding silk fibroin matrices [87]. The authors concluded that prerequisites include both established cell-to-cell contact and sufficient ECM before stress is applied.

The optimal mechanical stimulation regimes for tendon bioscaffolds are yet to be established, but some studies have demonstrated that loading results in a sixfold increased failure stress [88]. Future cellular bioscaffold design will require a multidisciplinary strategy combining cell technology, engineered scaffolds, and mechanical stimulation [31].

6. Summary

Current clinical treatments for tendon defects and chronic tendinopathy are only moderately effective. Tendons are poorly vascularized, relatively acellular, and have limited regenerative potential. Tendon healing is prolonged and results in biomechanically inferior scar tissue that is prone to reinjury. Surgical reconstruction or augmentation with current scaffolds is often associated with donor site morbidity and usually requires lengthy and costly postoperative rehabilitation. New therapies are required, and cell-based treatments offer great potential due to their ability to regenerate connective tissues, the improved understanding of the properties required for cell-seeded bioscaffolds, and the ease of precision implantation with minimally invasive percutaneous guided injection. Current studies range from pluripotent cells to fully differentiated tenocytes, but are yet to determine the ideal cell type for therapeutic tenogenesis. Cell lines such as ESCs have greater potency and proliferative properties, but also have the potential for more complications including tumorogenesis. MSCs offer some promise in tendon engineering due to their proliferative capacity and the potential of genetic modification to secrete tenogenic growth factors. MSCs are also immunosuppressive and are allogeneic, obviating the need for host biopsy if nontissue-matched cells are used. The ideal cell source for MSC harvesting for use in tenogenesis is yet to be determined. Ectopic bone formation has been reported in MSC-seeded tissue-engineered tendon bioscaffolds, but this complication appears to be due to in vitro factors [31]. Unintended differentiation has not been reported with intratendinous injection in large animal tendinopathy studies or with therapeutic use in thoroughbred horses. For the repair of tendon (and ligament) defects, the ideal tissue engineered bioscaffold, seeding density and preferred mode of mechanical stimulation for both in vivo and in vitro seeding are unknown.

Dermal fibroblasts are a nonhomologous cell, which have the advantage of easy harvesting with a minimally invasive biopsy. However, the lack of tenocyte markers and histological confirmation of current studies makes it difficult to determine whether the microscopic similarities between fibroblasts and tenoblasts result in true tenogenesis. The harvesting procedure for autologous tenocytes (ATI) is more invasive, and tenocytes have limited proliferation potential, but there is no risk of unintended differentiation. Even though the safety of ATI appears established, the results of randomized controlled trials to determine efficacy are awaited. In a study comparing tenocytes, tendon sheath fibroblasts, adipose tissue-derived MSCs, and bmMSCs in healing rabbit flexor tendon defects, little difference was noted in the ability to reseed a decellularized tendon scaffold [89]. However, several other studies have suggested that tendon sheath fibroblasts (tenoblasts) possess a greater rate of proliferation than tenocytes [49, 50].

There is a great deal yet to be discovered in our understanding of the role that cellular therapies will play in the treatment of tendon disorders, and at present there is insufficient data to conclusively prove that these treatments are safe and efficacious. However, this technology appears to hold great promise and will probably become an important clinical therapy in the near future in orthopedic, sports, and musculoskeletal medicine.

Conflict of Interests

No competing financial interests exist.

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Research Article

In Vivo Healing of Meniscal Lacerations Using Bone Marrow-Derived Mesenchymal Stem Cells and Fibrin Glue

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Fibrin glue created from a patient's own blood can be used as a carrier to deliver cells to the specific site of an injury. An experimental model for optimizing various permutations of this delivery system *in vivo* was tested in this study. Harvested equine meniscal sections were reapposed with fibrin glue or fibrin glue and equine bone marrow-derived mesenchymal stem cells (BMSCs). These constructs were then implanted subcutaneously in nude mice. After harvesting of the constructs, BMSC containing constructs showed significantly increased vascularization, and histology showed subjectively decreased thickness of repair tissue and increased total bonding compared to fibrin alone constructs. This model allowed direct comparison of different meniscal treatment groups while using a small number of animals. This *in vivo* model could be valuable in the future to optimize fibrin and cellular treatments for meniscal lesions in the horse and potentially humans as well.

1. Introduction

The meniscus is a C-shaped fibrocartilage structure within the femorotibial joint [1]. It is integral in providing stability to the joint [2], lubrication during movement, and absorption and dissipation of shock [2, 3]. Meniscal injuries can result from a variety of causes, most notably chronic wear and tear, acute damage, and cruciate or collateral ligament laxity [4]. Over time, negative effects of meniscal injury include joint instability and articular cartilage degeneration. Medial meniscal injuries comprised 81% of the damaged menisci reported in humans, and sports activities were the major cause [5]. Severe meniscal tears in the horse have resulted in a decreased return to function despite surgical treatment [6, 7]. Meniscectomy and partial meniscectomy of severely damaged menisci are not without their long-term effects on the joint, resulting in osteoarthritis (OA) and joint disability [8]. Treatment or repair of the meniscus while avoiding meniscectomy may allow the patient to recover with decreased long-term dysfunction in the knee or stifle [9, 10].

Efforts to create a synthetic replacement meniscus generally resulted in suboptimal performance of the implants with consequential degenerative joint changes in a matter of weeks to months in test subjects [11, 12] though some recent progress has been made [13]. Promising options have arisen using donor menisci as biologic scaffolding and incorporating these into the patient meniscal structure, or maintaining the patients' meniscus *in situ* and encouraging cellular repopulation and vascular in-growth through various methods [14–20]. Clinicians have begun to implant stem cells or platelet rich plasma into visible lesions via ultrasonography or arthroscopy. Fibrin glue derived from the patient's own blood can be used as a carrier to adhere the cells in the damaged region [17, 21]. A study in rabbits by Ishimura et al. [22] placed fibrin glue and whole bone marrow aspirate into defects in the avascular zone of the meniscus. This resulted in drastically more mature healing within 12 weeks than fibrin alone or empty defects. One version of this cellular/fibrin treatment has been pioneered by Scotti et al. [20] using chondrocytes implanted in fibrin glue to reappose cadaver meniscal sections. Their study showed increased bonding of the sections in the chondrocyte samples versus fibrin glue alone.

The aforementioned studies suggest the possibility that implanting viable cells in fibrin glue directly into a lesion may give rise to new advances in cell-based meniscal healing. Our

laboratory has used intra-articular injection of autogenous bone marrow-derived mesenchymal stem cells (BMSCs) in the treatment of meniscal lesions in the horse for the last 4 years [23, 24] and has seen encouraging results for horses with severe meniscal lesions. Intra-articular injection of adult stem cells from bone marrow expansion showed significant regeneration of meniscal tissue in a caprine experimental model and a human clinical case [25, 26]. Intra-articular injection of BMSCs is not injury site specific, relying on inflammatory homing mechanisms [21, 27, 28] for the cells to migrate to the damaged tissues. The current project was developed to further examine the effects of more directed site-specific cellular treatment using fibrin as a carrier. Utilizing the murine model published by Scotti et al. [20] offers a variety of benefits that make it a suitable step between *in vitro* and larger scale *in vivo* work. The model provides the ability to experiment with meniscal constructs of a controlled size, type, and treatment in sufficient numbers with cohort controls to provide adequate statistical power. Such a model allows testing of various treatment permutations in a relatively short amount of time. These include dose (cell numbers), type or concentration of fibrin, degree of differentiation in multipotent cell lines, and addition of growth factors or other biologic substances in a controlled *in vivo* setting. The result will enable researchers to determine the optimal treatment strategies utilizing these modalities in larger animal models. The purpose of the current study was to determine if beneficial healing effects from implantation of BMSCs in fibrin could be observed compared to fibrin alone.

2. Materials and Methods

All procedures involving live animals and collection of tissue for this study were approved by the Colorado State University Animal Care and Use Committee.

2.1. Meniscal Tissue Collection. Eight medial menisci were collected from four horses euthanized for reasons unrelated to the stifle joint or other factors that would adversely affect the study. Menisci were harvested in an aseptic manner from cadaver limbs within 6 hours following euthanasia (with a range of 30 minutes to 6 hours), held in phosphate buffered saline (PBS) (Invitrogen Corporation (Headquarters) Carlsbad, CA, USA), snap frozen in liquid nitrogen, and then stored at −80°C for a minimum of 1 week (with a range from 1 week to 8 months). Samples were thawed in a 25°C water bath, trimmed to retain only the axial 2/3 of the meniscus, and sectioned further into 0.4 cm wide triangular wedges. Sections were placed into PBS and PSA (penicillin, streptomycin, and amphotericin B) (Invitrogen Corporation (Headquarters) Carlsbad, CA, USA) and passed through two additional freeze-thaw cycles [29], changing the media to fresh PBS (Invitrogen Corporation (Headquarters) Carlsbad, CA, USA) with each freeze-thaw cycle, to ensure that no viable cells were present.

2.2. Bone Marrow. Bone marrow was collected from the ilium of a horse euthanized at CSUVTH (Colorado State University Veterinary Teaching Hospital) for unrelated reasons within 30 minutes of euthanasia with an 8-gauge Jamshidi trocar using sterile technique. Approximately 20cc bone marrow was drawn into 2–35cc syringes with 3,000 units of heparin (APP Pharmaceuticals LLC, Schaumbeurg, IL, USA) anti-coagulant in each syringe. Bone marrow mesenchymal stem cell colonies were obtained by culturing the nucleated cell fraction according to the technique described by Kisiday et al. [30]. After reaching 60–70% confluence, each culture was passaged by lifting the BMSCs with trypsin (Invitrogen Corporation (Headquarters) Carlsbad, CA, USA), reseeding at 10,000 cells/cm^2, and allowing the BMSCs to grow to 60–70% confluence. The BMSCs were cryopreserved in 95% autogenous serum/5% DMSO (Sigma Aldrich, St. Louis, MO, USA).

2.3. Fibrin. Four hundred milliliters of equine venous blood was collected in a sterile manner from the jugular vein of a donor into a blood collection set (Jorgensen Laboratories) with sodium citrate anti-coagulant and stored at 4°C for several hours. Whole blood was centrifuged at 1000 G for 10 minutes and the plasma removed and stored at −80°C until needed. Plasma was thawed at room temperature, and the fibrin extracted per the ethanol precipitation protocol described by Yoshida et al. [31].

2.4. Construct Preparation. Preprepared meniscal sections, BMSCs, and fibrin were thawed immediately prior to construct creation. Construct pairs for each mouse (Group 0 and Group 1) were created from sections of the same meniscus to control for variation. For Group 1 constructs, thawed cell suspension was pelleted, then resuspended with the minimal amount of fibrinogen free plasma (approximately 50 μL) needed to return cells to suspension. The cell suspension was then mixed with 225 μL fibrinogen precipitate and additional fibrinogen-free plasma (approximately 175 μL) until a final cell concentration of 10×10^6 cells per mL of fibrinogen/plasma/cell mixture was reached. Equal parts (0.02 mL) of the fibrinogen/plasma/cell mixture were mixed with an equal amount of bovine thrombin (MP Biomedicals, Santa Ana, CA, USA) (0.02 mL) and applied to the cut surfaces of Group 1 meniscal sections. A second meniscal section was placed on top of the fibrinogen/plasma/cell mixture and the fibrin allowed to set for 30 minutes.

Group 0 samples were created using 50 μL of PBS to replace the BMSC/plasma suspension. This was then mixed with 225 μL fibrinogen precipitate and 175 μL of fibrinogen free plasma. Equal parts (0.02 mL) of the fibrinogen/PBS/plasma mixture were mixed with bovine thrombin (MP Biomedicals, Santa Ana, CA, USA) (0.02 mL) and applied to the cut surfaces of Group 0 meniscal sections. A second meniscal section was placed on top of the fibrin/PBS/thrombin mixture and the fibrin allowed to set for 30 minutes. To coat the exterior of all constructs, fibrinogen precipitate (225 μL) was mixed with fibrinogen free plasma (225 μL) and mixed with equal volumes of thrombin. This coating was applied to decrease subcutaneous tissue invasion *in vivo* [20]. Approximately 0.1 mL of fibrin and thrombin

mixture was used to completely coat each construct. The exterior fibrin coating was allowed to set for 30 minutes before the completed constructs were transferred to a sterile dish for transport to the surgical suite. Constructs were assembled and completed in groups of 6 of each treatment group between 15 minutes and 45 minutes prior to implantation.

2.5. Surgical Procedure. Twelve 10-week-old male nude mice were anesthetized, maintained on isoflurane inhalant anesthesia, and administered 5 mg/kg carprofen (Pfizer Animal Health, Madison, NJ, USA) subcutaneously. Mice were placed in sternal recumbency and bilateral paralumbar areas were prepped with 2% Chlorhexidine scrub and sterile water. Surgical procedure was modeled after previously described protocols [20, 32]. A 1 cm horizontal cutaneous incision was made in each paralumbar area and a pocket bluntly dissected into the subcutaneous tissue. A meniscal construct was inserted into each pocket, one side randomly receiving a BMSC and fibrin-treated sample and a fibrin only control sample in the other [32]. The incisions were closed with wound closure clips (AUTOCLIP) (Becton, Dickson and Co. Franklin Lakes, NJ, USA). Ear notches were used to uniquely identify each mouse. Mice were recovered and were monitored twice daily for three days following surgery. Mice received 5 mg/kg of Carprofen subcutaneously for 3 days following surgery. After three days, they were monitored once daily for the remaining 4 weeks of the study for signs of postoperative complications and general well-being.

2.6. Harvesting of Constructs. At 4 weeks after surgery, mice were placed in a CO_2 gas chamber and humanely euthanized with a gradually increasing concentration of CO_2 (starting with room air) until the mice ceased to breathe. A lack of heart beat was confirmed before the mice were completely removed from the chamber. The constructs were removed, immediately fixed in 10% neutral buffered formalin (StatLab Medical ProductsMcKinney, TX, USA), and graded for presence of tissue adhesion, adherent vessels, and perceived strength of bond between the sections when bluntly probed. Twenty four meniscal samples in total were harvested from 12 animals, with a Group 1 and Group 0 harvested from each animal as modeled by Peretti et al. (2001) [32]. Grading scores and criteria are illustrated in the rubric in Table 1.

2.7. Scoring. A modified Rodeo et al. [33] scoring system was used. See Table 1 for the complete rubric. The outcome parameter "Adherent vessels" graded on a scale of 0–2 the extent of subcutaneous vascular adherence to each surface of the construct. The outcome parameter "Tissue adhesion" (scale 0–1) graded adherence of mouse subcutaneous tissue to the surface of the construct. The outcome parameter "Bond" scored the apparent adhesion between the two sections when gently probed along the junction of the two sections (0–2). A classification of "No bond" was determined if the two sections separated completely with gentle probing, "flexible bond" was determined as one that maintained apposition but the sections could be slightly shifted in relationship to each other, and a "firm" attachment was one where no movement was noted between the sections when probed.

2.8. Histology. Five micron sections were created from paraffin-embedded constructs in a plane perpendicular to the bonded edge. Two random tissue sections from the central area of each sample were selected and placed into two different staining groups. Tissues were applied to slides and stained with haematoxylin and eosin (H&E); (Anatech LTD; Battle Creek, MI, USA) and Safranin O-Fast Green (SOFG) (Electron Microscopy Sciences, Hatfield, PA, USA) stain and examined microscopically.

The outcome parameter "cellular ingrowth" graded the cellular infiltrate along the repair tissue interface to repopulate the acellular meniscal tissue (0–3). The outcome parameter "predominant cell type" assessed the population of cells present in the repair tissue and their relative frequency (1–3). The outcome parameter "fiber organization" graded the repair tissue as organized or disorganized (0–2 scale). The outcome parameter "fibrinous tissue between sections" graded the amount of fibrinous tissue noted between the meniscal sections at the time of analysis (0–1). This tissue was assumed to be remnants of the fibrin glue which was placed between the sections during construct creation. The outcome parameter "SOFG % positive staining" assessed the amount of the repair tissue that exhibited SOFG positive staining (0–3). The outcome parameter "thickness of repair tissue" assessed the thickness of the repair tissue that was present between the meniscal sections (0–2). The outcome parameter "cell repopulation" analyzed the presence of cells throughout the body of the meniscal section (0–2).

The outcome parameter "total percent bonded" was completed on a microscope at 20x–40x power and measured on digital capture images of the slide sections (Adobe Photoshop CS Extended Edition 10.0.1) Additional images for publication were obtained (Leica DFC 425 camera, LAS Core software, Buffalo Grove IL, USA). Slide images of the sections were measured for a total length of the repair area (area of interface between the cut sections). Images were then marked where evidences of bonding (bridging and incorporation of meniscal fibers into the repair tissue) were noted along the length of the repair. Bonded measurements were recorded and converted to a percentage of the total repair section. These total percentages were grouped into 4 groups and assigned a score of 0–3 based on Table 1.

2.9. Statistical Analysis. Fishers Exact Test and Chi-Square table analysis were performed on all data (SAS v.9.2., SAS Institute Incorporated, Cary, NC). Statistical significance was set at $P \leq 0.05$ and a statistical trend was defined as $P \leq 0.1$.

3. Results

There were no morbidities or mortalities in any of the study animals. At a 3 day post operative examination it was noted visually through the skin of two mice that two of the constructs had shifted so that sections were no longer in apposition on the cut surfaces (one was sitting edge to edge, one was sitting edge to cut surface). Both samples were in

Table 1: Explanation of grading rubric used in the current study and the coding used for the results.

Category/score	0	1	2	3
Gross observations				
Adherent vessels	Vessels on 1 side only	Vessels on 2 sides	Multiple vessels on multiple sides	
Tissue adhesion	No Tissue Adhesion	Areas of external tissue adhered		
Bond	No bond	Flexible bond	Firm attachment	
H&E section observations				
Cellular in-growth	Acellular along cut edge	Incomplete cellular repopulation	50% in-growth	Complete repopulation along cut edge
Predominant cell type		Small round cells (fibrocytes)	Larger less dense cells (inflammatory origin)	50-50 dispersed
Fiber organization	Disorganized	50% parallel fibers	Greater than 70% organization	
Fibrinous tissue between sections	Large amounts of fibrous tissue present	No large fibrous tissue sections present		
SOFG section observations				
SOFG % positive staining	None	Less than 50%	More than 50%	Near 100%
Thickness of repair tissue		Thin	Medium	Thick
Cell repopulation	Rare	Moderate	Frequent	
Total bond distance	25%	25–50%	50–75%	75–100%

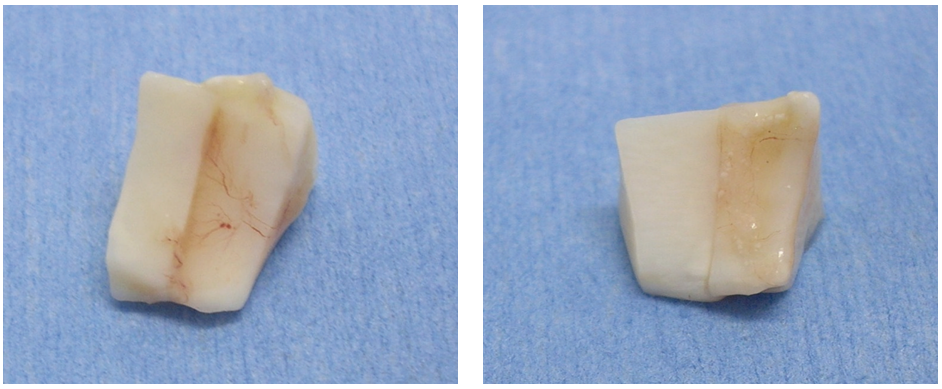
the cell-treated group (Group 1). Once cut in for histology, these samples had bonding evidenced between the sections and were graded along with the rest of the samples. For complete results of the scoring rubric for each sample, see Table 2.

3.1. Gross Observations. All constructs had fibrinous coating remaining, noted upon removal from the mouse. There was a statistically significant difference ($P = 0.0094$) in the presence of "Adherent vessels" to the construct surfaces. Group 0 had four (4/12, 33.3%) constructs with vessels present only on one side while Group 1 constructs all had vessels present on two sides (10/12, 83.3%) and multiple sides (2/12, 16.7%). Please see Table 2 for complete scoring results. See Figure 1(a) for an image of a Group 1 construct with multiple vessels adherent to the surface and Figure 1(b) for a Group 0 construct with minimal vessels present only on one surface. There was no statistical difference ($P = 1.0$) in external "tissue adhesion" between treatment groups. Group 1 constructs had no presence of tissue adhesion in 5/12 (41.7%) and did have presence of tissue adhesion in 7/12 constructs (58.3%). Group 0 also had 5/12 constructs with no evidence of external tissue adhesion and 7/12 constructs with presence of tissue adhesion. There was no significant statistical difference ($P = 0.64$) in the level of subjective of "Bond" between treatment groups. While some sections had a flexible bond, it was noted that all sections were adhered together and more resistant to separation with pressure applied at the cut edge than they had been prior to implantation, indicating the occurrence of bonding in all constructs. Please see Table 2 for a breakdown of the full results of these outcome parameters.

3.2. H&E-Stained Sections. There was no statistical significance ($P = 1.0$) in "cellular ingrowth" from the repair tissue into the meniscal sections between treatment groups. There was no statistically significant difference ($P = 0.822$) in the "predominant cell type" present in the repair tissue between the treatment groups. Some sections had very organized repair tissue with parallel fibers; however, these parallel fibers were mostly oriented perpendicular to the meniscal fibers. While there subjectively appeared to be a relationship between treatment group and fiber organization, this was not statistically significant ($P = 1.0$). There was no statistical difference ($P = 0.214$) in the amount of fibrinous tissue remaining in the repair area between sections when comparing the treatment groups. Subjectively, larger amounts of fibrinous tissue were present in non-cell-treated sections than in cell-treated sections with 7/12 (58.3%) of the Group 0 constructs having a large amount of fibrinous tissue present compared to only 3/12 (25%) in the Group 1 constructs. Figure 2(a) shows a histologic section from Group 1 illustrating the absent fibrinous tissue and showing vascular ingrowth into the repair tissue and progressing between the cut sections. Figure 2(b) shows a histologic section from Group 0 showing thick fibrinous tissue present. There is still some vessel ingrowth present in the fibrinous tissue. There was no statistical difference in the "total bond distance" between treatment groups ($P = 0.569$). However, Group 1 (Figure 3(a)) subjectively showed better bonding overall with 11/12 (91.7%) sections

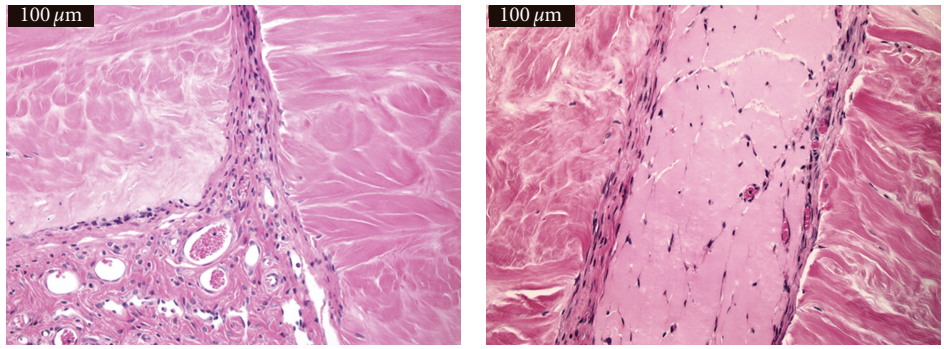
Table 2: Group 0 is fibrin only treatment; Group 1 is fibrin with BMSCs treatment. See Table 1 for grading score rubric.

Category/Score	Group	0	1	2	3	*P* value
Gross observations						
Adherent vessels	Group 0	4 (33.3%)	3 (25%)	5(41.7%)		0.0094
	Group 1	0 (0%)	10 (83.3%)	2 (16.7%)		
Tissue adhesion	Group 0	5 (41.7%)	7 (58.3%)			1.00
	Group 1	5 (41.7%)	7 (58.3%)			
Bond	Group 0		2 (16.7%)	10 (83.3%)		0.640
	Group 1		4 (33.3%)	8(66.7%)		
H&E section observations						
Cellular in-growth	Group 0	3(25.0%)	6 (50.0%)	3(25.0%)		1.000
	Group 1	3(25.0%)	5 (41.7%)	4 (33.3%)		
Predominant cell type	Group 0		8 (66.7%)	1(8.3%)	3(25.0%)	0.822
	Group 1		6(50.0%)	1(8.3%)	5(41.7%)	
Fiber organization	Group 0	3(25.0%)	7(58.3%)	2(16.7%)		1.000
	Group 1	2(16.7%)	7 (58.3%)	3(25.0%)		
Fibrinous tissue between sections	Group 0	7(58.3%)	5(41.7%)			0.214
	Group 1	3(25.0%)	9(75.0%)			
Total bond distance	Group 0	0	3(25.0%)	4(33.3%)	5(41.7%)	0.569
	Group 1	0	1(8.35)	7(58.3%)	4(33.3%)	
SOFG section observations						
SOFG % positive staining	Group 0	5(41.7%)	3(25.0%)	4(33.3%)	0	0.428
	Group 1	5(41.7%)	5(41.7%)	1(8.3%)	1(8.3%)	
Thickness of repair tissue	Group 0		5(41.7%)	6(50.0%)	1(8.3%)	0.680
	Group 1		8(66.7%)	3(25.0%)	1(8.3%)	
Cell repopulation	Group 0	5(41.7%)	6(50.0%)	1(8.3%)		0.520
	Group 1	7(58.3%)	3(25.0%)	2(16.7%)		



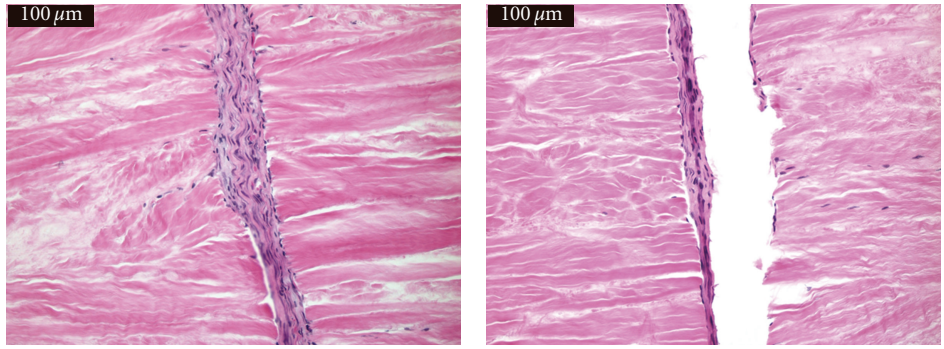
(a) (b)

Figure 1: (a) Multiple vessels externally adhered to Group 1 meniscal construct. The vessels can be seen entering the cut area between the two sections. (b) Group 0 construct with minimal evidence of vessels on only one surface.



(a) (b)

Figure 2: (a) shows thin repair tissue and vasculature ingrowth in Group 1 meniscal section. (b) shows a grouping of small vessels within a large amount of fibrinous tissue between two meniscal sections in Group 0 construct.



(a) (b)

Figure 3: (a) A cell-treated (Group 1) meniscal section with cellular repopulation of the acellular meniscal tissue and evidence of bridging between the two meniscal sections, especially in the upper aspect of the image. (b) A non-cell-treated section (Group 0) with separation of the repair tissue from the meniscal fibers on the right-hand side (indicative of a less robust attachment). While this detachment occurred during processing, a lack of bonding can be seen on the left side of the repair tissue as well.

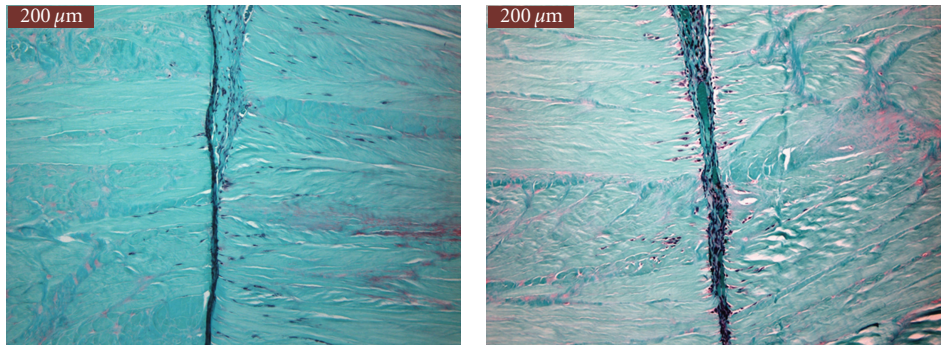
exhibiting bonding characteristics evident in greater than 50% of the repair area compared to 9/12 (75.0%) of Group 0 (Figure 3(b)) constructs with bonding characteristics over 50% or more of the repair area.

3.3. SOFG-Stained Sections. Figures 4(a) and 4(b) show two examples of SOFG-stained sections. There was no significant difference in "SOFG % positive staining" between groups ($P = 0.428$) with Group 0 exhibiting the same number of constructs with negative SOFG (i.e., green counter staining) (5/12, 41.7%) compared to Group 1 (5/12, 41.7%). While Group 0 had more constructs (4/12, 33.3%) that exhibited 50% or more positive SOFG staining compared to Group 1 (2/12, 16.6%), this difference was not significant. When examining "thickness of repair tissue" present and treatment group, there was no statistically significant difference between groups ($P = 0.680$). Based on subjective scoring, there appeared to be less repair tissue present between the sections in Group 1 (8/12 (66.7%) with thin repair tissue) compared to Group 0 (5/12 (41.7%) with thin repair tissue). Finally, there was no statistical difference noted when examining "cellular repopulation" in the body of the meniscal sections (away from the cut edge) between treatment groups ($P = 0.520$).

4. Discussion

This study allowed comparison of repair and bonding differences of acellular meniscal sections when treated with BMSCs and fibrin compared to fibrin alone in a controlled *in vivo* model. Overall, results showed significantly increased vascular adherence to BMSC and fibrin-treated sections ($P = 0.0094$). Subjectively the results showed improved characteristics of increased bonding and healing in constructs treated with stem cells and fibrin compared to constructs with fibrin only in the outcome parameters of "fibrinous tissue between sections", "thickness of repair tissue", and "total % bonded". It is to be noted that the statistical results of this study are likely limited by subtle differences between the treatment groups and small sample size.

The degree of bonding seen in constructs treated with fibrin only in the current study appeared different when compared with previously published work. Specifically, Scotti et al. [20] saw no evidence of bonding between sections in their fibrin only group and did not report evidence of



(a) (b)

Figure 4: (a) and (b) show SOFG-stained sections with little positive SOFG-stain uptake. There was no significant differences between the SOFG staining between groups. Cellular repopulation along the cut edge is evident in both sections.

vascular infiltration in any of the experimental constructs in their study. In contrast, the current study showed multiple signs of bonding within the fibrin only treatment group as well as the BMSC-added group. There were vessels present between the sections in both treatment groups in the current study. It is unclear what resulted in this discrepancy between the results of the two studies. Potentially it could have been due to the difference in the fibrin sources. Scotti et al. [20] used a commercially prepared porcine fibrin product whereas the current study utilized equine fibrin drawn and prepared on-site, as would be commonly performed in a clinical setting. Another potential difference between the studies was the thickness of the fibrin coating. Images published by Scotti et al. [20] showed thicker layers of fibrin surrounding the constructs than was achieved in the current study. A thicker layer of fibrin would potentially limit vascular invasion and alter the diffusion and delivery of exogenous oxygen, cytokines, and cells to the construct.

In humans, while the outer third of the meniscus is well vascularized due to connections to the joint capsule, the axial portion is essentially avascular [34, 35]. Due to this difference, the outer portion of the meniscus is more likely to heal with a viable repair [34, 35]. The thinner avascular portion and meniscal ligament attachments are more likely to have a suboptimal repair and more likely to become reinjured on return to strenuous activity [14, 34]. Treatment of the constructs with BMSCs appeared to result in a greater and more consistent revascularization of the construct tissues. There were significantly ($P = 0.0094$) more vessels externally adhered to Group 1 constructs compared to Group 0 constructs in the current study. For an example, please see Figures 1(a) and 1(b). It is possible that vascular growth factors released by the BMSCs [21, 36, 37] resulted in a stimulus for vessel in-growth in the cell-treated sections, and these growth factors were not present in the previous study by Scotti et al. [20] which utilized chondrocytes in their cell constructs. It is also plausible that these growth factors may have also affected the non-BMSC (Group 0) sections implanted in the same mouse. Having both Group 0 and Group 1 constructs in the same mouse would allow growth factors to be systemically absorbed or to spread locally through the subcutaneous space. This may have accounted for the increased vascularity seen in the current study compared to Scotti et al. [20]. Increased vascular formation is often a goal of meniscal healing treatments in order to provide physiologic support for repair tissues in the meniscus. Thus the increased vascularity seen here could be a benefit in clinical use [38, 39].

Scotti at al. [20] reported more linear fibrous repair tissue and less fibrin remnant in the chondrocyte-treated constructs. These results are mirrored in the results of the current study with the BMSC-treated constructs: thinner repair tissue (66.6% of Group 1 compared to 41.75% of Group 0 constructs with thin repair tissue) and less fibrinous remnants (25% of Group 1 constructs had large amounts of fibrinous material between section compared to 58.3% in Group 0). One explanation for this result is that the cell-treated samples created a repair tissue with less extraneous scar tissue than those without cells.

Mature meniscus contains live fibrochondrocytes despite the avascular nature of the axial portion. If an injury occurs and viable cells are still present, these cells can be recruited for healing. However, studies have shown that fibrochondrocyte apoptosis is closely related to, and may even precede, meniscal damage [40]. Apoptosis that occurs in an injured area greatly reduces the fibrochondrocytes available for repair, and lack of vascularity limits the opportunity for cells to migrate in from other locations [41]. In a large retrospective study of humans with injured menisci, Englund et al. [10] found that degenerative meniscal damage was less likely to have a satisfactory return to function compared to traumatically injured menisci. Most of the constructs in both Group 0 (9/12) and Group 1 (9/12) in the current study had cellular ingrowth from the cut edge and repair tissue present. These cells could contribute to repopulation of the avascular meniscal section and ultimately increase healing. This repopulation of the acellular meniscal tissue would also be critical in healing as presence of fibrochondrocytes would be necessary for long-term viability of the meniscal implant. It was beyond the scope of this study to determine the origin of the repopulated cells.

The study presented here aided in further describing this model and resulted in significant results that may have clinical implications. Utilizing this model could allow further investigations to better define optimal conditions for fibrin and BMSC treatment in live animals. Unpublished studies *in vitro* in our laboratory have suggested that specific concentrations of fibrin may be optimal for migration of BMSCs; however, these more dilute concentrations may not offer as much support to maintain tissue apposition during the early healing phases. Using this model and cellular markers [38, 42] to track migration of cells at different time points may help to determine the most physiologically effective concentration of fibrin to utilize *in vivo*. Ideal dosage of cells per surface area treated may be investigated as well. Further studies with cell marking techniques will also provide information on the source of cells participating in the repair tissue between the sections and aid in determining what cells are repopulating the meniscal tissue. This may enable medical personnel to better target treatment to increase positive outcomes in difficult meniscal injuries.

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Review Article

Ligament Tissue Engineering and Its Potential Role in Anterior Cruciate Ligament Reconstruction

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Tissue engineering is an emerging discipline that combines the principle of science and engineering. It offers an unlimited source of natural tissue substitutes and by using appropriate cells, biomimetic scaffolds, and advanced bioreactors, it is possible that tissue engineering could be implemented in the repair and regeneration of tissue such as bone, cartilage, tendon, and ligament. Whilst repair and regeneration of ligament tissue has been demonstrated in animal studies, further research is needed to improve the biomechanical properties of the engineered ligament if it is to play an important part in the future of human ligament reconstruction surgery. We evaluate the current literature on ligament tissue engineering and its role in anterior cruciate ligament reconstruction.

1. Introduction

Ligamentous tissue is composed of fibroblasts and an extracellular matrix. Whilst the fibroblast is the main cell type, the extracellular matrix is predominantly types I and III collagen with proteoglycans, water, and small amounts of elastin present [1, 2]. The fibroblasts secrete the extracellular matrix and also maintain, repair, and regenerate new tissue growth. Whilst there are no specific markers to differentiate ligament from tendon, the total amount of collagen, elastin, and proteoglycans varies between the two tissues as well as the types of collagen. The process of ligament healing is complex and growth factors play an important role [2]. Most studies concentrate on the anterior cruciate ligament (ACL) repair as it is one of the most common injuries and is therefore a good model for illustrating ligament repair or regeneration [3–7]. The ACL plays an essential role in the smooth motion and stability of the knee joint and, due to its poor vascularity, has limited healing capacity [4, 5]. Traditionally, ACL ruptures have been treated using autografts, allografts, and synthetic grafts made from polymers. All of these techniques have a number of disadvantages [4–8].

The speciality of orthopaedics lends itself to tissue engineering. Musculoskeletal tissues are often injured or lost in trauma and disease and demonstrate limited healing potential. Whilst orthopaedic surgery has advanced in the use of cartilage replacement, it remains to be seen whether there will be a shift from tissue replacement towards tissue regeneration [9]. Tissue engineering offers an unlimited source of natural tissue substitutes. By using appropriate cells, biomimetic scaffolds, and advanced bioreactors, it is possible that tissue engineering could be implemented in the repair and regeneration of tissue such as bone, cartilage, tendon, and ligament [10]. The use of cells significantly improves the construct quality, and in vivo injections of cells into the injured ligament can accelerate the repair process by laying down extracellular matrix, releasing growth factors, and triggering the necessary immune responses [4, 11]. Novel approaches are being tried including stem cell therapies, use of growth factors, mechanical loading, and gene therapy to achieve this end point [1, 2, 11–14].

2. Cell Source and Different Approaches in Ligament Tissue Engineering

Whilst it is imperative to use an appropriate cell type to achieve a functional ligament construct, little is known about

the optimal cell source for ligament tissue engineering [4]. Ligament cells from different sources vary in their growth, dexamethasone responsiveness, and cell surface marker expression. All of these factors are important in enabling the tissue engineers to carefully select the optimal cell source and hence maximise efficacy [15]. The actual source of the cell, the variation in the behaviour of cells from different species, the passage number, and the animal model experiments must also be considered [5, 16]. The options available are mesenchymal stem cells (MSC) or primary fibroblasts derived from ligaments such as the ACL or medial collateral ligament (MCL) [4, 16–19].

Stem cells are immature biological cells which have the ability to proliferate, differentiate, and regenerate tissues. The two main types of stem cells in mammals are embryonic stem cells (ESC), formed a matter of days after egg fertilisation, and nonembryonic stem cells (non-ESC). Nonembryonic stem cells are also referred to as adult stem cells and are usually obtained from the bone marrow of adults. There are two types of stem cells available from this source: haemopoietic, which differentiate into blood cells, and MSCs. The less mature sources of MSCs such as the placenta and the umbilical cord blood are still considered non-ESCs whilst fetal stem cells are considered an intermediate cell type [20, 21].

Since the discovery of MSCs in 1976, it has become more apparent that their capacity to repair tissue is due to their ability to secrete soluble factors which alter the tissue microenvironment. A number of chemokines and cytokine receptors have been implicated in guiding the MSCs to the zone of tissue injury to allow tissue repair to begin. Despite this, there is little evidence with regard to the mechanism of mobilisation of MSCs from the bone marrow. One of their most important features is their unexplained immunological properties. Adult MSCs express moderate levels of class I major histocompatibility complex (MHC) proteins but no class II proteins. Their nonimmunogenicity indicates that immunosuppressive therapy is not required if transplanted into an allogenic host [20].

Bone marrow is the most popular source used to acquire MCSs due to its relative ease of access. The MSCs of bone marrow have a greater transdifferentiation capability compared to the MSCs of different tissue origin. However, bone marrow aspiration is invasive, potentially painful, and associated with increased risks of morbidity and infection. Other sources for MSCs have been discovered including amniotic fluid, umbilical cord blood, adipose tissue, synovium, and tendon but it is currently unclear which lineages these MSCs can differentiate into [20, 22].

3. Mesenchymal Stem Cells in Ligament Tissue Engineering

The literature would suggest that MSCs are the preferred method of ligament tissue engineering because they can easily differentiate into ligament fibroblasts after two weeks [1, 2, 4, 5, 12, 14, 16, 18, 23–26]. In large animal model experiments involving pigs, MSCs (passage 2) exhibited fibroblast phenotype and differentiation at 24 weeks postoperatively with silk-based scaffolds [5]. Most of the studies use MSCs at passage 2 [5, 12, 27, 28]. Following passages 2 and 3 at 25–30 days, rabbit MSCs have been shown to have stopped proliferation, increased in size, and assumed an irregular morphology. However, this might not be true for hMSCs [4]. Interestingly, Ge et al. found no difference in collagen production between passage 1 and 2 using rabbit MSCs [4]. Furthermore, it has been reported that MSCs lose their potential for osteogenic differentiation after passage 5-6 due to senescence or fibroblast contamination [29]. This may have important implications in ligament tissue engineering where MSCs are expected to differentiate into fibroblastic lineage and therefore achieve pure MSC culture. There are currently no specific markers that can reliably distinguish between MSCs and fibroblasts [29].

There is also the issue regarding the source of MSCs. Traditionally, MSCs have been harvested from bone marrow and other sources such as adipose tissue and cord blood. The potential role for harvesting MSCs from synovial fluid in ligament regeneration has also been reported [25, 30]. The number of MSCs is known to increase following any ligament injury and in degenerative disorders such as osteoarthritis [30, 31]. Cheng et al. reported better outcomes from the stem cells derived from the ACL itself compared to bone-marrow-derived MSCs [32].

A bioreactor uses various combinations of chemical, mechanical, electrical, or magnetic stimulation to accelerate the process of differentiation of MSCs into the fibroblastic lineage and facilitate the development of a de novo tissue construct that is comparable to the desired tissue [33].

There are number of key bioreactor principles that must be obeyed for the bioreactor to function successfully. The bioreactor should be designed to operate under strict sterile conditions in order to prevent contamination of the neotissue with microorganisms. The bioreactor should maintain accurate control of the physiological environment of the tissue culture. This ensures control of parameters such as pH values, oxygen concentrations, temperature and metabolite concentrations. The bioreactor should provide the culture with the fundamental nutrients and gases. Finally, the bioreactor should be able to accommodate the culture of more than two cell types at the same time. This is particularly relevant when engineering complex tissues [33].

Chemical stimulation techniques employ a cocktail of polypeptides known as growth factors. Growth factors such as transforming growth factor-β (TGF-β) and epidermal growth factor (EGF), TGF-β and insulin (maybe in a sequential approach), insulin-like growth factor (IGF-I), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), or growth and differentiation factor (GDF) can expedite the MSCs to differentiate into fibroblasts and also improve the cell proliferation and extracellular matrix deposition [1, 12, 25, 27, 34]. Sustaining sufficient quantities of growth factor within the local tissue has been difficult until the introduction of gene transfer technology [21]. Wei et al. experimented by transfecting bone-marrow-derived MSCs with adenovirus vector encoding TGF-β1, VEGF, or TGF-β1/VEGF before surgical implantation into experimental ACL grafts [24]. They found that this combination significantly improved

the performance of the MSCs by promoting angiogenesis. The best mechanical properties were achieved at 24 weeks.

Mechanical conditioning is another method used to induce differentiation of MSCs into the fibroblast lineage. Triggering the cell surface stretch receptors results in activation of the intracellular signalling cascades leading to synthesis of the necessary extracellular matrix proteins [12, 27, 28]. Altman et al. developed a specialised bioreactor for this purpose and found that helically organised collagen fibres formed in the direction of the load [28]. Cocultures are rapidly becoming popular to promote MSC differentiation by growing them together with fibroblasts [12, 35, 36]. The mechanism of action is based both on the cell-to-cell interactions between the fibroblasts and MSCs and on the cytokines released within the 3-dimensional environment. The differentiated MSCs are also stimulated to secrete more extracellular matrix [12]. In one study, fascia was wrapped around the MSC-seeded ACL tissue construct and, whilst this promoted extracellular matrix production, it did not enhance the ultimate tensile load and stiffness [37].

Experiments using electromagnetic stimulation techniques have been carried out and demonstrate positive findings. In one study, a single shot of low-energy laser therapy was administered to the medial collateral ligament of a rat resulting in a significant increase in the collagen fibril size. Another study reported an increase in osteoblastic and alkaline phosphatase activity when electrical stimulation was applied to rabbit bone marrow [33].

Oe et al. studied ligament regeneration in rats following intra-articular injection of either fresh bone marrow cells (BMCs) or cultured MSCs 1 week after partial ACL transection. At 4 weeks, donor cells were detected within the transected ACLs in both the BMC and MSC groups and the ACLs exhibited almost normal histology. Furthermore, there were significantly more mature spindle cells, near normal biomechanical properties and higher levels of TGF-β in the ACL tissue of the BMC group. They concluded that direct intra-articular bone marrow transplantation is an effective treatment for partial ruptures of the ACL [3]. Similar results using intra-articular injections have been reported by other researchers [38]. Lim et al. performed ACL reconstructions in adult rabbits using hamstring tendon autografts which were coated with MSCs in a fibrin glue carrier. At 8 weeks, good osteointegration was observed and they performed significantly better on biomechanical testing than the controls [39].

In summary, MSCs of low passage number are a good source of cells for use in ligament regeneration. The advantages include: the use of autologous cells, the relative ease of procurement and growth in the lab and the ability to differentiate into fibroblasts at around 2–4 weeks and secrete the extracellular matrix.

4. Primary Fibroblasts in Ligament Tissue Engineering

Fibroblasts are another choice of cell and can be harvested from different sources. Cooper et al. concluded that ACL-derived fibroblasts were the most suitable cells for the further study and development of tissue-engineered ligament as opposed to the cells derived from the MCL, achilles tendon, or patellar tendon [40]. Another study compared the performance of fibroblasts extracted from both intact and ruptured human ACLs. They observed that cells extracted from the ruptured ACL were more useful in ligament tissue engineering [17]. Fibroblasts from other sources such as the skin are also being tested for their use in ligament tissue engineering. However, there is debate as to how well they are able to function given the change from their normal physiological environment [4, 41].

5. Comparison between Mesenchymal Stem Cells and Primary Fibroblasts

Ge et al. compared the performance of fibroblasts isolated from the ACL and MCL to that of bone-marrow-derived MSCs in rabbits. He found that the proliferation rate and collagen production were higher with MSCs (passage 1–37.1 mg/mL and passage 2–36.4 mg/mL) than with fibroblasts (ACL 23.2 mg/mL and MCL 19.8 mg/mL). The cells survived at least 6 weeks in the knee joint and in the MSC group, survivorship was due to the protection of the surrounding fascial covering which led to a mild immune response. All three groups expressed equal amounts of collagen I, collagen III, and α-smooth muscle actin [4]. Liu et al. also found that MSCs grew faster than fibroblasts on silk scaffolds. Furthermore, the gene expression for transcripts and production was increased in the MSC group compared to the fibroblast group [16].

6. Response of Cells to Different Biomaterials in Ligament Tissue Engineering

Stem cells are commonly seeded or implanted into a construct that is capable of providing structural support to three-dimensional tissue growth. These constructs or scaffolds facilitate tissue formation by enabling cell migration, proliferation, and differentiation [9, 22].

The ideal scaffold should have some key properties. The scaffold should be able to bridge any complex three-dimensional anatomical defect, and this can be achieved using surgical experience or through sophisticated computer mapping systems. The scaffold must provide temporary mechanical support until the three-dimensional neotissue has regenerated to a mature enough state that the tissue is able to bear load. A scaffold that is biodegradable is often desirable because absorption by the surrounding tissue prevents the need for surgical removal. However, the rate of absorption must mirror the rate of neotissue formation. This allows the scaffold to provide a temporary structural mechanical support until the newly formed tissue takes over the mechanical load. Porous scaffolds enhance tissue regeneration by delivering biofactors. Pore diameter is important in facilitating cell migration, proliferation, and growth factor movement. It is important to get the right balance between tissue regeneration and the mechanical properties of the scaffold. Whilst

smaller pores are inefficient, larger pores can compromise the mechanical properties of the scaffold [21, 22].

Scaffolds can be composed from naturally occurring material or from synthetic material. Studies have evaluated the ability of different components within the extracellular matrix to support cell growth. Proteinaceous material such as collagen and polysaccharidic material such as glycosaminoglycans (GAGs) have been found to be suitable with regard to cell compatibility, but immunogenicity remains a potential problem. Polylactic acid (PLA) is a commonly used synthetic scaffold which easily degrades within the human body by forming lactic acid. Materials such as polycaprolactone(PCL) and polyglycolic acid (PGA) degrade in a similar way to PLA but exhibit different rates of degradation. Research continues in the quest to create a scaffold that combines the advantages of both groups of biomaterials [21, 22].

Scaffolds that are used in ligament tissue engineering should mimic the extracellular matrix both by providing appropriate mechanical support and by promoting cellular adhesion and proliferation [5, 25]. The biomaterials commonly used are silk, a variety of polymers (especially polyhydroxyesters), and natural substrates such as collagen, gelatine, small intestinal submucosal extracellular matrix, and even decellularised ligaments [5–7, 17, 23, 25, 34, 42, 43]. In a study by Ouyang et al., the adhesion, proliferation, and morphology of rabbit ACL cells and MSCs were investigated using different biodegradable polymeric films. They found that high molecular weight poly (DL-lactide-co-glycolide) was most favourable for cell attachment and proliferation and that MSCs performed better than ACL cells [44]. Knitting, braiding, and electrospinning are all popular techniques used in the manufacture of biomimetic fibrous scaffolds for ligament tissue engineering [5, 6, 19, 23, 42, 43]. The cells are proven to spontaneously orientate along the direction of the fibres leading to abundant extracellular matrix secretion rich in collagens I and III [5, 42].

Silk is becoming increasingly popular due to its good biocompatibility, slow degradability, and excellent mechanical properties [5, 6, 34, 45]. Altman et al. popularised silk scaffolds for ligament tissue engineering [45]. They showed a significant increase in the number of cells and matrix production after culturing human MSCs for 14 days. Furthermore, mRNA analysis demonstrated a similar gene expression to native ligament cells. Composite scaffolds comprising silk are extremely biocompatible and both MSCs and primary fibroblasts can attach to them within 18 hours and proliferate profusely to secrete extracellular matrix [5, 16, 23, 25, 46]. Sahoo et al. developed a composite electrospun nanofibrous PLGA on knitted microfibrous silk scaffolds. They were coated with bioactive bFGF, the controlled release of which was dependent on the degradation of the fibres, facilitating MSC attachment, cell proliferation, and fibroblastic differentiation. This was proven by the upregulation of ligament specific extracellular matrix proteins by 14 days [25, 26]. bFGF is known to stimulate MSC proliferation and differentiation by acting synergistically with the mechanical stimulation and nanotopographic cues of the scaffold [25].

7. Methods of Characterising the Tissue Engineered Ligament

Cellular proliferation, protein synthesis, and extracellular matrix production are important aspects of ligament tissue engineering. Characterisation can be performed by histotechniques, quantifying the extracellular matrix protein (perhaps by immunostaining), scanning electron microscopy, or quantitative polymerase chain reaction for gene expression of relevant ligament-related proteins such as collagen type I and relevant transcription factors such as scleraxis, tenomodulin, and tenascin-C [5, 14, 16, 23, 27, 34, 42, 46–48]. In the case of MSCs, the protein transcription levels increase by 2 weeks [16]. Stress-strain tensile testing can be performed for tissue engineered ligaments to assess their mechanical efficiency [42].

8. Conclusion

Stem cells, growth factors, mechanical loading, biomimetic scaffolds, and gene therapy all play important roles in the quest to engineer the ideal ligament neotissue. Whilst repair and regeneration of ligament tissue has been demonstrated in animal studies, further research is needed to improve the biomechanical properties of the engineered ligament if it is to play an important part in the future of human ligament reconstruction surgery.

Ultimately, randomised controlled trials on human populations will be required to demonstrate the clinical application of the engineered ligament. Furthermore, a cost-benefit analysis will be necessary to justify its use over conventional ACL reconstruction surgery.

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Review Article

Tissue Engineering Strategies in Ligament Regeneration

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Ligaments are dense fibrous connective tissues that connect bones to other bones and their injuries are frequently encountered in the clinic. The current clinical approaches in ligament repair and regeneration are limited to autografts, as the gold standard, and allografts. Both of these techniques have their own drawbacks that limit the success in clinical setting; therefore, new strategies are being developed in order to be able to solve the current problems of ligament grafting. Tissue engineering is a novel promising technique that aims to solve these problems, by producing viable artificial ligament substitutes in the laboratory conditions with the potential of transplantation to the patients with a high success rate. Direct cell and/or growth factor injection to the defect site is another current approach aiming to enhance the repair process of the native tissue. This review summarizes the current approaches in ligament tissue engineering strategies including the use of scaffolds, their modification techniques, as well as the use of bioreactors to achieve enhanced regeneration rates, while also discussing the advances in growth factor and cell therapy applications towards obtaining enhanced ligament regeneration.

1. Introduction

Fibrous connective tissue bands connecting two or more bones are called ligaments. Ligaments augment joint stability and resist to forces to prevent excessive motion. Extracellular matrix (ECM) forms 80% of the tissue volume and fibroblasts make up the remaining 20%. The dry weight of a ligament consists of collagen (75%), elastin (1%), proteoglycans, and glycoproteins [1]. 90% of the collagen is type I and 10% is type III.

Although ligaments sustain excessive mechanical loads, they have a poor regeneration capacity with their low cell density and low nutrient and oxygen requirements. Thus, ligaments are repaired by a weaker and disorganized tissue which is prone to reinjury [2]. Using autografts for ligament reconstruction remains the gold standard with their high mechanical strength and compatibility, besides having high revascularization and remodeling capacities [3]. However, donor site morbidity and damage, thus, pain, and altered harvest site biomechanics that sometimes require a second invasive procedure are the drawbacks of autografts [4–6]. Allografts, on the other hand, exclude the risks associated with autografts, such as donor site morbidity; however, they carry additional risks of disease transmission, infection, and allergic reactions in addition to their lower early cellularity and less revascularization [7]. These circumstances drive attention to other techniques for ligament reconstruction, such as the use of biomaterials, cell therapies, and tissue engineering strategies, to promote a more functional healing. Preserving the native insertions and proprioceptive functions of the ligaments are advantages of these techniques leading to functional healing, over the surgical reconstruction of the tissue.

Tissue engineering strategy involves the use of biodegradable and biocompatible biomaterials with adequate structural and mechanical properties to mimic the organization of the native tissue, along with cells isolated from the healthy proportion of the patients own ligament, or other alternative cell sources such as stem cells, and growth factors to regulate the function of these cells. Conceptually, tissue engineering aims to improve the quality of the processes associated with the healing of the ligaments by creating viable artificial substitutes in the laboratory and their transplantation to the patient after in vitro maturation. Therefore, tissue engineering holds promise for the future in terms of decreasing the need for ligament grafting procedures while reducing

the risks associated with them, such as rejection and tissue mismatch, as the construct would carry the patient's own cells.

From the clinical point of view, the main advantages offered by the use of tissue engineered ligament could listed to be minimal patient morbidity, simpler surgical technique, reliable fixation methods, rapid return to preinjury functions, minimal risk for infection or disease transmission, biodegradation at a rate that provides adequate mechanical stability, and supporting host tissue ingrowth [8]. Cellular adherence and matrix formation are also included in the design factors of ligament tissue engineering [9].

Another important aspect that should be taken into account in the clinical translation of tissue engineered ligament is the ligament-bone interface, which consists of a multilayered transition zone. The tissues involved in this interface display distinct mechanical properties; the ligament is strong in tension and bone is strong in compression [10, 11]. Therefore, interface is challenging for tissue engineers to mimic creating one of the current field of interests in ligament tissue engineering.

Anterior cruciate ligament (ACL) and medial collateral ligament (MCL) as well as the glenohumeral ligaments are the most frequently practiced ligament tissues to date, while all ligaments are in the pursuit of tissue engineering and studies are being carried out to create functional biological replacements of these tissues.

2. Growth Factor and Cell Therapies in Ligament Repair

Growth factors are regulators of cellular activities and several of them, including insulin like growth factor I (IGF-I), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet derived growth factor (PDGF), were shown to be effective in the healing of ligament repair. In vitro and in vivo studies have shown that these growth factors have the capability to improve ligament cell proliferation and matrix formation alone or in combination [12–21]. Deie et al. have demonstrated that rabbit MCL fibroblasts are responsive to TGF-β1 and EGF [12] and Hildebrand et al. suggested that use of PDGF may improve the quality of ligament healing [13]. In a similar study, TGF-β1 and IGF were shown to modify the metabolic activity of cells of healing ligaments in rabbit MCL [14]. Marui et al. have reported that topical application of TGF-β1, alone or in combination with EGF, may strengthen the ligament by increasing matrix synthesis during its healing processes [15]. Age and fibroblast origin were also found to be important factors in determining the proliferative response to PDGF and bFBF [12, 16]. Schmidt et al. also reported that PDGF, bFGF, and IGF-I can stimulate cell proliferation in ligaments [18]. Kobayashi et al. demonstrated that the application of bFGF enhances neovascularization and the formation of granulation tissue in lacerated canine ACL [22]. Kobayashi et al., in a rabbit study, have developed a quantitative method to assess cell migration and their findings supported previous qualitative observations [23]. They used a mathematical method to analyze cell densities in a wound model and showed that cells moved into cell-free areas [23]. DesRosiers et al. have combined EGF, PDGF, TGF-β1, and IGF-I two by two and analyzed their effects on cell proliferation and proteoglycan synthesis. Their results showed that EGF and PDGF had a greater effect than TGF-β1 and IGF-I on cell proliferation and proteoglycan production was increased by all four factors, with TGF-β1 having the strongest effect [19]. Others have demonstrated that combination of growth factors can have synergistic effects [20, 21]. Letson and Dahners demonstrated that ligaments treated with a combination of PDGF plus IGF-I and PDGF plus bFGF had increased rupture force, stiffness, and breaking energy [20]. A similar study showed that synergistic effect of combination of bFGF, TGF-β1, bovine insulin, and PDGF was as much as 20-fold of the effects of individual factors [21].

Platelet rich plasma (PRP) and collagen-PRP-complex (CPC) were also shown to be effective in the improvement of ligament healing. In a study of Liu et al., platelet concentration was shown to have a dose-response relationship with proliferation of mesenchymal stem cells (MSCs), fibroblasts, and production of collagen type I in vitro [24]. Thus, PRP is attributed to be an effective agent for ligament healing [25, 26]. Studies have supported this idea, showing effects of PRP on intraarticular ligament homogeneity [27], increase in load at yield, maximum load, and linear stiffness [28]. However, other studies concluded that application of PRP did not yield any advantage over standard ACL reconstruction procedures [29, 30]. In contrast, the use of CPC on ACL allografts was reported to inversely correlate with sagittal plane laxity [31]. Murray et al. used CPC to fill the ACL wound site at the time of suture repair in pigs and concluded that CPC scaffolds result in improved biomechanical properties [28].

Cell sources used in the repair of ligaments include MSCs which have revealed great potential in tissue engineering as a cell source that can differentiate into various connective tissue cell types including fibroblasts [32–35]. It was shown that MSC concentration at the site of injury can be augmented by allogenic MSCs delivered via the bloodstream [33]. Dermal fibroblasts are another possible cell source [36] which is easier to harvest and also display low donor site morbidity. Presence of such cells was reported to enhance peak breaking stress of hybrid collagen biomaterials in an in vitro study [37].

Functioning of certain cells can be altered via introducing DNA fragments using retroviral, adenoviral or liposomal carriers [38]. After successfully introducing a marker gene and detecting its expression in rabbit MCL and ACL [39], potential therapeutic genes such as TGF-β1, IGF-1, PDGF, and bone morphogenetic protein- (BMP-) 12 were also successfully introduced and expressed [40–43]. Steinert et al. have investigated the transfer of IGF-I genes using an adenovirus vector to a collagen hydrogel inserted between the cut ends of the ACL and reported promising results [40]. PDGF gene introduction was shown to enhance and accelerate matrix synthesis in a rat study and therefore claimed to be a useful tool for improving ligament repair [41]. Collagen hydrogels were used to augment ACL healing in a bovine

model and increased cell accumulation was reported with TGF-β1-transferred hydrogels [42]. BMP-12 gene transfer was reported to result in a twofold increase of tensile strength and stiffness of repaired tendons in a chicken model [43].

3. Scaffolds in Ligament Reconstruction

Scaffolds are important components of tissue engineering strategy as they define the ultimate shape of the construct while providing the required mechanical strength during regeneration and proper cell attachment sites. Although there are alternate views on the ideal material, and the structure and composition of it, for ligament tissue engineering, it is generally believed that a scaffold that allows immediate load bearing and degrades at a comparable rate with the tissue regeneration would form the ideal engineered ligament [9].

Natural and synthetic materials have widely been used as ligament replacements in the forms of gels, membranes, or 3D scaffolds. Collagen, silk, hyaluronic acid (HA), ECM bioscaffolds such as porcine small intestine submucosa (SIS), and urinary bladder membrane (UBM) and polyhydoxyalkanoates (PHAs) such as poly(β-hydroxybutyrate) (PHB), poly-3-hydroxybutyrate-co-hydrovalerate (PHBV), and poly-3-hydroxy-10-undecenoate (PHUE-O3) are examples of potential natural replacements, whereas Dacron polyester, poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), poly(lactic acid-co-glycolide) (PLGA), high molecular weight PLGA (HMW PLGA), poly(ε-caprolactone) (PCL), poly(ethylene oxide) (PEO), and poly(urethane urea) (PUUR) are examples of synthetic materials.

Collagen was one of the first natural scaffold materials to be used in ligament reconstruction as it is the natural component of the native tissue and has great ability to support ligament fibroblast growth under static tension [44]. However, collagen scaffold alone was found to be ineffective to enhance suture repair of the ACL [45]. Fibroblast-seeded collagen scaffolds, on the other hand, were more effective in ligament regeneration [44, 46–48]. It was shown in vitro that such scaffolds are consolidated with ECM and that DNA content increased rapidly over the first weeks [46]. In vivo studies have shown that fibroblast-seeded ligament analogs remain viable after implantation into the knee joint [47, 48]. Collagen scaffolds were also combined with PRP [25, 28] and various autologous and allogenic cell types [49, 50] to achieve enhanced mechanical properties and repair.

Silk was also effectively used as a ligament replacement material. It has a relatively slow rate of degradation within the body compared to collagen and other most widely used natural biomaterials which could possess an advantage in load-bearing applications. Its main advantage is its remarkable tensile strength and toughness compared to most natural materials although being lower than native human ACL [51]. The use of pure silk was shown to include problems associated with the sericin protein it contains as it may lead to allergic reactions [52]. This issue was tried to be overcome by the use of virgin silk, in which this allergen was extracted [53, 54].

Silk fibroin is a protein excreted by silkworms and isolated from sericin [55]. This protein has surface amino acids for cell adhesion and slowly degrades in aqueous solutions [56]. It can be fabricated into gels, films, and fibers. In animal models, silk fibroin has been reported to regenerate ligaments, thus claimed to be an excellent natural biomaterial alternative to collagen [9]. MSC-seeded silk fibroin scaffolds [57] and hybrid silk fibroin-silk sponge scaffolds [58] were also developed and demonstrated to be good alternatives for in vivo ligament replacement.

Composite natural scaffolds have also been fabricated using silk and collagen and then seeded with cells [59]. Due to its rapid tissue ingrowth, this chimeric silk-collagen sponge matrix was suggested to be an effective treatment for MCL transactions [59].

ECM bioscaffolds such as SIS and UBM are composed of collagen and contain cytokines and growth factors [60, 61]. ECM bioscaffolds were found to support ligament regeneration and repair and claimed to be effective candidate tools for ligament tissue engineering [62–67]. Dejardin et al. used SIS to promote regeneration of large fascial defects in adult dogs and reported promising results [62]. In a goat model, Badylak et al. reported that SIS holds promise as a resorbable bioscaffold for ACL repair [63]. MCL was shown to have better mechanical properties when SIS is applied in the healing process [64, 65]. Musahl et al. reported that SIS treatment improves not only the mechanical properties but also the histological appearance of the MCL [66]. In a more recent study, Woo et al. demonstrated that SIS enhances the fibril morphology and the collagen composition of healing MCL in rabbits [67].

In a rabbit study, Wiig et al. bilaterally lacerated ACL in the midsubstance and injected hyaluronic acid in one and saline to the other knee. The results showed increased synthesis of type III collagen in the hyaluronic acid treated injured ACL [68]. Recently, it was reported that chitosan-hyaluronan hybrid fibrous scaffolds enhance type I collagen production and improve mechanical strength in the engineered ligament in vivo [69]. PHB, PHBV, and PHUE-O3 were also reported to be good candidates for ligament tissue engineering [70].

The use of synthetic polymeric biomaterials has several advantages over the natural ones, such as they are more readily available, can be produced in large scale with low cost, and are easier to process. Moreover, their mechanical strength is mostly higher compared to natural biomaterials which offer an advantage in the engineering of tissues which are required to handle mechanical forces such as ligaments. On the other hand, they may have some disadvantages such as involving unnatural degradation byproducts and may lack functional chemical cellular binding groups [9].

Among the synthetic polymeric biomaterials used in ligament regeneration, Dacron, which is basically poly(ethylene terephthalate) (PET), is a nondegradable ligament prosthesis. It was shown that seeding of fibroblasts allowed for a more uniform connective tissue encapsulation [71].

Polyhydroxyesters that degrade by hydrolysis such as PLLA and PGA are biodegradable polymers that are popularly used in ligament repair [72, 73]. Braided PLGA scaffolds were claimed to have great promise for ligament engineering [74] while PLLA scaffolds were shown to be a more appropriate choice for ligament tissue engineering because of their

slower degradation rate [75]. HMW PLGA was reported to allow more MSC attachment and proliferation than PLGA [76]. To optimize degradation rates and hydrophilicity that determines cell adhesion, composites of these materials are often fabricated [77].

A newer synthetic polymer, PUUR, was shown to have a similar loading profile as a postmortem-tested human ACL [78], and no relaxation or fatigue was observed after 50 repetitive cyclic loading [79]. PUUR was reported to keep at least 50% of its original tensile strength at body temperature for more than 9 months. Taking into account together with the strength and stiffness properties, PUUR was claimed to fulfill the desired properties for ACL reconstruction [80]. It was also shown that native cells have migrated into the implanted PUUR and that neovascularization between its fibers was detected, indicating that it is well tolerated by the host [79]. In a rabbit study where PUUR was used as a full ACL prosthesis, the knee function was reported to be intact even after 24 months [79].

4. Engineering the Ligament-Bone Interface

The ligament-bone interface consists of four distinct but continuous regions: ligament, noncalcified fibrocartilage, calcified fibrocartilage, and bone [81–83]. It is well known that the native interface is not regenerated in case of an injury [84, 85]. For recreating this multi-zone organization, it is essential to have a stratified or multi-phased scaffold that exhibits a continuing increase in mechanical properties through the scaffold phases [86]. In addition to such stratified, multiphased or 3D braided scaffolds, stem cell applications, cytokines, BMP-2, and BMP-12 are also considered in order to improve regeneration of this interface.

Coating of tendons with calcium phosphate layer [87], TGF-β [88], and BMP-2 [89–91] was found to improve osteointegration between ACL and bone tunnel, however not the fibrocartilage interface.

Multiphased, porous knitted silk [92], 3D braided PLLA [93], and poly(ethylene glycol) hydrogel incorporating HA scaffolds [94] were engineered to mimic ligament-bone interface and promising results were reported. Another innovative triphasic scaffold that has three distinct yet continuous phases including chondrocytes along with fibroblasts and osteoblasts was developed intending to regenerate the fibrocartilagineous interface [95, 96]. A further engineered ligament equivalent is reported to be a fibroblast-embedded fibrin gel with cast brushite anchors [97]. A multiphase tissue-engineered construct for ACL grafts using bone marrow origin MSCs was presented as a viable option for ACL replacement [98] using sheep as a model system.

5. Physical and Chemical Modification of Ligament Tissue Engineering Scaffolds

It is important to optimize cell-biomaterial interactions to achieve enhanced regeneration, mainly in terms of cell attachment and ability of cell proliferation and matrix secretion. Cell surface integrin receptors typically mediate cell-matrix interactions and the most common peptide sequence is arginine-glycine-aspartic acid (RGD) which has been used in a number of studies to achieve enhanced cell attachment [99].

In general, phosphate, amide, and sulfonate groups are used as functionalizing groups in tissue engineering applications [9]. To enhance scaffold-mediated tissue repair, growth factors are also used for signaling [100]. In a porcine model, it was shown that adding CPC to a suture repair enhances biomechanical and histological properties of the ACL via increasing cellularity within the healing ligament [26]. Silk fibroin was functionalized with MSC seeding and blending it with hyaluronan [101]. Functionalization of PET scaffolds was done with poly(sodium styrene sulfonate) (PNaSS) which was reported to have more fibroblastic adherence than nonfunctionalized fibers [102].

6. Bioreactors in Ligament Tissue Engineering

The body itself can be used as a "bioreactor" when cell-scaffold composites are directly implanted into the injured site or ex vivo bioreactors can be used for a period of time to achieve mature constructs prior to transplantation [103]. Ex vivo bioreactors allow application of controlled biochemical and physical regulatory signals to guide differentiation, proliferation, and tissue development [103].

Initial bioreactors applied uniaxial forces to tethered constructs [104]. More recent bioreactors are capable of applying multiaxial and cyclic strains, which better mimics the native physiology [8, 105–107]. Although the use of such bioreactor systems is a relatively new practice in ligament tissue engineering, the results are promising, which positively affects the cell proliferation and differentiation of stem cells towards musculoskeletal lineages in most if not all cases [9]. However, type, magnitude, and duration of mechanical stimuli and, thus, the ideal stimulation regime have not yet been described [9].

Application of mechanical loading was reported to have positive effects on cellular proliferation in various studies [104, 108, 109]. Mechanical loading was also shown to effect cellular morphology and alignment [104, 108–111]. The differentiation of MSCs in the presence of mechanical loads was shown to tend towards the ligament lineage [108, 112–115]. ECM synthesis and remodeling [109, 116–120] is another factor that is shown to be affected by mechanical loading. Studies have also reported that enzyme activity and growth factor expression [109, 121–123] and (6) Collagen type I, collagen type III, elastin, and tenascin-C expression in MSCs [108, 112–114] were increased with the application of mechanical loads.

7. Conclusion and Future Directions

This manuscript provides an overview of the previous applications and current concepts in ligament tissue engineering. Combining different approaches seems to be mandatory in order to assemble ligament-like tissue structures. Such combinations may include braiding, stratifying, knitting, or 3D braiding scaffolds as well as merging scaffolds with sponges, merging different material types in a single multiphased

scaffold, aligning or cross-linking its cellular content, functionalizing its surface, and adding mechanical ex vivo stimulation. Scaffolds further need to possess a porous structure and full pore interconnectivity to allow ingrowth of native cells and connective collagen fibers.

Biomimicry is the main strategy with the intention of developing functional artificial tissues in tissue engineering. To mimic the structure as well as function of the native tissue, the interface is a great challenge and multiphased scaffolds constitute a promising option to fulfill this need.

Various types of growth factors, stem cells, cytokines and plasma ingredients; gene delivery; a range of natural and synthetic materials, and effects of mechanical loading and functionalizing have been and are being investigated. Clearly, much work remains but there are exciting and promising advances. Important future targets should include developing scaffolds that match tissue ingrowth rate with its degradation rate, matching native biomechanical properties, and should have improved strength and biological integrity as well as being able to mimic the properties of tissue interfaces.

Although many steps have been taken, to date, tissue engineering is probably still far from producing the ideal bioscaffold to replace, repair, or regenerate injured ligaments. Clinician-scientist coordination is indispensable for achieving such a goal. Along with this multidisciplinary approach, interdisciplinary contribution from biologists, chemists, biomaterial scientists, and tissue engineers is needed for meeting patients' demands.

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Review Article

The Potential of Stem Cells in the Treatment of Skeletal Muscle Injury and Disease

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Tissue engineering is a pioneering field with huge advances in recent times. These advances are not only in the understanding of how cells can be manipulated but also in potential clinical applications. Thus, tissue engineering, when applied to skeletal muscle cells, is an area of huge prospective benefit to patients with muscle disease/damage. This could include damage to muscle from trauma and include genetic abnormalities, for example, muscular dystrophies. Much of this research thus far has been focused on satellite cells, however, mesenchymal stem cells have more recently come to the fore. In particular, results of trials and further research into their use in heart failure, stress incontinence, and muscular dystrophies are eagerly awaited. Although no doubt, stem cells will have much to offer in the future, the results of further research still limit their use.

1. Introduction

Skeletal muscle is the most abundant tissue of the human body, it is highly dynamic and has the ability to regenerate. Loss of skeletal muscle through trauma, tumour ablation, or prolonged denervation is a common clinical challenge.

Despite the ability of muscle fibres to regenerate, muscle function is often compromised after injury due to the formation of dense fibrotic scar tissue. This may be induced by a rise in TGF-B1 and IGF-1, causing postnatal muscle-derived stem cells (MDSCs) and other myogenic cells to differentiate into myofibroblasts, producing type 1 collagen, the major component of fibrotic tissue [1–5]. Although some studies have looked at the effects of blocking components of the inflammatory cascade, such as agents that block TGF-B1, these treatment methods have potential deleterious side-effects [6–8].

Skeletal myopathies present a different challenge. Muscular dystrophy is caused by a defective gene encoding dystrophin, which links the extracellular matrix in muscle to the intracellular cytoskeleton. As skeletal muscle is composed of large multinucleated fibres whose nuclei cannot divide, cell therapy has to restore gene expression in hundreds of millions of postmitotic nuclei [9].

Clinical application of skeletal muscle engineering in human subjects thus far has been limited, with clinical trials on humans concentrating on cardiac disease, stress incontinence of the bladder, and muscular dystrophies. This in part is due to the challenges of transferring ex vivo to in vivo tissue engineering. It is also due to the complexity of the microenvironment needed to ensure stem cell integration and function. This review will focus on the potential of stem cells for skeletal muscle engineering; their sources, microenvironment, and clinical applicability.

2. Anatomy

The formation of skeletal muscle begins during the fourth week of embryonic development as specialised mesodermal cells, termed myoblasts, begin rapid mitotic division. The cytoplasmic fusion of myoblasts forms what is known as myotubes, and by week nine of development these can

be identified as multinucleate skeletal muscle cells, termed muscle fibres. By month five, the muscle fibres are accumulating protein filaments important in muscle contraction. As growth of the muscle fibres continues, aggregation into bundles occurs, and by birth myoblast activity has ceased.

The electromicroscopic structure of a muscle fibre reveals a structured longitudinal arrangement of proteins—named myofilaments. These are arranged in groups within the muscle fibre known as myofibrils. The major myofilaments are actin, and myosin. These form functional sub-units known as sarcomeres. Muscle contraction on a subcellular level is a complex process in the sarcomere involving influx of calcium ions into the muscle fibre, and interaction between myosin, actin and the proteins troponin and tropomyosin. This results in the myofilaments sliding relative to one another, generation of ATP, shortening of the sarcomere, and subsequent contraction of the muscle belly.

Contracting muscle fibres would be ineffective if they worked as isolated units. Each fibre is bound to adjacent fibres to form bundles. An accumulation of muscle bundles forms the muscle belly itself. Supporting connective tissue is present, surrounding, and within the muscle. The endomysium surrounds individual fibres, the perimysium encloses the fascicles, and the epimysium surrounds the muscle belly itself.

3. Sources

Stem cells may be totipotent, pluripotent, or multipotent, depending on tissue type. Totipotent cells form all the cells and tissues that contribute to the formation of an organism. Only the embryo itself is totipotent. Pluripotent cells can form most cells of an organism from all three germ cell layers. Embryonic stem cells present in the fertilised oocyte, zygote, and morula [10]. Pluripotent cells have the ability to expand in vitro almost indefinitely and form tissues from ectoderm, mesoderm, or endoderm. Concerns about tumour formation in vivo and ethical concerns regarding their harvest have thus far restricted their use.

Multipotent cells form a number of cells or tissues that are usually restricted to a particular germ layer. Multipotent cells are derived from specific tissue compartments in the adult. The two main types of multipotent stem cell are haemopoietic and mesenchymal type, both usually derived from adult bone marrow, but occasionally from fat, skin, periosteum, and muscle, as described below. Mesenchymal stem cells (MSCs) are multipotent, capable of differentiating into several connective tissue types including osteocytes, chondrocytes, adipocytes, tenocytes, and myoblasts [11]. Mesenchymal stem cells have the advantage of being easily obtainable in adult tissue, and with the appropriate microenvironment, can differentiate into various target tissue types.

For skeletal muscle engineering, most research thus far has focused on the satellite cell. The satellite cell was first described by Mauro in 1961, who observed them as mononuclear cells between the basal lamina and plasma membrane (sarcolemma) of the muscle fibre [12]. In response to injury, satellite cells are activated, differentiate into myoblasts, and proliferate. They either fuse with themselves, damaged muscle fibres, or remain quiescent as satellite cells at the sarcolemma. Satellite cells are characterised by expression of the muscle-specified paired box (Pax) transcription factor Pax7 [13]. They also consist of a majority of Myf5+ cells which act as an initiator of myogenic differentiation, marking the commitment of this cell population to the myogenic lineage [14].

Satellite cell usage has been promising. Studies have demonstrated their ability to regenerate large parts of musculature in vivo with low tumourgenic potential [15, 16]. Extracellular factors are necessary for the function of the satellite cell, and ex vivo studies have shown rapid dedifferentiation after a few cell cycles [17]. These cells have potential for the treatment of muscular dystrophy. Early studies in mice lacking the gene for dystrophin production, showed that an injection of normal satellite cells into the muscle belly resulted in fusion with host fibres and extensive production of dystrophin [18]. Later studies however showed an immune response to the satellite cells and poor survival [19]. More efficient methods of delivery have been researched including transplanting individual muscle fibres (containing at least seven satellite cells) or isolating "purer" sources of satellite cells. Although some of these studies have shown promising results, the inability of these cells to cross the endothelial cell wall makes systemic delivery impossible, which impacts on their use to heal diseased diaphragm and cardiac muscles [20].

MSCs can be obtained from a variety of different sources which can harbour myogenic potential. The first evidence of this was reported in 1998 in transgenic mice, showing that transplantable progenitors in bone marrow could be recruited to injured muscle and take part in repair [22]. Many studies have shown their potential in differentiating ex vivo into skeletal muscle under the right conditions [23–25]. Some studies have shown a low incorporation rate of MSCs into myofibres [26]. MSCs can however impose an additional paracrine effect on differentiation and tissue regeneration via cytokine pathways [27]. MSCs, unlike satellite cells maintain their stem-cell characteristics when systemically delivered and pass through vascular walls into target tissues [28]. There are a number of other tissue sources of stem cells for skeletal muscle engineering which are summarised in Table 1. MSCs are recognised using a range of cell surface markers as shown in Figure 1 [21].

4. Matrices

In vivo, the extracellular matrix of muscle provides fibres with the architecture to support development and function. It is a highly organised tissue with high cell density, with the parallel orientation of muscle fibres generating longitudinal contraction [29]. During tissue engineering, therefore, a scaffold is needed to mimic this matrix. In vivo studies have shown that stem cells with extracellular matrix, when injected into damaged muscle such as gastrocnemius, can significantly improve functional recovery when compared to matrix alone [30]. There are many different permutations to matrix structure and material. Matrix structure can be two-dimensional or three-dimensional. Scaffold material can be biodegradable or nonbiodegradable. Biodegradable matrices

Table 1: Potential of other cell sources for skeletal muscle engineering.

Cell type	Source	Potential advantages
Skeletal muscle side population	Skeletal muscle	Can be delivered systemically May have increased capacity to incorporate into stem cell of muscle
Muscle-derived stem cells	Skeletal muscle	Can undergo myogenic and osteogenic differentiation Can repopulate haematopoietic lineage
Mesoangioblasts	Other mesodermal tissues, for example, dorsal aorta	Can be delivered systemically May be able to efficiently regenerate normal skeletal muscle
Pericytes	Basement membrane adjacent to endothelial cells Pancreas, adipose tissue, placenta	May improve the physiological performance of skeletal muscle Can be easily manipulated in vitro to reduce host's immune response
Adipocytes	Adipose tissue	Proven good differentiation into myogenic cells in vitro and in vivo
Embryonic stem cells and induced-pluripotent stem cells		Can regenerate acutely and chronically injured muscle but concerns of tumourigenic potential and ethical concerns

can be synthetic or natural. There are relative advantages and disadvantages to each.

The extracellular matrix of muscle in vivo is three-dimensional. Traditional cell culture has made use of 2D (two-dimensional) surfaces for ex vivo cell growth and is valuable in identifying cell structure and differentiation. In such environments, cells are forced to adopt unnatural characteristics, including aberrant flattened morphologies. 2D culture is not suitable for engineering 3D muscle tissue. Advantages of 3D over 2D culture include enhanced proliferation and differentiation of stem cells. In addition, 3D culture is more likely to accurately reflect the in vivo tissue environments from which cultured cells are derived.

Recent research on 2D "cell sheet technology" has shown promise, however, using temperature-responsive 2D scaffolds made out of a polymer, poly (N-isopropylacrylamide). Cell layers (with their extracellular matrix) can separate out with increasing temperature, obviating the need for enzymes [31]. Parallel alignment of fibres can be reached by techniques such as "electrospinning" and "microgrooving." Microgrooving uses abrasives to create microgrooves within the matrix and has shown promising results in the orientated cell growth of myoblasts [32]. Electrospinning technique uses electrical current to form fibres as well as proteins of the natural extracellular matrix and can uniquely mimic the structure of the natural extracellular matrix [33]. Out of these 2D cell sheets, 3D matrices can be made, from 2D layering on a vascular bed. One disadvantage of cell-sheet technology is the inability of myoblasts to proliferate and differentiate more than 150 micrometres from a nutrient source [34]. Also, the electrospinning of nanofibres can often lead to them being densely packed, which can lead to poor cell infiltration [35].

The vast majority of scaffolds developed are biodegradable. When these degrade, remodelling to the natural muscular extracellular matrix can occur [36]. 3D scaffolds made from synthetic material such as polylactic-co-glycolic acid (PGA) fibre mesh sheets can provide rigidity and connection [37]. The nano- and microscale features of a polymer scaffold cause alignment of myoblasts and cytoskeletal proteins and promote myotube assembly to mimic the organisation seen in skeletal muscle. The surface stiffness in the polymer can help in the differentiation of satellite cells [38]. Parallel alignment can be induced by applying a strong magnetic field or mechanical strain [39].

Natural biodegradable 3D scaffolds also contain aligned topographical features causing alignment of myoblasts and proteins. Fibrin can be used, mixed with satellite cells and a growth medium. The original fibrin matrix is eventually taken over by the muscle progenitor cells, which produce their own extracellular proteins. Fibrin has the advantage of being able to bind growth factors such as IGF-1. In vitro models have been encouraging, showing that normal skeletal muscle in structure and function can be produced [40]. Collagen has also been used as a biodegradable 3D scaffold in some studies to good effect [41]. The type of proteins used for the scaffold is important. A recent study in mice showed that stem cell proliferation and differentiation using laminin and Matrigel was superior to collagen type 1, fibronectin, and gelatin [42].

In summary, although a pluripotent cell source is desirable, the tumour-forming potential in the use of these cells at present likely represents an unacceptable risk. Therefore, taking into account the literature discussed, a satellite cell source in a 3D matrix with a biodegradable scaffold appears to be the current optimum method of skeletal muscle tissue engineering.

5. Clinical Applications

Clinical trials on human subjects are limited due to the difficulties encountered with satellite cells, and the myogenic potential of alternative progenitor cells, delivery methods of these cells and the search for the "ideal" matrix. Highlighted below are the main clinical findings from human trials.

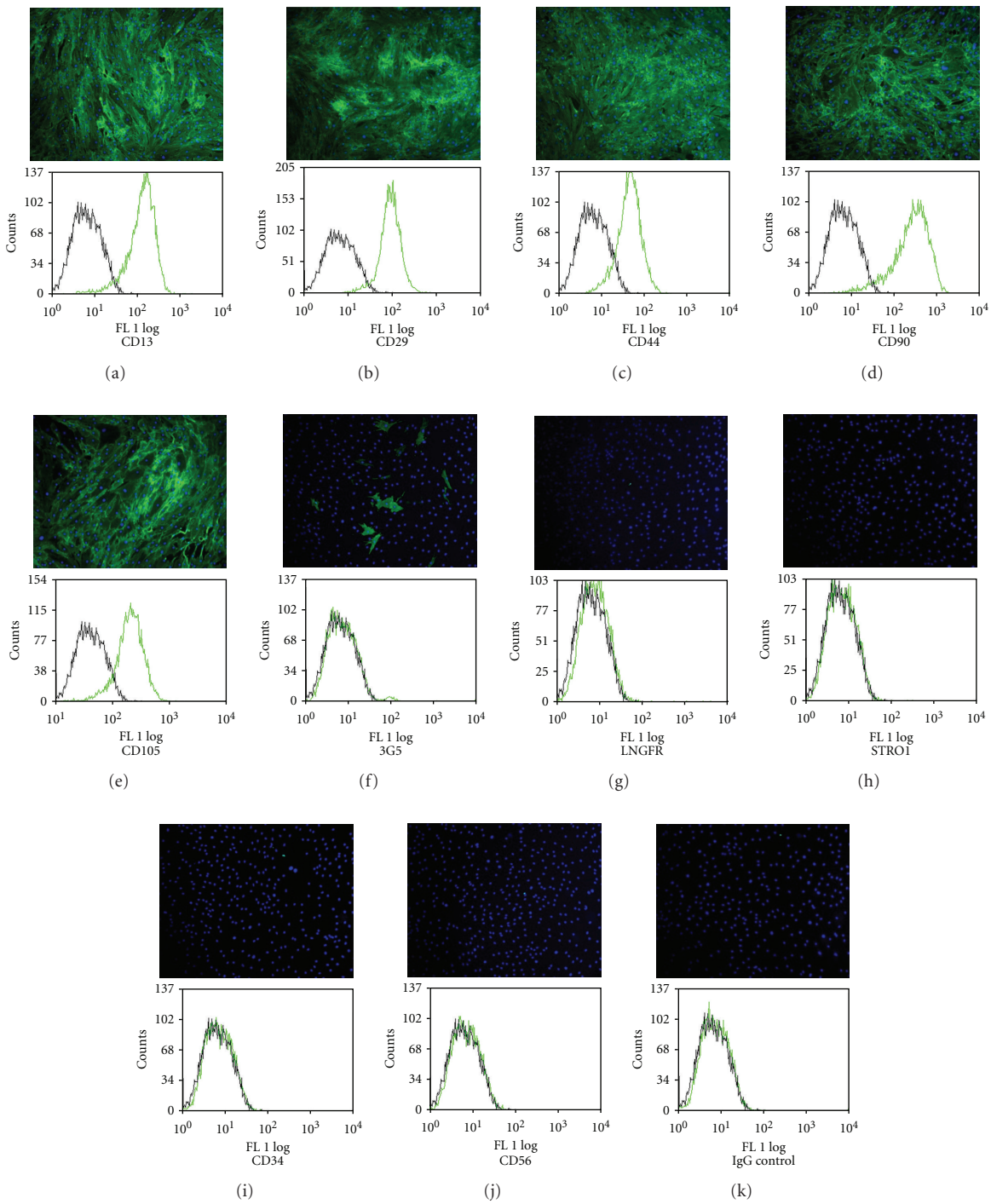

Figure 1: Cell surface epitope characterisation of passage 2 infrapatellar fat-pad-derived stem cells using a panel of antibodies. Cell surface staining using FITC-conjugated secondary antibody (green) and DAPI (blue) shows that the cells stain strongly for CD13, 29, 44, 90, and 105, and poorly for 3G5, LNGFR, STRO-1, and CD34 and 56. No staining was observed for the IgG control. The staining pattern is confirmed by flow cytometry and shows the increase in fluorescence (green) compared with the autofluorescence (black) [21].

5.1. Muscular Dystrophies. These are a group of heterogeneous disorders producing progressive weakness, muscle wasting, and in the case of Duchenne muscular dystrophy usually paralysis and death in the patient's early 20s. Traditional treatment was limited to pharmacological suppression of the immune response with cortocosteroids.

Since the 1990s, from the first clinical trial in humans, it has been shown that stem cell transplantation via intramuscular injection can lead to dystrophin production. In 1991, Law injected extensor digitorum brevis (EDB) muscle in each of three Duchenne muscular dystrophy (DMD) boys with myoblasts. He demonstrated increases in isometric twitch

and voluntary contractions whereas sham-injected EDBs showed reductions. All patients expressed dystrophin in their muscles following injection [43]. The next landmark study by Law involved 21 patients, with intramuscular injections of myoblasts. At 3 months, of the 69 muscle groups tested for isometric force generation in these subjects, 43% showed a mean increase of 41.3%. Eighty-one percent of the muscles tested showed either an increase in strength or did not show continuous loss of strength [44].

Several other studies began to question the longevity of muscle function following the intramuscular injections. Karpati et al. [45] showed that 12 months following multiple intramuscular bicep injections in 8 patients, only 3 had improved muscle strength. Tremblay et al. [46] showed in 5 patients that one month after myoblast transplantation into tibialis anterior (TA), the percentage of dystrophin-positive fibres ranged from 0–36%, compared to 0–4% on the control side. The expression of dystrophin in these fibres was generally low and most likely less than 10% of the normal level. In the biceps brachii on both sides 6 months after the transplantation, less than 1.5% of dystrophin-positive fibres were detected. No patients had improved strength at followup.

Mendell et al. injected donor myoblasts once a month for six months into the biceps brachii muscle of one arm in 12 boys with Duchenne's muscular dystrophy. Six months after treatment, there was no significant difference in muscle strength between the arms injected with myoblasts and sham-injected arms. In one patient, 10.3 percent of muscle fibers expressed donor-derived dystrophin after myoblast transfer. Three other patients also had a low level of donor dystrophin (<1 percent); eight had none [47].

Neumeyer et al. evaluated myoblast implantation therapy in three subjects with Becker muscular dystrophy. Each patient received 60 million myoblasts implantated into one TA muscle. They had begun cyclosporine immunosuppression two months prior to implantation and this was continued for 1 year. Results showed that myoblast implantation did not improve strength of the implanted TA muscles [48]. Skuk et al. showed similarly disappointing results after myoblast transplantation in the TA of 9 patients with the percentage of myofibers expressing donor's dystrophin varying from only 3.5% to 26% at 4-week follow-up [49].

Several other studies have shown a similar trend, but with no significant improvement in muscle function [50]. One of the problems with intramuscular injections for systemic conditions is the need to perform large numbers of injections to target different areas of muscle in order to gain a clinical response. Secondly, as already highlighted, vital muscles, such as the diaphragm for respiration, are not suitable for this form of treatment.

It was shown by Gussoni et al. [51] in mice that a marrow-derived cell could migrate into areas of muscle degeneration, undergo myogenic differentiation, thereby participating in muscle repair. Systemic delivery obviously holds the advantage of negating the need for multiple injections into the muscle belly, although an immune response to these cells is possible. Recent developments in the field of gene transfer therapy promise hope for future treatment possibilities. Cassano et al. recently showed that electrotransfer of "Magic"-Factor-1 gene into adult mice promoted muscular hypertrophy, improved running performance, and accelerated muscle regeneration after injury [52]. Phase I trials after gene transfer in patients with Duchenne muscular dystrophy have shown no adverse events [53].

It is likely that a strategy for treatment of these disorders will require a combination of stem cell and gene transfer techniques and we await the results in a few years time from ongoing trials.

5.2. Heart Failure. Like skeletal muscle cells, myocardial cells are striated; containing actin and myosin filaments arranged in the form of sarcomeres. They differ in that they interconnect through gap junctions to transfer electrical impulses. Muscle-derived myoblasts are considered an optimal cell therapy for heart failure, as they can be easily obtained from the same patient, rapidly expanded in vitro, and transplanted back into the patient's heart [20].

Several randomised controlled trials have shown benefits after transepicardiol injections of skeletal myoblasts [54–56]. Patients have benefited through an increased left ventricular ejection fraction, end-systolic volume, and subsequent symptomatic improvement. Concern remains about the increased occurrence of ventricular tachycardia following treatment. Ex vivo studies have shown embryonic stem cells to be of value in the development of new myocardial tissue [57].

5.3. Stress Urinary Incontinence. Stress urinary incontinence (SUI) is characterised by the loss of small amounts of urine when intra-abdominal pressure increases through laughing, coughing, or exercising. Muscle, connective tissue, and nerve damage during childbirth appears to be the most important risk factor [58]. Traditional treatment options of pelvic floor muscle training, pharmacological agents, and surgical solutions have had limited success. Recently, stem cell treatment has focused on treating the connective tissue and skeletal muscle component of the rhabdosphincter—thought to be the structure most important in controlling continence [59].

Most clinical trials in humans have involved muscle-derived stem cells injected under transurethral ultrasound guidance, together with a fibroblast/collagen suspension followed by pelvic floor exercises and transvaginal electrical stimulation [60, 61]. Numerous studies have shown benefit in females with stress incontinence, showing one-year cure rates up to 93% [60–63]. These patients have shown increased electromyelogram activity in the rhabdosphincter and increased urethral thickness. There have been reported benefits of using autologous-derived adipose stem cells in some patients [64]. Studies have also shown benefits of using stem cells in men for postprostatectomy stress incontinence [65].

6. Summary

Stem cells are emerging as a potential source of tissue repair and regeneration in many musculoskeletal tissues [66–80].

Although most advances have been made with bone, cartilage, tendon, and ligaments [81–109], this review shows that the application of stem cells in skeletal muscle regeneration following injury and disease is slowly emerging. Although satellite cells have attracted much interest due to their commitment to the myogenic lineage, their ability to cross the endothelial junction is limited, thus meaning locally-delivered transplantations are required. An appropriate matrix is needed to cultivate stem cells prior to their delivery in vivo. Human trials thus far have concentrated mainly on patients with muscular dystrophies, heart failure, and stress urinary incontinence. While successful results have been shown in patients treated with myoblast transplantation in heart failure and urinary incontinence, stem cell use in muscular dystrophies has so far been limited. Recent studies using gene therapy in combination with stem cell transplantation has shown some promise. Treatment using stem cells for skeletal muscle regeneration should combine a systemically delivered progenitor cell with controlled differentiation into myoblasts in vivo which can cross the endothelial lining of the blood vessel and target damaged muscle. We look forward to future studies developing current techniques and highlighting potential uses in the regeneration of skeletal muscle following trauma and disease.

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Clinical Study

Treatment of Chronic Patellar Tendinopathy with Autologous Bone Marrow Stem Cells: A 5-Year-Followup

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The purpose of this study is to determine if patients with chronic patellar tendinopathy will improve clinically after the inoculation of bone marrow mononuclear cells (BM-MNCs). Eight patients with chronic patellar tendinopathy were included. Patients averaged 24 years old (range 14–35). All patients were refractory to conservative treatment for at least 6 months before the procedure. BM-MNCs were harvested from the iliac bone crest and inoculated under ultrasound guide in the patellar tendon lesion. Improvement was assessed through established clinical scores and ultrasound. At 5-year followup, statistically significant improvement was seen for most clinical scores. Seven of eight patients said they would have the procedure again if they had the same problem in the opposite knee and were completely satisfied with the procedure. Seven of 8 patients thought that the results of the procedure were excellent. According to our results, inoculation of BM-MNCs could be considered as a potential therapy for those patients with chronic patellar tendinopathy refractory to nonoperative treatments.

1. Introduction

Chronic patellar tendinopathy is a common and disable disease. Despite improvements in early detection, evaluation, and advance rehabilitation techniques, treatment is long and tedious. Multiple approaches, both surgical and nonsurgical, have been suggested.

The precise mechanism by which tendinopathy develops in humans is not quite understood. Histopathology studies have consistently shown that tendinopathy in humans is typically due to tendinosis [1]. Tendinosis is characterized histologically by tissue degeneration with failed reparative response and absence of inflammatory cells. The pathological region is distinct from normal tendon with both matrix and cellular changes. Instead of clearly defined, parallel, and slightly wavy collagen bundles, tendinosis is associated with relative expansion of the tendinous tissue, loss of the longitudinal alignment of collagen fibers, and loss of the clear demarcation between adjacent collagen bundles. Multiple cellular changes coexist with these matrix changes. The most obvious is hypercellularity resulting from an increase in cellular proliferation. There is atypical fibroblast and endothelial cellular proliferation and extensive neovascularization. It has been suggested that fibroblast in tendinosis had an abnormal respond to healing stimulus, probably due to cell transformation [2]. Increased expression of MMP has also been shown in diseased tendons [3].

Recent work *in vitro* has focused on the use of bone marrow mononuclear stem cells (BM-MNCs) combined with growth factors to improve the quality and speed of healing in tendinosis repair. Clinically, PRP or platelet-rich plasma has shown to improve pain and function over time in patients with chronic patellar tendinopathy [4–6].

BM-MNCs are pluripotential cells and are believed to play an important role in connective tissue repair such as tendon, ligament, bone, and cartilage. Several animal studies have shown that the inoculation of these cells can accelerate tendon healing [7–9]. A combination of BM-MNCs and anabolic growth factors would seem an attractive approach for improving connective tissue repair.

Table 1: Sonographic grading of the patellar tendinopathy. Grade ratio between injured area and whole tendon section at US scan.

Grade	
1	Injured area is less than 20% of the whole tendon section
2	Injured area is between 20% and 50% of the whole tendon section
3	Injured area is more than 50% of the whole tendon section
4	Subtotal or total tear, respectively, with partial and total tendon retraction

The objective of this study is to evaluate the outcomes of inoculation of BM-MNCs in patients with chronic patellar tendinopathy of the knee through established outcomes scales, clinical assessment followup, and ultrasound images.

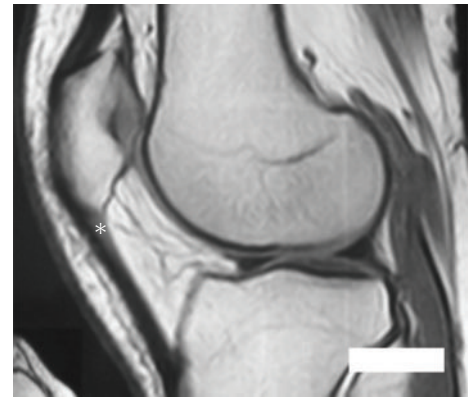


Figure 1: Preoperative magnetic resonance imaging showing hypodens area in the proximal aspect of the patellar tendon (*).

2. Materials and Methods

2.1. Patient Selection. Between June 2005 and September 2006, 8 consecutive patients with chronic patellar tendinopathy treated with mononuclear BM-MNCs were included. Approval for the study was obtained by the institutional review board at our institution, and all patients signed informed consent to participate. Indications for BM-MNCs were history of pain (more than 6 months), tenderness on patellar tendon palpation, and imaging findings of degenerative changes (MRI). All patients had failed previous nonoperative treatment for at least six months including all or a combination of conventional stretching exercises and strengthening, and were classified as grade III-B according to the criteria originally described by Blanzina and later modified by Warden et al. [10] (patients unable to participate in sports at the same level as before the onset of symptoms). Exclusion criteria were systemic disorders such as diabetes, rheumatoid arthritis, coagulopathies, cardiovascular diseases, infections, immunodepression, patients in therapy with anticoagulants, and use of NSAIDs in the five days before bone marrow aspiration. All patients had an ultrasound (US) done before surgery and at 6 months postoperatively. Based on US findings, four grades of patellar tendinopathy were identified (Table 1) [11].

2.2. Bone Marrow Aspiration. Under general anesthesia, bone marrow was aspirated from anterior iliac crest using a bone marrow harvest needle (Medical Device Technologies, Inc., Gainesville, Fla, USA) with 20-mL syringes prefilled with anticoagulant citrate dextrose (1.5 mL) to prevent blood clotting. Ficoll-Paque Premium (3 mL) was added to centrifuge tubes. Then the bone marrow was carefully layered in the Ficoll-Paque Premium, making sure that the bone marrow sample did not mix with the Ficoll-Paque Premium. Then centrifugation was done, and the upper layer containing plasma and platelets using sterile pipette was drawn off leaving the mononuclear cells undisturbed at the interface. The layer of mononuclear cells was transferred to a sterile centrifuge tube. These cells were washed by centrifugation. Supernant was removed and the mononuclear cells were resuspend in 6 to 8 mL balanced solution appropriate for application.

2.3. Haematological Analysis. Haematological analysis was performed according to a previously published method [12]. The numbers of nucleated cells in BMA (bone marrow aspiration) were determined before and after concentration with an automated haematology analyzer. The concentration ratio was determined as follows: concentration ratio = number of nucleated cells in BMA after concentration/number of nucleated cells in BMA before concentration.

2.4. Inoculation. The area of injury was identified, and the zone was clearly marked based on physical examination, imaging studies, and area of maximal tenderness. An ultrasound-guided injection was used to better identify the pathologic area. (Figures 1, 2, and 3). After the injection, the patients were sent home with instructions to limit the use of the leg for at least 24 hs and use cold therapy for pain. Then, patients started with stretching exercises and mild activities (such as bicycle exercises and mild exercised in the pool). After a month, patients were allowed to do recreational sports or activities as tolerated.

2.5. Outcomes Assessment. Only patients with a minimum 24 months followup were included for analysis. Questionnaires were administered preoperatively, 1 year postoperatively and then annually. Subjective measures were based on several scoring systems including Lysholm, Tegner, Cincinnati, international knee documentation committee (IKDC), knee injury ad osteoarthritis outcome score (KOOS) and Short Form-12 (SF12) [13–15]. The KOOS holds 5 separately scored subscales: pain, other disease-specific symptoms, activities of daily living (ADL) function, sport and recreation function, and knee-related quality of life (QOL) [12]. Patients were also asked to rate the overall condition of their knee at the time of the last followup: 0 to 2 poor (significant limitations that affect activities of daily living); 3 to 4, fair (moderate limitations that affect activities of daily living, no sport possible); 5 to 6, good (some limitation

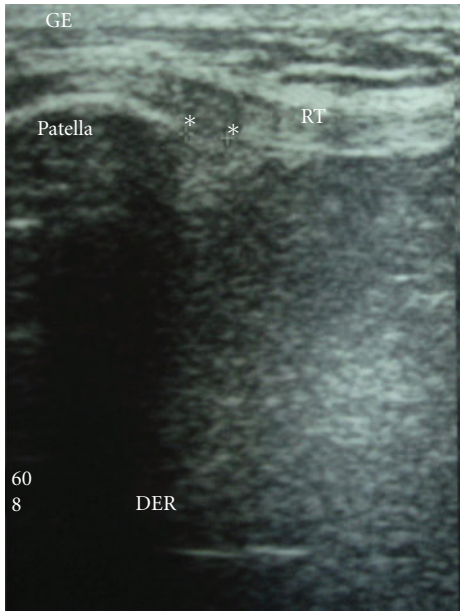


Figure 2: Preoperative US (ultrasound) of the patellar tendon showing areas of edema (hypoechoic) (**). RT: rotulian or patellar tendon.

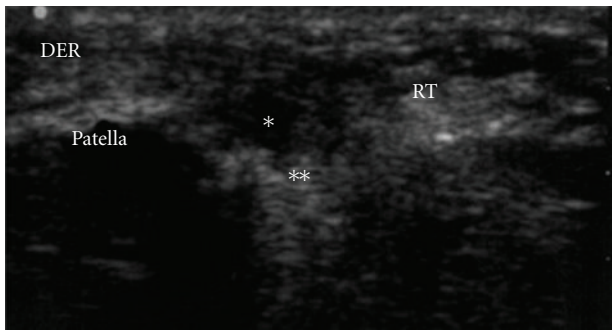


Figure 3: Under US guide, the cells were inoculated. Observe the hypoechoic area (*) where the cells were inoculated. An acustic shadow is evident after the inoculation of the cells (**). RT: rotulian or patellar tendon.

with sports but I can participate, I compensate); 7 to 8, very good (rare limitations, able to participate); 9 to 10, excellent (able to do whatever I wish no problems). Patient satisfaction with the procedure was elicited with the following scale: completely satisfied, mostly satisfied, somewhat satisfied, and unsatisfied. Finally, the patients were asked if, based on their experience, they had the same problem in the opposite knee, would they have the same surgery again.

An ultrasound was performed in all patients prior to inoculation and at 6 months after the injection to objectively evaluate tendon healing.

2.6. Statistical Analysis. Descriptive statistics were calculated according to standard methods, including frequencies, means, standard deviations, and ranges when appropriate. Clinical outcome scores were analyzed at 2 time points: preoperatively and at the most recent followup. Score improvement was calculated using a paired t test. A statistical significance was set at $P < 0.05$. Statistics were performed using GraphPad software (GraphPad Software, La Jolla, Calif, USA).

Table 2: Outcome score changes from preoperative results to final results, with P value for all outcome scales.

Knee scoring system	Preoperative	Postoperative	P value
Tegner	2	8	0.0061
Lysholm	33	53	0.1043
IKDC	36	69	0.047
KOOS			
Pain	47	63	0.2399
Symptoms	44	71	0.0086
ADL	63	90	0.0246
Sport	24	63	0.0078
QOL	50	71	0.0825
SF-12			
Mental	52	57	0.5589
Physical	41	44	0.438

IKDC: international knee documentation Committee; KOOS: knee injury and osteoarthritis outcome score; ADL: activities of daily living; QOL: quality of life; SF-12: short form-12.

3. Results

The mean age of the patients was 24 (range 14–35). Four patients were female and four male. No complications were recorded. The average total number of cells inoculated injected was 30×10^3. The average patient followup was 5 years (range, 3–6). Overall, statistically significant improvement (preoperative to postoperative) for the Tegner (2 to 8, $P = 0.006$), IKDC scores (36 to 69, $P = 0.047$), KOOS symptoms (44 to 71 $P = 0.0086$), KOOS ADL (63 to 90, $P = 0.0086$), KOOS sport (24 to 63 $P = 0.0078$). No statistical improvement was seen for the Lysholm (33 to 53 $P = 0.1043$), KOOS pain (47 to 63 $P = 0.2399$), KOOS QOL (50 to 71 $P = 0.0825$), SF-12 mental (52 to 57 $P = 0.5589$) and SF-12 physical (41 to 44 $P = 0.438$). Detailed overall results are shown in Table 2. Considering each time followup, there was significant improvement at 2 years and plateau till last follow-up at 5 years (Figure 4).

Seven of eight patients said they would have the procedure again if they had the same problem in the opposite knee. Seven of eight patients were completely satisfied with the procedure, one patient was somewhat satisfied. Seven of 8 patients thought that the results of the procedure were excellent (10, scale from 0–10). None of the patients had additional procedures.

3.1. Cells Inoculated. The number of nucleated cells obtained from bone marrow aspiration was 37×10^3 cells (± 10). After concentration (cell recovery 85%) the average total numbers of BM-MNCs were 45×10^3 (± 5).

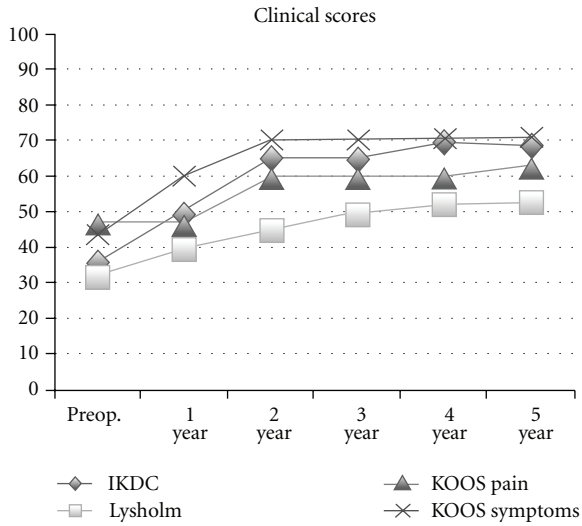
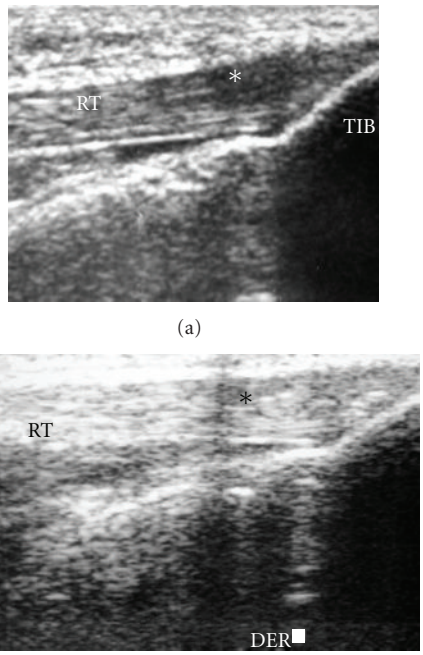


Figure 4: Preop. and yearly time follow-up for IKDC, Lysholm, KOOS pain and symptoms.



(a)

(b)

Figure 5

3.2. Ultrasound Evaluation. All patients were grade 2-3 before inoculation. At 6 months 8 of the nine patients had grade 1. Only one patient had grade 3 (Figure 5).

4. Discussion

In this study, 8 patients with chronic patellar tendinopathy were treated with autologous BM-MNCs. We compared the outcomes of patients before and at 5 years after the inoculation, showing statistical improvement in most of the outcomes scores at the time of followup. Patients reached a plateau after one year followup. Although this can be argued as may be a self-limiting process, none of our patients had a recurrence which is normally reported to be between 12 to 27% in patients treated nonoperatively [9]

The major limitation of this study is the lack of control group, resulting in a low level of evidence study (Level 4) and the few number of patients that were included. Although we did not compare MRI pre- and postoperative to assess healing, we used ultrasound to assess healing before and at last followup. Warden et al. compared the accuracy between MRI and US in confirming clinical diagnosis of patellar tendinopathy. They suggested that US is more accurate than MRI confirming clinical diagnosed patellar tendinopathy (83% versus 70% resp.) [10]. To our knowledge, there have not been published articles that assess the effect of BM-MNCs for the treatment of patellar chronic tendinopathy in patients.

Treatment of chronic patellar tendinopathy is challenging secondary to the low capacity of healing that the tendon has resulting in long and tedious treatments. Identifying alternative strategies is a priority. The tendon itself is relatively cell poor, with a low turnover rate. Recently, it has been proposed that adult stem cells would be good candidates for cell-based tendon regeneration [9, 16]. The exact role of implanted stem cells on tendon healing remains uncertain. One possibility is that they become differentiated into tenocytes within the healing tendon environment and participate in healing through collagen production and remodeling. Alternatively, it has been suggested that BM-MNCs may contribute to healing by acting as "growth factors pumps" rather than through terminal differentiation [11–17].

Chong et al. studied in 57 rabbits the effect of inoculation of BM-MNCs in an Achilles tendon injury model. A transection in the Achilles tendon was performed and either treated with Kessler suture with or without the addition of MSCs. Histological, immunohistochemistry, morphometric, and mechanical testing was performed. The BM-MNCs improved mechanical and histological parameters only at early stages (3 weeks), suggesting the effect on accelerating healing at early time period [8]. Multiple questions still remain uncertain. Time is crucial for biological therapies. It is not clear at what time point the inoculation should be considered and the number of applications needed. Should be wait for 6 months of nonresponding medical treatment or try this therapy earlier? Should be inoculate these cells alone or combine them with growth factors such as PRP? Shall we give only one injection or try serial inoculations? In all our patients, we did one inoculation of BM-MSC after 6 months of failed nonoperative treatment. However, in the one patient that did not improve, probably a second inoculation of BM-MNCs or inoculation of PRP would may have been the answer.

We believe that this therapy could be considered as an alternative treatment for those patients who have failed nonoperative treatment before surgical intervention is considered. Further control studies will be needed to determine if inoculation of BM-MNCs can improve tendon healing in patients with chronic patellar tendinopathy. According to

this study, inoculation of BM-MNCs for the treatment of patellar tendinopathy is a promising therapeutic approach.

5. Conclusions

This study investigates the use of BM-MNCs for the treatment of chronic patellar tendinopathy. Patients showed statistically clinical improvement at 5-year followup. Inoculation of BM-MNCs could be considered as a potential therapy for those patients with chronic patellar tendinopathy refractory to nonoperative treatments.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Biological Strategies to Enhance Healing of the Avascular Area of the Meniscus

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Meniscal injuries in the vascularized peripheral part of the meniscus have a better healing potential than tears in the central avascular zone because meniscal healing principally depends on its vascular supply. Several biological strategies have been proposed to enhance healing of the avascular area of the meniscus: abrasion therapy, fibrin clot, organ culture, cell therapy, and applications of growth factors. However, data are too heterogeneous to achieve definitive conclusions on the use of these techniques for routine management of meniscal lesions. Although most preclinical and clinical studies are very promising, they are still at an experimental stage. More prospective randomised controlled trials are needed to compare the different techniques for clinical results, applicability, and cost-effectiveness.

1. Introduction

The menisci of the knee are two semilunar fibrocartilaginous structures sitting between the joint surfaces of femoral condyles and the tibial plateau. Meniscal injuries are a common and important source of knee dysfunction. Repair should be considered depending on the type and location of the meniscal tear [1–3], as meniscal healing principally depends on the vascular supply of the zone that has been injured [4]. A rich network of arborizing vessels within the peripheral capsular and synovial attachments supplies vascularization to the menisci. This perimeniscal network provides radial branches to the meniscus. The outer third of the meniscus is vascularised, showing a good healing capacity. Given its abundant vascularization, this zone is also called "red-red zone." The remaining two-thirds of the meniscus, respectively called "red-white zone" and "white-white zone," have a scanty vascular supply and present a limited ability to heal spontaneously [4–7].

A meniscal lesion followed by disruption of the structure in the avascular zone impairs load distribution and initiates erosion of the adjacent articular surfaces, causing osteoarthritis (OA) [8–11]. The most common treatment for lesions of the avascular part of the meniscus is arthroscopic partial meniscectomy, which reduces symptoms but similarly predisposes patients to OA [12]. Studies have demonstrated that healing of the knee is inversely related to the amount of resected meniscal tissue [10, 11, 13, 14]. Meniscal repair techniques in the avascular zone are in continuous evolution.

This paper covers current knowledge on biological strategies for the stimulation of meniscal healing after repair.

2. Abrasion Therapy

Rasping of the damaged meniscus in the vascularized parameniscal synovium promotes an injury response and is one of the most simple and effective strategies to favour healing [3]. A small incision is performed to produce a vascular channel that redirects the blood flow from the vascular zone into the avascular one. Several studies showed a significant difference in healing between menisci treated

with abrasion therapy and control groups [15, 16]. The most common techniques of abrasion therapy are rasping or trephination, in which radially oriented channels are performed to encourage vascular and cellular migration from the peripheral vascular portion to the tear site [17–19]. Rasping increases the production of interleukin-I-alpha (IL-1-alpha), transforming-growth-factor-beta 1 (TGF-beta1), platelet-derived growth factor (PDGF), and proliferating cell nuclear antigen (PCNA). This protein network improves vascular induction and meniscal healing [20]. Nevertheless, trephination and rasping procedures may damage the normal meniscal structure by an additional full thickness-transverse tear, resulting in poor meniscal function.

3. Fibrin Clot

Fibrin is a fibrous protein produced in response to bleeding that plays an important role in blood clotting. Fibrin clots may be used topically or by injection as an haemostatic agent, binding to several adhesive proteins of different cells. The fibrin clot technique acts as a chemotactic and mitogenic stimulus for reparative cells because of the presence of several growth factors [21–24]. The fibrin clot attaches to the exposed collagen caused by the tear and induces proliferation of fibrous connective tissue. This stimulates the development of fibrocartilaginous tissue. The fibrin clot technique can be used in combination with abrasion therapy or with meniscal sutures. Two studies in animal models showed that organized fibrous connective tissue developed into cartilaginous tissue after a period of 12–24 weeks [25, 26]. A potential disadvantage of the fibrin clot technique is the difficulty of keeping fibrin clots on the tear without immobilizing the operated leg [27].

4. Organ Culture

Organ culture is a useful model to assess the intrinsic healing potential of the meniscus excluding the influence of microvasculature and the synovium [28–30]. The effects of cultured meniscal explants in a rabbit model have been reported [30]. After gross evaluation, each meniscal explant underwent histological evaluation to study the relationship between the graft and recipient tissue. Application of this technique has demonstrated that meniscal tissue presents an intrinsic healing ability, which is greater in the peripheral zone of the meniscus than in the inner zone [30]. Regional differences in healing potential and extrinsic factors, such as blood supply, could explain good meniscal healing in the peripheral zone.

5. Cell Therapy

Human menisci are populated by different cell types, responding differently to various stimuli released from the matrix [31, 32]. Different cells have already been used in studies on meniscal healing: mesenchymal stem cells (MSCs) deriving from synovial or bone marrow, chondrocytes, and fibrochondrocytes. MSCs are pluripotent cells able to differentiate into specific therapeutic cell types (developmental plasticity) [33–35]. The effects of bioactive molecules, which are secreted by MSCs, determine a regenerative microenvironment that promotes healing of meniscal lesions [36, 37]. The combination of suturing and MSC treatment, combined or not with fibrin glue, seems to be the most effective treatment [38].

Zellner et al. [37] reported the efficacy of mesenchymal stem cells in the repair of meniscal defects in the avascular zone. Nonprecultured mesenchymal stem cells in hyaluronan-collagen composite matrices stimulated the development of completely integrated meniscus-like repair tissue in defects produced in the avascular zone of rabbit menisci [37].

Further studies confirm the production of abundant extracellular matrix around the cells, restoring a meniscal-like tissue in the avascular zone [37, 39–42]. These results are supported by early studies which demonstrated the efficacy of the association between growth factors and mesenchymal stem cells within scaffold implants to increase proteoglycans and/or collagen synthesis [37, 43]. Articular autologous and allogenic chondrocytes have also been used to induce repair in the avascular part of the meniscus [44, 45]. Peretti et al. described a porcine chondrocyte model where implantation of such cells was performed in the avascular part of the meniscus using an allogenic scaffold seeded with autologous chondrocytes. These chondrocytes were effective in promoting healing meniscal tears [44]. Fibrochondrocytes showed potential for initiating a reparative response in meniscal defects through the production of new extracellular matrix (ECM) [46, 47]. When seeded into a porous collagen scaffold, fibrochondrocytes harvested from the inner avascular part of the meniscus produce more glycosaminoglycans (GAGs) than fibrochondrocytes from a peripheral fibrous location [48, 49]. Although these findings are encouraging, the application of autologous fibrochondrocytes in meniscal tissue engineering is limited by the difficulty in harvesting a sufficient number of cells.

6. Growth Factors

Growth factors act as signalling molecules on target cells to stimulate the regeneration of damaged tissue [6]. Furthermore, they can induce the synthesis and inhibit degradation of ECM by a mechanism of downregulation of proteases [50]. Several studies in vitro and in vivo evaluated the effects of treatment with specific growth factors. Two categories of growth factors in consideration of their biochemical attributes are generally considered: anabolic and catabolic growth factors.

6.1. Anabolic Growth Factors

6.1.1. Fibroblast Growth Factor (FGF). basic FGF was used to stimulate type II collagen and aggrecan mRNA production in cellular and tissue development [51, 52]. In an ovine experimental model, meniscal fibrochondrocytes responded to bFGF by proliferating and producing new extracellular

matrix [46]. Another FGF type, FGF-2, stimulates proliferation of the joint chondrocytes, mesenchymal stem cells, osteoblasts, and adipocytes. Furthermore, it maintains the ability of any cell types to differentiate [53, 54]. Moreover, a hyperexpression of FGF-2 and alpha-smooth muscle actin (alpha-SMA) through recombinant adeno-associated virus (rAAV) enhanced cell proliferation and increased survival rate compared with control groups. However, FGF did not significantly increase the synthesis of major extracellular matrix components or DNA contents [55].

6.1.2. Transforming-Growth-Factor-Beta-I (TGF-Beta-I). TGF-beta-I seems to have several regulatory activities, stimulating collagen and proteoglycan production to increase the attachment of the cells in repaired meniscal tissue. Nevertheless, it has no effect on cell proliferation [6, 56–59].

6.1.3. Bone Morphogenetic Proteins (BMPs). BMPs are a group of growth factors belonging to the TGF-β superfamily playing an important role during embryogenesis and tissue repair in relation to their osteoinductive properties [60, 61]. BMP-2 acts as a stimulus in the differentiation of mesenchymal cells. It also presents a migratory effect in endothelial cells or smooth muscle cells, but rarely in chondrocytes [62]. BMP-7 regulates matrix homeostasis and inhibits the processes of degradation. BMP-7 acts with different chondrogenic agents and is more effective than BMP-2 in chondrogenic differentiation of MSCs in promoting meniscal healing [63].

6.1.4. Insulin-Like-Growth-Factor-I (IGF-I). This is considered the main anabolic growth factor for articular cartilage [64, 65]. Unlike TGF-beta-I, IGF-I increases cell proliferation significantly but has no effect on the attachment [51]. Therefore, a mixture of growth factors in association with IGF-I could induce an extensive cellular response to mediate avascular meniscal healing [56].

6.1.5. Vascular Endothelial Growth Factor (VEGF). The induction of angiogenesis is important to stimulate healing of meniscal tears. Vascular endothelial growth factor (VEGF) may promote better healing, stimulating angiogenesis to improve the healing capacities of meniscus tissue. In adults, VEGF expression is downregulated by endostatin, mostly in the avascular zone [66]. However, the local application of VEGF did not show an improvement of meniscal healing [67].

6.1.6. Platelet-Derived Growth Factor-AB (PDGF-AB). PDGF-AB plays an important role in the angiogenesis and cell development [68, 69]. The application of PDGF-AB in the peripheral part of the menisci showed a better healing response than the application in the central part [70]. However, this anabolic growth factor increased both cell proliferation and ECM formation in all zones of the meniscus, including the avascular zone [71].

6.2. Catabolic Growth Factors

6.2.1. Endostatin. Endostatin is an antiangiogenic factor expressed by fibrochondrocytes in the avascular zone of menisci. Endostatin concentrations were higher when fibrochondrocytes were in coculture with MSCs, suggesting that meniscal cell growth is inhibited by the proliferation of MSCs [7].

6.2.2. Interleukin-I (IL-I). This is a proinflammatory cytokine that stimulates the development of a local inflammatory reaction. Meniscal explants treated with IL-I have failed to show any signs of regeneration [72]. These findings suggest that relevant expression of IL-I in association with higher levels of tumor-necrosis-factor-alpha (TNF-alpha) inhibit meniscal repair [73].

7. Platelet Rich Plasma

Platelet-rich plasma (PRP) is an autologous substance rich in platelets that releases growth factors from both alpha and dense granules. These growth factors have been associated with the initiation of a healing cascade leading to cellular chemotaxis, angiogenesis, collagen matrix synthesis, and cell proliferation [74]. Ishida et al. reported the effects of PRP on meniscal tissue regeneration, both in vitro and in vivo, in a rabbit model. In the in vitro study, monolayer meniscal cell cultures were prepared and proliferative behaviour, extracellular matrix (ECM) synthesis, and fibrocartilage-related messenger ribonucleic acid (mRNA) expressions were assessed in the presence of PRP. PRP stimulated DNA synthesis, ECM synthesis, and mRNA expression of biglycan and decorin. In the in vivo study, full-thickness defects were produced in the avascular region of rabbit meniscus. Gelatin hydrogel (GH) was used to deliver PRP into the defects. At histology 12 weeks after surgery, significantly better meniscal repair was evident in animals that received PRP with GH than in the control groups [75].

In contrast, Zellner et al. evaluated several cell and biomaterial-based treatment options for repair of defects in the avascular zone of rabbit menisci by producing circular meniscal punch defects in the avascular zone of rabbit menisci. The defects were left empty or filled with hyaluronan-collagen composite matrices without cells loaded with platelet-rich plasma, autologous bone marrow, or autologous mesenchymal stem cells. Neither bone marrow nor platelet-rich plasma loaded in matrices induced improvement in meniscal healing [37].

8. Discussion

In the last few decades, many studies on meniscal healing have focused on methods to enhance the healing capacities of the meniscus after repair. Abrasion of the torn meniscus and synovial tissue or the establishment of vascular channels to redirect blood flow into the avascular zone seems to be the preferred treatment [3, 15, 16]. However, the healing potential depends on the type and location of the tear and

its distance from the peripheral vascularised zone. The use of a fibrin clot can also be an effective technique to support a reparative response in the avascular zone of the meniscus [21, 22, 24]. Findings demonstrated that the rasping technique is more effective than fibrin clot application to improve meniscal healing [76]. Kobayashi et al. reported healing rates in the peripheral zone of the menisci in an on-organ culture model. Regional differences in healing potential and extrinsic factors, such as a blood supply, could explain the good meniscal healing potential in the peripheral zone [30].

Cell-based therapy for meniscal tears has significantly contributed to an increasing number of patients treated with repair techniques rather than meniscectomy. Different cell types have already been used in studies on meniscus healing: MSCs, articular chondrocytes and autologous fibrochondrocytes [37, 44, 48, 49]. Progenitor cells such as mesenchymal stem cells present the advantage of being easily expandable without losing their differentiation potential into a variety of mesenchymal tissues including bone, tendon, cartilage, muscle, ligament, fat, and marrow stroma [31, 33, 35]. The application of MSCs and their stimulation with growth factors in combination with a mechanically loadable scaffold have been proposed as the focus of future studies [77, 78].

Several studies reported the efficacy of mesenchymal stem cells in the repair of meniscal defects in the avascular zone, with production of abundant extracellular matrix around the cells and restoration of a meniscal-like tissue [37, 39–42]. Early studies demonstrated the efficacy of the association between growth factors and mesenchymal stem cells within scaffold implants to increase proteoglycan and/or collagen synthesis. Therefore, the healing response of mesenchymal stem cells seems to produce additional repair qualities besides the delivery of growth factors [37, 43].

Many studies have shown the importance of growth factors in the treatment of meniscal tears of the avascular portion, but there is a very complex interplay among a variety of factors that influences healing processes. Growth factors that promote cell differentiation and chondrocytic proliferation include both anabolic growth factors (TGF-beta-I, BMPs, IGF-I, FGF, VEGF, and PDGF-AB) and catabolic growth factors (endostatin, IL-1, and TNF-alpha). Anabolic growth factors could be of additional value in improving the healing of meniscal lesions [6, 46, 51–57]. However, the application of growth factors remains very limited in clinical settings [6, 51]. Future research should focus on the use of tissue-engineered constructs in association with different growth factors. A preparation rich in growth factors could produce better results than the use of isolated growth factors. Only a few studies to date have evaluated the effectiveness of a preparation of platelet-rich plasma (PRP), but there is some evidence that PRP can improve healing of the menisci [70, 71]. The release of growth factors from platelets has been associated with the initiation of a healing cascade leading to cellular chemotaxis, angiogenesis, collagen matrix synthesis, and cell proliferation [74, 75]. In contrast, a study in an animal model reported that application of PRP did not produce improvements in meniscal healing [37].

9. Conclusion

Patients with meniscal tears report pain and functional limitation of the knee joint. Partial meniscectomy is the most common treatment option, but it represents a predisposing factor for osteoarthritis [12]. To date only limited scientifically proven management modalities are available. A better understanding of meniscal healing mechanisms will allow specific treatment strategies to be developed. Although most preclinical and clinical studies are very promising, they are still at an experimental stage. Further prospective trials are necessary to compare the different techniques for efficacy, applicability, and cost-effectiveness in the management of lesions of the avascular region of the meniscus.

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Review Article

Scaffolds in Tendon Tissue Engineering

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Tissue engineering techniques using novel scaffold materials offer potential alternatives for managing tendon disorders. Tissue engineering strategies to improve tendon repair healing include the use of scaffolds, growth factors, cell seeding, or a combination of these approaches. Scaffolds have been the most common strategy investigated to date. Available scaffolds for tendon repair include both biological scaffolds, obtained from mammalian tissues, and synthetic scaffolds, manufactured from chemical compounds. Preliminary studies support the idea that scaffolds can provide an alternative for tendon augmentation with an enormous therapeutic potential. However, available data are lacking to allow definitive conclusion on the use of scaffolds for tendon augmentation. We review the current basic science and clinical understanding in the field of scaffolds and tissue engineering for tendon repair.

1. Introduction

Tissue engineering techniques using novel scaffold materials offer potential alternatives for managing tendon disorders [1]. Tissue engineering strategies to improve tendon repair healing include the use of scaffolds, growth factors, cell seeding, or a combination of these approaches [1]. Scaffolds have been the most common strategy investigated to date [1]. The rationale for using a scaffold device for tendon repair may include mechanical augmentation, improving the rate and quality of biologic healing, or both. Scaffolds with robust mechanical and suture retention properties, applied in a surgically appropriate manner, may have the ability to "off-load" the repair at time zero and for some period of postoperative healing, depending on the rate and extent of scaffold remodeling. Despite the growing clinical use of scaffold devices for tendon repair, there are numerous questions related to their indication, surgical application, safety, mechanism of action, and efficacy that remain to be clarified or addressed [1].

The use of scaffolds alone in flexor tendons has not been highly studied, but they have been combined with tenocytes in an effort to engineer an autologous tendon graft [2, 3]. However, the use of scaffolds in flexor tendon repairs may have a detrimental effect on tendon gliding, due to their size, and the lack of space within a repaired synovial sheath.

Scaffolds for both the Achilles tendon and the rotator cuff have been investigated both as structural supports and as delivery systems for other tissue engineering modalities. Available scaffolds for tendon repair include both biological and synthetic scaffolds. In this paper we review the current basic science and clinical understanding of scaffolds and tissue engineering for tendon repair. We underline benefits and limitations of the available scaffolds for augmentation of tendon disorders and discuss the implications of these data on future directions for the use of these scaffolds in tendon repair procedures.

2. Biological Scaffolds

Biological scaffolds are obtained from mammalian (human, porcine, bovine, and equine) tissues [4]. To remove any noncollagen components, thus, minimizing the risk of host

rejection while retaining its natural collagen structure and mechanical properties, small intestine submucosa (SIS), dermis, and pericardium are processed through cascade steps, including general cleaning, removal of lipids or fat deposits, disruption of cellular and DNA materials, crosslinking, and sterilization [4]. The final scaffolds are composed mainly of naturally occurring collagen fibres, predominantly type I collagen, and several of them have a surface chemistry and native structure that is bioactive and promotes cellular proliferation and tissue ingrowth [4].

The Restore graft (Depuy, Warsaw, IN) is a circular implant consisting of 10 not crosslinked layers of porcine small intestinal submucosa (SIS). It is more than 90% collagen with approximately 5–10% lipids and a small amount of carbohydrate [5, 6]. Iannotti et al. [7] tried to determine the effectiveness of porcine SIS to augment the repair of rotator cuff. They randomized 30 shoulders with a chronic two-tendon rotator cuff tear (9 with a large tear and 21 with a massive tear of rotator cuff) that was completely repairable with open surgery to be managed with either augmentation with porcine SIS or no augmentation. The rotator cuff healed in 4 of the 15 shoulders in the augmentation group compared with 9 of the 15 in the control group. The authors concluded that augmentation of the surgical repair of large and massive chronic rotator cuff tears with porcine SIS did not improve the rate of tendon healing or the clinical outcome scores. On the basis of their investigation, they do not recommend using porcine SIS to augment repairs of massive chronic rotator cuff tears performed with the surgical and postoperative procedures described in this study. Metcalf et al. [8] conducted a 2-year followup of 12 patients who underwent arthroscopic repair of massive chronic rotator cuff tears using Restore SIS as an augmentation device. Postoperative magnetic resonance imaging (MRI) scans showed significant thickening of the cuff tendon with the incorporation of the SIS graft in 11 patients. In 1 of 12 patients, clinical failure was observed within 12 weeks with complete resorption of the graft. There was no evidence of local or systemic rejection or infection in any patient. The mean postoperative University of California, Los Angeles, (UCLA) score was 19.9 on a scale of 35, a significant improvement over the preoperative score of 9.9, but the shoulder function remained far below normal in these patients. This study demonstrated improved postoperative outcomes for patients managed with the Restore graft augmentation compared with their preoperative condition. However, the lack of a control group makes it difficult to conclude that the functional improvements in the study were the result of SIS augmentation. Sclamberg et al. [9] evaluated clinical and MRI at 6 months in 11 patients undergoing open repair of large or massive rotator cuff tears augmented with Restore. MRI showed a retear in 10 of 11 patients. Zheng et al. [10] performed a study to evaluate the safety and efficacy of the Restore SIS membrane. The Restore orthobiologic implant was examined by histology and the nested PCR technique using porcine immunoreceptor DAP12 gene to examine if SIS membrane contained porcine cells or DNA, respectively. The material was also implanted into mice and rabbits for the evaluation of biological reaction and inflammatory response. Restore SIS was found to contain multiple layers of porcine cells. Chloroacetate esterase staining showed that some of these cells were mast cells. Nested PCR of the DAP12 gene demonstrated that Restore SIS contained porcine DNA material. Subcutaneous implantation of Restore SIS membrane in mice, and in rabbits for rotator cuff tendon repair, showed that the membrane caused an inflammatory reaction characterized by massive lymphocyte infiltration. The authors concluded that Restore SIS is not an acellular collagenous matrix, and contains porcine DNA, contradicting the current view that Restore SIS is a cell-free biomaterial and that no inflammatory response is elicited by its implantation. Walton et al. [11] compared a group of patients who had undergone rotator cuff repair with xenograft augmentation with a group repaired without augmentation. Four patients in the xenograft group showed a severe postoperative reaction requiring surgical treatment. Two years postoperatively, MRI documented retears in 6 of the 10 tendons repaired with a xenograft and in 7 of the 12 nonaugmented tendons; the patients with a xenograft also had less strength than the controls and had more impingement in external rotation, a slower rate of resolution of pain during activities, more difficulty with hand-behind-the-back activities, and a lower rate sports participation.

The Zimmer Collagen Repair Patch (Tissue Science Laboratories, Covington, Ga, USA, licensed to Zimmer) is a single layer porcine skin xenograft. It is an acellular crosslinked collagen sheet of crosslinked porcine dermis. Soler et al. [12] used Zimmer Collagen Patch as a bridging device to repair massive rotator cuff tears. After a good postoperative period, between 3 and 6 months, the graft began to fail, and the patients showed signs and symptoms of retear, with also signs of inflammation. MRI scans showed inflammatory changes, resorption of the graft, fluid pooling in the subdeltoid bursa, and loss of continuity of the remaining graft material. Histology of the debris revealed necrotic fibrinous material on a background of chronic inflammation. Badhe et al. [13] prospectively evaluated 10 patients with extensive rotator cuff tear treated with Zimmer Collagen Patch (Permacol). All patients experienced significant pain relief and improvement in abduction power and range of motion. Ultrasound imaging at the final followup identified intact grafts in eight and disrupted grafts in two patients.

GraftJacket (Wright Medical Technology, Inc.) is a commercially available acellular dermal matrix obtained from tissue bank human skin, composed of collagen types I, III, IV, and VII, elastin, chondroitin sulfate, proteoglycans, and fibroblast growth factor. It has an intact basement membrane complex and preserved vascular channels to allow rapid infiltration of fibroblasts and vascular tissue, with minimal host inflammatory response [2, 6, 11]. Barber et al. [14] compared the failure mode of supraspinatus tendon repair with and without GraftJacket augmentation in a human cadaveric model. No significant displacement occurred during the cyclic phase, and no anchors failed. During the destructive testing phase, the mean load-to-failure strength of the control construct was 273 + 116 N. The load-to-failure strength of the supraspinatus tendon augmented with GraftJacket was 325 + 74 N. The constructs failed by

two different mechanisms: tendon-suture interface failure (8/10 nonaugmented repairs and 6/10 augmented repairs) and suture breakage (2/10 nonaugmented repairs and 4/10 augmented repairs). Bond et al. [15] treated 16 patients with massive rotator cuff tears with arthroscopic implantation of a GraftJacket allograft. At a mean followup of 26.7 months, 15 of 16 patients were satisfied with the procedure. The mean UCLA score increased from 18.4 preoperatively to 30.4 postoperatively. The mean pain score improved from 4.6 to 9.8 postoperatively. The mean constant score increased from 53.8 to 84.0. Statistically significant improvements were noted in pain, forward flexion, and external rotation strength. MRI scans showed full incorporation of the graft into the native tissue in 13 patients. Chronic Achilles tendon rupture, repaired with GraftJacket, showed early return to activity and good plantarflexion strength [16]. Two studies [17, 18] evaluated GraftJacket as an augmentation device in the Achilles tendon repair. In the first study [17], nine patients with chronic Achilles tendon ruptures were followed up. There were no reruptures or recurrent pain at 20–30 months postoperatively, and the average return-to-activity time was 15.2 + 1.7 weeks. In the second study [18], 11 patients with acute tendon ruptures were followed up for 20 to 31 months. At 20 months, there were no reruptures or recurrent pain; the average return-to-activity time was 11.8 + 0.75 weeks. Significant increase in strength and stiffness of Achilles tendon repair augmented with GraftJacket was also observed in a human cadaver model (12.99 + 5.34 N/mm versus 4.29 + 0.83 N/mm of the control group) [19].

3. Synthetic Scaffolds

Because allograft materials may cause inflammatory responses in the host, there is notable interest in developing synthetic extracellular matrix (ECM) grafts for surgical use. Synthetic ECMs may still serve as an adequate scaffold for cellular and fibrotic growth, while running a smaller risk of provoking an inflammatory response than allograft ECMs. Several animal studies have investigated the benefit of augmenting rotator cuff repair with synthetic ECMs.

Yokoya et al. [20] used a polyglycolic acid (PGA) sheet to augment rotator cuff repairs of infraspinatus tendons in Japanese white rabbits, showing histological improvement in fibrocartilage layering but only a slight improvement in tensile strength when compared to control tendons augmented with another slowly absorbing synthetic material [20]. In a similar study, Funakoshi et al. [21] demonstrated increased fibroblast presence and collagen formation when synthetic ECM was surgically applied to rotator cuff tears. In this experiment, a 10-mm surgical defect was created at the humeral insertion of the infraspinatus tendon in 21 Japanese white rabbits. In one shoulder, the 10-mm defect was covered with chitin, a biodegradable polymer, sutured into the bone trough, and attached to the free end of the infraspinatus tendon. The contralateral shoulder was left untreated as a control. Throughout the experiment, tendon-to-bone junctions covered with chitin fabric demonstrated greater cell number, better collagen fiber alignment, and greater mechanical strength than the tendon-to-bone junctions left free as control [21]. In another study, MacGillivray et al. [22] used polylactic acid patches in goats, showing no observable difference between the treated and control groups [22]. A similar experiment, using a woven poly-L-lactide device, was performed by Derwin et al. [23] in a dog model. The superior 2.3 of each infraspinatus tendon was removed from the rotator cuff and then repaired in both shoulders. In one shoulder, a woven poly-L-lactide device was placed over the repair. In the other shoulder, the repair was left unaugmented. The augmented rotator cuff repair resulted in fewer tendon retractions, greater strength, and increased stiffness when compared to the contralateral untreated rotator cuff repairs. A recent study demonstrates that the application of the X-Repair device significantly increased the yield load and ultimate load of rotator cuff repairs in a human cadaveric model and altered the failure mode but did not affect initial repair stiffness [24].

4. Tissue Engineering with Mesenchymal Stem Cells (MSCs)

Technological advances in biology and engineering have resulted in marked improvements in the design and manufacture of tissue-engineered substitutes that can modify and maintain living tissue [25, 26]. Tissue engineering is an emerging field made up of the combination of scaffold, cell and stimulation or their stand-alone application [27, 28]. MSCs are capable of differentiating into a variety of specialized mesenchymal tissues including bone, tendon, cartilage, muscle, ligament, fat, and marrow stroma [27, 28]. Tissue engineering can be divided into two subtypes: the *in vivo* approach and the *ex vivo, de novo* one [25, 26]. The *in vivo* approach permits the self-regeneration of small tissue lesions. The *ex vivo, de novo* approach is designed to produce functional tissue that can be implanted in the body [29]. Tissue engineering is a multidisciplinary field founded on three fundamental principles: (I) the use of healthy multipotent cells that are nonimmunogenic, easy to isolate, and highly responsive to distinct environmental cues, (II) the development of carrier scaffolds that provide short-term mechanical stability of the transplant and a template for spatial growth of the regenerate tissue, and (III) the delivery of growth factors that drive the process of cell differentiation and maturation [25, 26]. MSCs can be applied directly to the site of injury or can be delivered on a suitable carrier matrix, which functions as a scaffold while tissue repair takes place [30].

The ideal scaffold for tendon engineering would possess the basic structure of the tendon, native extracellular matrix, and capability of cell seeding [31]. Decellularized multilayer tendon slices were seeded with BMSC, harvesting BMSC and infraspinatus tendons from dogs. Histology showed the alignment of the seeded cells between the collagen fibers of the tendon slices. qRT-PCR analysis showed higher tenomodulin and MMP13 expression and lower collagen type I expression in the composite than in the BMSC before seeding, suggesting that BMSC might express a tendon

phenotype in this environment [31]. Delivering MSC-contracted, organized collagen implants applied to large tendon defects can significantly improve the biomechanics, structure, and probably the function of the tendon after injury [32]. A tissue prosthesis made up of cultured, autologous, marrow-derived MSCs suspended in a collagen gel delivery vehicle and contracted onto a pretensioned suture was implanted into a 1-cm-long gap defect in a rabbit Achilles tendon [32]. Load-related structural and material properties evaluated 4, 8, and 12 weeks later were greater than in the control repairs, which contained suture alone with natural cell recruitment. Furthermore, the treated tissue showed a significantly larger cross-sectional area, and their collagen fibers appeared to be better aligned than those in the controls. The use of MSCs to enhance allograft osteointegration is a novel method offering the potential of more physiologic and earlier healing [33]. MSCs derived from synovium have a higher proliferation and differentiation potential than the other MSCs. Their potential to accelerate the early remodelling of tendon-bone healing histologically by producing more collagen fibers at 1 week and forming more oblique collagen fibers connecting the bone to tendon resembling Sharpey's fibers at 2 weeks has been shown [34]. Moreover, MSCs do not interfere with tendon-bone healing at 4 weeks [34]. MSCs have been investigated in the management of tendinopathy. MSCs and IGF-I genes enhanced MSCs (AdIGF-MSCs) on the healing of a collagenase-induced bilateral tendinopathy lesions in an equine flexor digitorum superficialis injury model. Both MSC and AdIGF-MSC injections resulted in significantly improved tendon histological scores [35].

Tissue engineering techniques using novel scaffold materials offer potential alternatives for managing irreparable rotator cuff tears. A chitosan-based hyaluronan hybrid scaffold, with seeded fibroblasts to repair infraspinatus tendons defects produced in rabbits, demonstrated an enhanced type I collagen production and a significant improvement in tensile strength and tangent modulus from 4 to 12 weeks postoperatively [36]. *In vivo*, the effect of auto-crosslinked HA gel on adhesions and healing of injured and surgically repaired rabbit digital flexor tendons was studied, demonstrating a significantly faster increase in breaking strength with an accelerated tissue repair response after injury, but unaffected adhesions formation [37]. In rabbits, MSCs expanded in culture, suspended in type I collagen gel, and implanted into a surgically induced defect in the donor's right patellar tendon demonstrated significant increases in maximum stress, modulus, and strain energy density [38]. Changes in nuclear morphology of the MSCs in response to physical constraints provided by the contracted collagen fibrils may trigger differentiation pathways toward the fibroblastic lineage and influence the cell synthetic activity [39]. Controlling the contraction and organization of the cells and matrix will be critical to successfully produce tissue-engineered grafts. Seeded collagen gels with rabbit bone-marrow-derived MSCs and contracted onto sutures were implanted into full thickness, full length, central defects in the patellar tendons of the animals [38]. Repair tissues containing the MSC-collagen composites showed significantly higher maximum stresses and moduli than natural repair tissues at 12 and 26 weeks post surgery [38]. Autogenous tissue-engineered constructs were fabricated in culture between posts in the wells of silicone dishes [40]. Constructs were implanted in bilateral 2-cm-long gap defects in the rabbit's lateral Achilles tendon. At 12 weeks after surgery, no significant improvement was observed in any structural or mechanical properties or in histological appearance compared with control. The same authors tried also to determine how a tensile stimulus affects the gene expression of stem cell-collagen sponge constructs used to repair rabbit central patellar tendon defects [40]. MSCs were introduced into a gel-sponge composite showing cellular alignment comparable with that of normal tendon [41]. Cao et al. [3] tested the feasibility of engineering tendon tissues with autologous tenocytes to bridge a tendon defect in either a tendon sheath open model or a partial open model in the hen. FDP defects were bridged either with a cell-scaffold construct in the experimental group or with scaffold material alone in the control group. At 14 weeks, the engineered tendons resembled the natural tendons grossly in both colour and texture and displayed a typical tendon structure hardly distinguishable from that of normal tendons. The same authors also explored the feasibility of *in vitro* tendon engineering using the same type of cells and scaffold material [42]. Unwoven polyglycolic acid (PGA) fibers were arranged into a cord-like construct and fixed on a U-shape spring, and tenocytes were then seeded on PGA fibers to generate a cell-PGA construct. The results showed that tendon tissue could be generated during *in vitro* culture. In addition, the tissue structure and mechanical property became more mature and stronger with the increase of culture time. Alginate-based chitosan hybrid polymer fibers showed much improved adhesion capacity with tenocytes compared with alginate polymer fiber [43]. The rAAV-Gdf5 vector significantly accelerates wound healing in an *in vitro* fibroblast scratch model and, when loaded onto freeze-dried flexor digitorum longus tendon allografts, improves the metatarsophalangeal joint flexion to a significantly greater extent than the rAAVlacZ controls do [44]. In an experimental study on rabbits, a sharp complete midsubstance transection of the Achilles tendon was immediately repaired using a modified Kessler's suture and a running epitendinous suture. Both limbs were used, and each side was randomized to receive either bone-marrow-derived MSCs in a fibrin carrier or fibrin carrier alone (control). At 6 and 12 weeks, there were no differences between the groups with regard to morphometric nuclear parameters. Biomechanical testing showed improved modulus in the treatment group as compared with the control group at 3 weeks, but not at subsequent time periods [45].

Costa et al. [46] tried to optimize tenocyte proliferation in three tendon cell populations using growth factor supplementation. They isolated cells of the synovial sheath, epitenon, and endotenon from rabbit FDP tendons and maintained them in culture. For all three tendon cell populations, proliferation at 72 hours was greater in the presence of individual growth factors as compared with controls. In

addition, a synergistic effect was observed. The combination of growth factors resulted in greater proliferation as compared with maximal doses of individual growth factors. Synthetic oligo[poly(ethylene glycol)fumarate]-(OPF-) based biomaterials were tested as a mean to deliver fibroblasts to promote regeneration of central/partial defects in tendons and ligaments. To further modulate the swelling and degradative characteristics of OPF-based hydrogels, OPF crosslinking via a radically initiated, mixedmode reaction involving poly(ethylene glycol) (PEG) diacrylate (PEG-DA) and PEG-dithiol was investigated. After encapsulation, tendon/ligament fibroblasts remained largely viable over 8 days of static culture. Although the presence of PEG-dithiol did not significantly affect cellularity or collagen production within the constructs over this time period, image analysis revealed that the 20% PEG-dithiol gels did appear to promote cell clustering, with greater values for aggregate area observed by day [47].

The use of a PEG-DA hydrogel incorporated with hydroxyapatite (HA) and the cell-adhesion peptide RGD (Arg-Gly-Asp) was tested as a material for determining an *in vitro* tissue interface to engineer intact ligaments. Incorporation of HA into PEG hydrogels reduced the swelling ratio but increased mechanical strength and stiffness of the hydrogels. Further, HA addition increased the capacity for cell growth and interface formation. RGD incorporation increased the swelling ratio but decreased mechanical strength and stiffness of the material [48].

A novel fabrication system for photopatterning and assembling cell-laden OPF : PEG-DA hydrogels with high spatial fidelity and thickness using a controlled, inert nitrogen environment was described [49]. Cross-linking was performed using Irgacure-2959 photoinitiator and 365 nm light (7 mW/cm^2) to form gels ranging from 0.9 to 3 mm in width. Employing an N^2 environment increased gel thickness up to 240%, generating gels greater than 1 mm thick prior to swelling. This technique was further applied for spatially controlled patterning of primary tendon/ligament fibroblasts and marrow stromal cells in a single 1.5-mm-thick laminated hydrogel construct. Cells encapsulated using this technique maintained viability over 14 days in culture.

5. Discussion

The emerging field of tissue engineering holds the promise to use materials in tendon injury repair, namely, artificial polymers, biodegradable films, and biomaterials derived from animals or human (ECM devices) [5]. The most innovative strategy in tendon injury repair is the use of ECM matrices. In contrast to traditional polymeric and metallic orthopaedic devices, intended to restore mechanical function and remain unchanged for the life of the patient, ECMs are temporary scaffold aimed to enhance and accelerate the biology of tissue repair [50, 51]. They undergo host cell infiltration and constructive tissue remodelling at variable rates [52]. Potential advantages of the use of ECM grafts include the capability to decrease the *in vivo* mechanical forces on the tendon repair during postoperative healing, to prevent repair gap formation or failure, to allow host cell infiltration and ideally even enhance the biology healing, and to be replaced by organized host tissue over time. Additional research studies are required to verify these issues. The ideal scaffold should induce host-tissue ingrowth and tendon regeneration during the process of degradation, which varies dramatically among the commercially available scaffolds [53]. The capability of inducing host-tissue ingrowth is superior when using biological scaffolds, even though this process appears uncontrolled and nonspecific [54]. The interaction between scaffold surface and host cells is a key aspect of the use of scaffolds for tendon reconstruction. In the first phase of cellular ingrowth, multiple attachment points are established by the cells through the interaction between transmembrane proteins and proteins at the scaffold surface [4], later strengthened by accumulating integrin receptors, eventually forming a focal adhesion which acts as a connection between the actin cytoskeleton of the cell and the surface [4]. The cell proliferation cycle and cell migration start after the formation of focal adhesions and spreading of cells on the surface [4]. Cell attachment, proliferation, and migration is facilitated by the porosity of scaffolds [55]. The surface of biological scaffolds is mostly composed of natural type I collagen protein, which determines a higher affinity to host cells and, therefore, promotes cellular adhesion, proliferation, migration, and tissue induction [4].

On the other hand, the surfaces of synthetic scaffold are composed of macromolecules lacking a well-defined structure that allows host cell to produce a strong binding point and start growing [4].

Even though biological scaffolds are becoming more popular, clinical well-conducted human studies are lacking, and little data describing the complications or adverse events associated with the use of these products are available. ECMs fabricating in parallel with other materials may increase their mechanical properties, such as natural ECMs seeded with bone marrow stem cells or tenocytes. However, clinical evidence in this field is scanty. Major concern about both biological and synthetic scaffolds is the biocompatibility and the inflammatory response associated with foreign body rejection [4]. To decrease the bioburden and the risk of inflammatory or foreign body reactions, all tissues, regardless of their origin, are extensively purified to remove proteins, cells, and lipids. Some graft options have been artificially crosslinked to decrease antigenicity, by decreasing their sensitivity to collagenases. Although rare, aseptic, nonspecific inflammatory reactions and foreign body-like reactions have been reported with certain xenografts [5, 6, 10, 56, 57]. Aseptic reactions were reported in 16–22% [57] of implantations, always with negative aspirates and cultures, destroyed xenografts, and histopathological evidence of inflamed granulation tissue with abundant neutrophils, but no foreign body reaction, as documented by the absence of organisms, crystals, or giant cells [6, 57, 58]. Valentin et al. [53] examined the host-tissue morphologic response to five commercially available extracellular matrix-derived biological scaffolds (GraftJacket, Restore, CuffPatch, TissueMend, Permacol) used for orthopaedic

soft-tissue repair in a rodent model. Each device elicited a distinct morphologic response that differed with respect to cellularity, vascularity, the presence of multinucleated giant cells, and organization of the remodelled tissue. More rapidly degraded devices such as Restore and autologous tissue showed the greatest amount of cellular infiltration, especially at the early time points. Devices that degraded slowly, such as CuffPatch, TissueMend, and Permacol, were associated with the presence of foreign-body giant cells, chronic inflammation, and/or the accumulation of dense, poorly organized fibrous tissue. Depending on the product, processing may involve acellularization treatment, chemical cross-linking, lamination of multiple layers or lyophilization [59]. These biomaterials have incomplete acellularization [10, 50], and the clinical implications are still not clear. Acellularization treatment aims to reduce antigenicity, by disrupting cells and removing water-soluble cellular proteins. Acellularization may also enhance host cell infiltration with phenotypically appropriate cells [60] and possibly prevent transmission of infectious genomic vectors [61]. Further biochemical and immunologic investigations are required to establish whether and how much acellularization treatment increases the safety and efficacy of these implants. The use of biological scaffolds manufactured from human or animal tissue carries also the risk of disease transmission, which, even though not reported to date, remains a theoretical concern. Obviously, there is no risk of disease transmission with the use of synthetic scaffolds [4]. One of the advantages of biomaterials is that exogenous growth factors, gene therapy approaches, or cell delivery can be used together with these biomaterials. Several chemical cross-linking agents (i.e., glutaraldehyde, polyepoxy compound, carbodiimide, genipin, isocyanate, and proanthocyanidin) have been used to stabilize the collagen structure of the scaffold, maintaining the mechanical properties. Clinical studies have not confirmed the expected beneficial effect of chemical cross-linking scaffolds. Further investigations are warranted to establish the *in vivo* benefit of chemical cross-linking in biocompatibility and mechanical properties on the scaffolds. As Chen et al. [4] proposed, another reason of concern is that available scaffolds are produced to mimic the tendon or ligament extracellular microenvironment to stimulate cell proliferation and tissue ingrowth, largely ignoring the healing process at the enthesis. The repair procedure often involves reconstruction of the junction, and failure of surgery is frequently caused by osteolysis and scaffold pullout. Further investigations are required to better understand how to promote the healing of bone-tendon junction.

6. Conclusion

Tendon disorders are frequent and cause significant morbidity both in sports and workplace [62–64]. Several conservative and surgical procedures are available for tendon healing, but one of the major problems encountered when dealing with tendinopathies is that etiology is largely unknown [65–69].

Preliminary studies support the idea that scaffolds can provide an alternative for tendon augmentation with an enormous therapeutic potential. However, available data are lacking to allow definitive conclusion on the use of scaffolds for tendon augmentation. Additionally, the prevalence of postoperative complications encountered with their use varies within the different studies. Further investigations are required to evaluate the role of scaffolds in the clinical practice.

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Review Article

Stem Cells for Augmenting Tendon Repair

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Tendon healing is fraught with complications such as reruptures and adhesion formation due to the formation of scar tissue at the injury site as opposed to the regeneration of native tissue. Stem cells are an attractive option in developing cell-based therapies to improve tendon healing. However, several questions remain to be answered before stem cells can be used clinically. Specifically, the type of stem cell, the amount of cells, and the proper combination of growth factors or mechanical stimuli to induce differentiation all remain to be seen. This paper outlines the current literature on the use of stem cells for tendon augmentation.

1. Introduction

Tendons function to transmit force from muscle to bone, with the ultimate effect of actuating motion. Tendon tissue has been designed to endure large tensile loads. When tendons are injured, normal tendon histology is not restored and, therefore, neither is function. Tendons heal with an intervening layer of scar tissue. This scar tissue has material properties that are inferior to native tendon. This makes surgical repairs of torn tendons prone to failure. It also makes them susceptible to adhesion formation due to excess fibrous tissue formation. Therapies that can augment regeneration of normal tendon and limit the amount of scar tissue that is formed in response to injury may improve clinical outcomes.

Stem cells have great promise in enhancing the biologic healing process since they provide a self-renewing population of pluripotent cells. However, several questions still remain before stem cells can be used clinically for augmenting tendon healing. Specifically, the type of stem cell, the amount of cells, the combination of growth factors and mechanical stimuli, and the ideal delivery vehicle all still need to be determined. The purpose of this paper is to outline the current research on developing a clinical stem cell therapy for the augmentation of tendon healing.

2. Types of Stem Cells

Stem cells are defined as a population of cells that can self-renew through symmetrical mitotic division, form daughter cell lines, and generate a broad range of tissue lineages through terminal differentiation [1, 2]. Stem cells can be derived from a number of sources, and thus different stem cell categories exist. These categories include embryonic, peri-natal (obtained from the umbilical cord or from amniotic tissue), somatic adult, or induced pluripotent stem cells (iPSCs). While there is some overlap, the most common categories of adult stem cells are mesenchymal stem cells (MSCs) and hematopoietic stem cells which are both defined based upon specific stem cell surface markers [3]. iPSCs are not adult stem cells in origin but mature adult cells that are modified resulting in cell pluripotency and the characteristics of embryonic cells [4, 5]. This exciting technique holds great promise for the future, but there has been very little investigation of their usefulness for orthopaedic interventions. In view of ethical concerns and current regulatory issues associated with embryonic or perinatal stem cells, orthopaedic stem cell research has predominantly focused upon MSCs.

MSCs (also referred to as mesenchymal stromal cells) are defined by their ability to self-renew and their multipotentiality [6]. MSCs are defined by three characteristics: (i) an ability to adhere to plastic, (ii) presentation of stem cell

specific antigens, and (iii) the potential to form multipotent mesenchymal cells which can differentiate into a number of cell lines interesting to musculoskeletal medicine such as osteocytes, chondrocytes, and adipocytes [7–9]. No stem-cell-specific marker has been isolated to date, although numerous stem-cell-associated positive and negative markers have been identified. Stem cell associated positive markers include CD 31, 34, 40, 49c, 53, 74, 90, 106, 133, 144, and 163, as well as cKit and Slams [10–13]. Negative stem cell markers indicate other cell lineages such as hematopoietic and endothelial cells and include CD 14, 31, 34, and 45 [14–17]. Induction of MSCs into specific cell lineages such as tenocytes is determined by culturing processes as well as growth and media conditions (Figure 1).

Bi et al. identified a tendon progenitor stem cell (TPSC) population in both mice and humans [14]. A greater propensity of TPSC was identified in "niches," or specialized tendon microenvironments, that contain an array of growth factors such as biglycan and fibromodulin. TPSC can be differentiated from tenocytes by the presence of stem cell markers such as Oct-4, tenomodulin, and SSEA-4 [16]. Multidifferentiation potential is maintained within the TPSC population as they can differentiate into tenocytes, chondrocytes, osteocytes, and adipocytes. Prostaglandin E_2 (PGE_2), BMP-2, BMP-12 and -13 TGF-β_3, and platelet-rich plasma releasate are proposed to be important mediators for promoting stem cell differentiation into tendon tissue as opposed to adipocyte and osteocyte formation [15, 18]. Tendon progenitor stem cells decrease with age, which may contribute to the age-related reduction in tendon repair seen in rotator cuff tears [19]. Increasing the pool of tendon stem may stimulate increased tendon healing with tendon regeneration rather than reactive scar formation.

MSCs have been isolated from a number of different tissue sources. The most common sources of MSCs for musculoskeletal applications are bone marrow and adipose tissue due to their accessibility and the ability to obtain large numbers of viable cells [20]. Bone-marrow-derived MSCs have a greater ability to differentiate into chondrocytes and osteocytes compared to adipose-derived MSCs [21, 22], although the latter provides greater ease of access and decreased donor site morbidity [23]. However, human bone marrow consists of very low yields of MSCs, accounting for only 0.001–0.01% of total nucleated cells [8]. Less common sources of MSCs include tendon, muscle, synovium, cartilage, skin, peripheral blood, periodontal tissue, hair follicles, and scalp tissue [18, 24–26]. While MSCs are relatively easy to harvest, there is concern about their ability to efficiently differentiate into tendon. Research into embryonic, perinatal, and iPSCs is fastly emerging, though their usefulness for tendon augmentation remains to be seen.

3. Untreated Stem Cells

As MSCs possess the potential to differentiate into tenocytes, MSC application to torn tendons is proposed to recapitulate tendon development signals and improve tendon healing capabilities. MSC-mediated tendon regeneration has been studied in numerous animal tendon models.

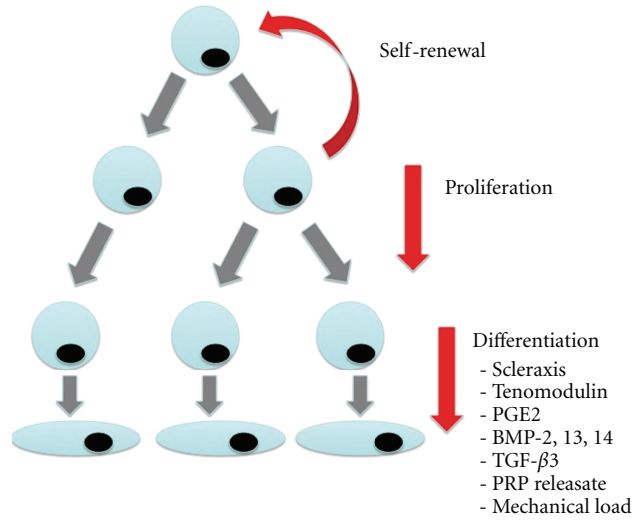

Figure 1: Pathway of mesenchymal stem cell differentiation into tenocytes.

The addition of MSCs to semitendinosus tendons in bone tunnels during anterior cruciate ligament (ACL) reconstructions promoted improved healing with higher failure loads and stiffness after 8 weeks [27]. Healing occurred through fibrocartilagenous tissue that resembled the native tendon-to-bone insertion site rather than the scar tissue typically seen following injury. Lim et al. also delivered MSCs in a fibrin glue carrier to tendon grafts in a rabbit ACL reconstruction model [28]. At 2 weeks, large numbers of immature fibrocartilage cells were noted at the tendon enthesis, and, by 8 weeks, the enthesis resembled a normal ACL insertion with a mature fibrocartilagenous zone as well as improved load to failure and stiffness properties. In a similar study by Soon et al., synovium-derived MSCs were added to rabbit ACL repairs [20]. Compared to the control group without cells, MCS-coated grafts produced a fibrocartilagenous zone resembling a normal ACL insertion. While the MSC-treated group produced significantly higher load to failures, it also had lower stiffness and Young's modulus values suggesting that MSCs mediated a more physiologic regenerative response.

Awad et al. demonstrated that bone-marrow-derived stem cells delivered to a patellar tendon wound site resulted in improved mechanical and some histological properties [24]. The addition of MSCs resulted in increased maximum stress, modulus, and strain in addition to improved mature collagen fibers and cells. However, there was no significant improvement in microstructure compared to the control group, and ectopic bone formation was also noted.

Another study used a rabbit Achilles model with a 1 cm tendon defect that was augmented with MSCs suspended in collagen gel that had been contracted onto pretensioned sutures [29]. MSC treatment resulted in superior load properties, collagen fiber alignment, and cross-sectional area at up to 12 weeks. Despite the increase in tissue volume, the regenerated tissue was composed of fibroblast-like cells rather than tenocytes. A study by Chong et al. reported an

early improvement in mechanical and histologic properties at three, but not six weeks following MSC delivery to a rabbit Achilles tendon model [25]. Labeled bone-marrow-derived MSCs were noted to be viable and localized to the intratendinous region at six weeks, before displaying a more diffuse spread. This suggested that any MSC-mediated acceleration in healing occurs during the early phase of healing. Synovium-derived MSCs have also been shown to accelerate early remodelling of Achilles tendons by stimulating increased collagen fiber production at 1 week and fibers resembling Sharpey's fibers between the tendon and bone at 2 weeks [26].

A number of animal studies suggest that simply delivering stem cells alone not appear to improve tendon healing. Gulotta et al. showed that the application of bone-marrow-derived MSCs alone was insufficient to improve rotator cuff healing in a rat model [30]. The effectiveness of MSCs may be determined by achieving targeted and sustained stem cell delivery to the site of tendon defects, as well as through appropriate modulation of growth factors, cytokines, mechanical environment, and cell concentration.

A few studies have tried to address the effect of different stem cell densities. MSCs were seeded onto collagen matrices and contracted onto sutures at different cells densities of 1, 4, and 8 million cells/mL prior to implantation into rabbit patellar tendon defects [31]. The repairs with MSC collagen composites demonstrated significantly higher maximum stresses and moduli, as well as faster healing than repairs without MSCs. The mechanical improvements did not correlate with improved histological appearance, although greater tissue volume was noted. Interestingly, no difference was detected between the different cell densities, suggesting that higher MSC concentrations do not necessarily translate to improved healing. Of concern was the fact that 28% of the MSC-mediated repairs developed ectopic bone formation at the repair site. A similar study by Juncosa-Melvin et al. investigated the effect of seeding different MSC densities onto tissue-engineered constructs to repair patellar defects in rabbits [32]. No differences in mechanical properties were detected between the two cell densities or between patellar tendon defects treated with or without cells.

Some studies have tried to address whether stem cells mediate a superior effect on tendon healing compared to alternative types of differentiated cells. Hankemeier et al. used a patellar tendon defect model to compare the effects of stem cells and human fibroblasts that were both delivered using a fibrin matrix [33]. Stem cells were found to produce better tendon healing with a significantly increased mean collagen fibril diameter and area covered by collagen fibrils after 10 but not 20 days, in addition to increased collagen 1 and 3 mRNA expression at 20 days. However, the different cell types did not produce a difference in mechanical properties, as measured by ultimate stress.

4. Growth and Differentiation Factors and Stem Cells

While most animal studies have shown encouraging results with the simple application of untreated stem cells, a growing consensus exists that optimal therapy will include treatment of these cells with growth factors. Many studies have demonstrated that mesenchymal stem cells differentiate in response to bone morphogenetic proteins, transforming growth factors and fibroblast growth factor [34].

Gulotta et al. have studied the effect of various growth or differentiation factors and stem cells on tendon healing in a rat model of rotator cuff repair [35–37]. They did not find any improvement in histologic and biomechanical outcomes when MSCs were transduced with BMP-13 when compared to untransduced MSC controls [36]. However, they did find improved healing parameters when MSCs were transduced with two genes present during tendon development during embryogenesis, scleraxis [35], and membrane type 1- matrix metalloproteinase (MT1-MMP) [38]. When MSCs transduced with either scleraxis or MT1-MMP were added to the healing site, improved histology scores and biomechanical strength were seen as early as 4 weeks following repair. This led the authors to conclude that certain growth or differentiation factors will be necessary, in addition to MSCs, in order to optimize their effectiveness.

In another study, investigating the role of bone morphogenetic proteins, Rui et al. modeled calcifying tendinopathy with an in vitro model that applied uniaxial cyclic loading to tendon-derived stem cells grown on plates coated with collagen type I [39]. Increased repetitive tensile loads led to increased expression of BMP-2 as well as increased cell alignment along the direction of externally applied tensile forces. In addition, BMP-2, when added to tendon derived stem cells, was demonstrated to promote osteogenic differentiation and led to ectopic calcification.

While growth factor therapy in addition to MSC seems promising, the question remains as to what factor yields the best results. The various possible combinations of factors are daunting, which has led researchers to pursue platelet rich plasma (PRP) as a way of delivering multiple factors in a single therapeutic agent. PRP has been shown to influence the behavior of stem cells. Using tendon stem cells derived from rabbit patellar tendons, Zhang and Wang demonstrated that increased PRP releasate caused tendon stem cells to become larger and elongated [18]. PRP releasate increased tendon stem cell proliferation, induced tendon stem cell differentiation into tenocytes, and increased protein expression and collagen type I and type III production.

5. Mechanical Load and Stem Cells

In addition to growth factors, altering the mechanical environment is another way to induce stem cell differentiation into tenocytes. The mechanical loading of stem cells appears to guide differentiation. Sharma and Snedeker combined mechanical and molecular cues by culturing bone marrow stromal stem cells on a hydrogel matrix with a gradient of mechanical compliance as well as gradients of the ligands fibronectin and collagen type I [40]. These cells differentiated towards osteogenic precursors on fibronectin and tenogenic precursors on the collagen substrate. In addition, osteogenic differentiation increased on stiffer fibronectin substrates and

decreased on collagen substrates. Tenogenic differentiation was only observed on collagen substrates within a narrow range of stiffness. Yin et al. further demonstrated that cells sense matrix topography and that this information effects gene expression and differentiation [41]. Human tendon progenitor cells were seeded on aligned or randomly oriented poly nanofibers. The expression of tendon-specific genes, such as integrin alpha 1, alpha 5, and beta 1 subunits and myosin B was found to be higher in cells growing on aligned nanofibers than those on randomly oriented nanofibers. In addition, randomly oriented nanofibers induced osteogenesis, as demonstrated by increased alkaline phosphatase activity, while aligned scaffolding hindered osteogenesis. This work demonstrates that nanotopography, with an aligned organization, induces tendon stem cells to form tendon-like tissue in vivo.

As work progresses on the development of biomimetic scaffolds for tendon tissue, the mechanical loading of tissue has also been demonstrated to influence stem cell differentiation [42]. Zhang and Wang recently demonstrated, in an in vitro model, how the magnitude of mechanical loading influences the route of differentiation of tendon stem cells [43]. Low mechanical stretching at 4% strain directed tendon stem cells into tenocytes, while stretching at 8% directed stem cells into adipogenic, chondrogenic, and osteogenic lineages. Zhang and Wang also reported, in a live animal rat study, that treadmill running doubled proliferation rates of tendon stem cells in both the patella and Achilles tendon as well as cellular production of collagen [44]. In addition, the lab demonstrated that stem cells isolated from the treadmill group produced more collagen when cultured with tenocytes than stem cells isolated from the cage control group. This indicates that the proper mechanical loading conditions increase the proliferation of tendon stem cells as well as cellular production of collagen.

While mechanical signals have been shown to affect mesenchymal stem cells fate, the characteristics of an effective loading regimen for tendon-to-bone healing have yet to be fully determined. Sen et al., using an in vitro model in which mesenchymal stem cells underwent two twenty-minute episodes of low intensity vibration or high magnitude strain, demonstrated that adipogenesis was suppressed and osteogenesis was amplified when there was at least a one-hour refractory period between bouts [45]. The group further showed that the effect was enhanced with increasing lengths of the refractory periods. This work shows that the scheduling of loading events is at least as important as the magnitude of the loads.

6. Clinical Applications of Stem Cells

A number of clinical applications for augmenting tissue healing with stem cells in humans have been reported. MSCs have been used for human craniofacial tissue regeneration with varying reports of success [46]. Orthopaedic stem cell studies in humans have predominantly focused upon enhancing bone healing, particularly in spine, foot and ankle, and fracture surgery [47, 48]. Centeno et al. treated 227 patients with autologous MSCs that were cultured and then injected into peripheral joints ($n = 213$) or intervertebral discs ($n = 13$) [49]. Patients underwent disease surveillance for an average of 10.6 months, and no malignant transformations were reported. One patient was diagnosed with cancer which the authors believe was "certainly unrelated" to the MSC therapy. Seven patients had complications related to the injection, and 3 possible stem-cell-related complications were reported. Forty-five of the patients had serial MRIs for up to 2 years, and none of the patients showed any evidence of tumor formation, suggesting that MSC therapy is a relatively safe and well-tolerated procedure. However, the few clinical stem cell studies have only reported short-term outcomes and ideally longer-term followup is required to determine safety.

Despite the promising MSC-mediated effects on tendon healing noted in a number of animal studies, there are no clinical studies examining the efficacy and safety of stem cells on human tendon repair. However, other cellular therapies have been shown to clinically improve tendon healing. Clarke at al. applied skin-derived tenocyte-like cells to 33 patients with patellar tendinopathy and compared it to 27 patients who were treated with plasma [50]. The group receiving stem cell treatment noted a significant improvement in Victorian Institute of Sport Assessment (VISA) scores. While both the cell and plasma groups resulted in an improvement in tendon hypoechogenicity on ultrasound and tear size, only the cell group showed a significant decrease in tendon thickness. A pilot study of 12 patients with refractory elbow epicondylitis showed a significant improvement in the patient-rated tennis elbow evaluation scale and ultrasound tendon appearance [51].

A number of issues regarding the clinical application of stem cells still need to be addressed. The method of stem cell aspiration may affect the viability and function of the cells. Stem cell therapies typically require in vitro culturing and expansion of cells to obtain sufficient numbers of viable cells prior to reimplantation (Figure 2). This raises a number of ethical and regulatory concerns. A one-step approach is increasingly advocated, wherein a sufficient number of stem cells are harvested intraoperatively, concentrated, and then reimplanted within the same procedure. This technique is considered an intraoperative procedure using autologous tissue, and as such does not require Food and Drug Authority (FDA) approval. Hernigou et al. have successfully used this technique to treat patients with femoral osteonecrosis and fracture nonunions [52, 53]. A number of commercial bone marrow aspirate concentration systems are available which can concentrate cells within an hour.

The source of stem cells may help to determine stem cell effectiveness. Locally derived stem cells may most likely to be effective in promoting tendon regeneration, particularly in view of the importance of tendon "niches" in tenocyte differentiation humans [14]. Progenitor cells with osteogenic potential were aspirated from the proximal humerus of 23 patients during arthroscopic rotator cuff repairs [54]. No adverse effects of stem cell aspiration were detected on clinical outcomes, although the aspirated cells were not reinjected into the patients. The authors proposed that the proximal

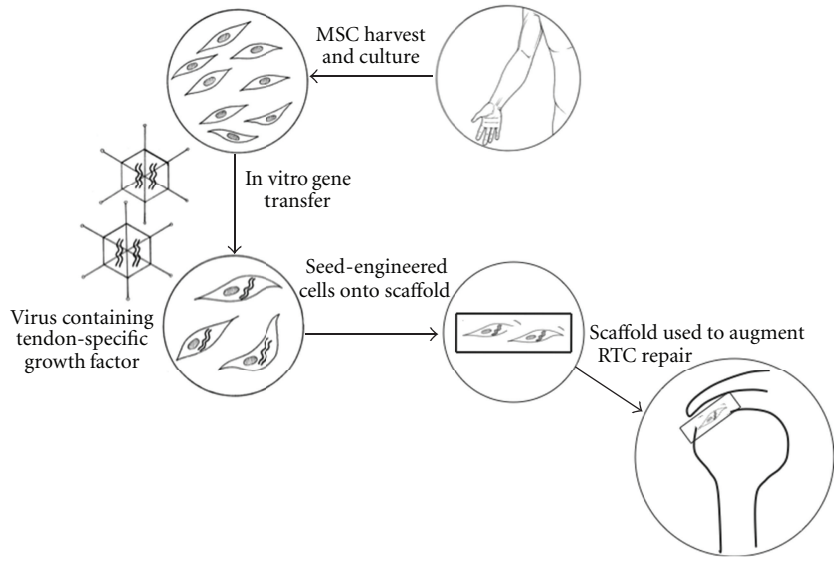

Figure 2: One potential clinical application of stem cell technology for tendon repair.

humerus is an efficient and safe site for harvesting progenitor cells during rotator cuff repairs. Using a reamer-irrigation-aspirator (RIA) during reaming of long bones was shown to permit isolation of MSCs which were comparable to iliac crest bone-marrow-derived cells in terms of differentiation potential and daughter line phenotypes [55]. However, the RIA produced greater numbers of colony forming units and total cell numbers compared to collecting cells from the iliac crest.

Effective translation of promising stem-cell-mediated tendon healing in animals has not yet been proven in the clinical setting. A number of concerns exist about MSC use within humans, such as tumor-like growth of MSCs and modulation of the immune system. Regardless of the source of MSCs, there is a risk of differentiation into undesirable lineages which could result in ectopic tissue formation and calcium deposition. Systemic injections of allogeneic MSCs have been shown to disseminate to a number of organs in baboons [56]. Some of the potential concerns are associated with the culturing process of MSCs and include the risk of genetic alterations, phenotypic drift, as well as transmission of zoonotic infections from the use of fetal bovine serum during culturing.

7. Conclusions

Stem cells are an attractive option for the augmentation of tendon repairs. Embryonic stem cells appear to offer the best differentiation potential, but their use is controversial, and no studies have evaluated their clinical usefulness. Several studies have evaluated the use of MSCs with some success, but profound results have been lacking when these cells are applied untreated. Molecular cues, such as the addition of BMP's, MT1-MMP, and scleraxis, and mechanical cues such as changes in strain and nanotopography, have shown promising results in their ability to drive MSCs into tenocytes differentiation. Utilizing this knowledge to develop a clinically useful therapy has yet to be accomplished, but our understanding of stem cell biology continues to expand with the hope of one day finding one.

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Review Article

Tissue Engineered Strategies for Skeletal Muscle Injury

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Skeletal muscle injuries are common in athletes, occurring with direct and indirect mechanisms and marked residual effects, such as severe long-term pain and physical disability. Current therapy consists of conservative management including RICE protocol (rest, ice, compression, and elevation), nonsteroidal anti-inflammatory drugs, and intramuscular corticosteroids. However, current management of muscle injuries often does not provide optimal restoration to preinjury status. New biological therapies, such as injection of platelet-rich plasma and stem-cell-based therapy, are appealing. Although some studies support PRP application in muscle-injury management, reasons for concern persist, and further research is required for a standardized and safe use of PRP in clinical practice. The role of stem cells needs to be confirmed, as studies are still limited and inconsistent. Further research is needed to identify mechanisms involved in muscle regeneration and in survival, proliferation, and differentiation of stem cells.

1. Introduction

Skeletal muscle injuries are common causes of severe long-term pain and physical disability, accounting for up to 55% of all sports injuries [1]. Contusions and strains are the most frequent muscle lesions, representing more than 90% of all sports-related injuries [2]. Mechanisms of muscle lesion can be divided into direct and indirect trauma. Direct injuries include lacerations and contusions, while indirect injuries include complete or incomplete muscle strains [3]. A muscle contusion takes place when a sudden, heavy compressive force is applied to the muscle [4]. A muscle strain occurs when an excessive tensile force is applied to the muscle leading to the overstraining of the myofibers up to a rupture near the myotendinous junction [5]. Muscle injuries can also result from a combination of these mechanisms. Finally, skeletal muscle can be damaged when compartment syndromes occur because of vascular and/or neurologic impairment [3, 6]. Injuries can counter the beneficial effects of sports participation because of the residual effects. The associated morbidity, including painful contractures and muscle atrophy, can result in prolonged loss of activity and increased risk of recurrent injury [7]. In some instances, muscle injuries leads to inability of athletes to continue to practice sport.

Therefore, there is a need to improve skeletal muscle injury management. Conservative management is commonly accepted, according to the principle that "muscle injuries do heal conservatively." It follows the RICE protocol (rest, ice, compression, and elevation). Other therapies include the local application of heat and passive motion exercises. Drug therapy typically consists of nonsteroidal anti-inflammatory drugs (NSAIDs) and intramuscular corticosteroids.

Operative management is required only in selected patients, such as athletes with a large intramuscular hematoma, a complete strain of a muscle with no agonist muscles, or a partial strain when more than 50% of

the muscle belly is damaged, or persisting extension pain (>6 months) in a previously injured muscle [8].

As current therapy does not seem to obtain complete restoration of preinjury status, new biological therapies could represent interesting and more effective strategies to manage muscle injuries. Biological therapies include cell therapy, tissue engineering, and the administration of growth factors with the goal of enhancing current therapies.

This paper provides an overview on current biological strategies for the management of patients with muscle injuries. The rationale behind these therapies and the best available evidence therapeutic options are reported.

2. Growth Factors

The healing process of the injured skeletal muscle is characterized by several bioactive molecules, including proinflammatory cytokines, transforming growth factor-beta (TGF-β) superfamily members, and angiogenic factors. For this reason, the growth factors and the cytokines represent a potential therapeutic option to improve the regeneration/repair process of injured skeletal muscles. These signaling molecules accelerate the regeneration of injured muscular tissue, providing a mitogenic stimulus activating myogenic precursor cells [9].

Each of these molecules shows specific biological activities. The transforming growth factor-beta (TGF-β) stimulates mesenchymal cell proliferation [10], promotes the proliferation of fibroblasts [11] and the biosynthesis of extracellular matrix, particularly type I collagen [12], regulates endothelial cell activity and angiogenesis [13], and inhibits satellite cell proliferation and differentiation [9]. Fibroblast growth factor (FGF) promotes proliferation of fibroblasts [14], stimulates satellite cells proliferation but inhibits their differentiation [15], and promotes the mitogenesis of mesenchymal cells [9]. Epidermal growth factor (EGF) stimulates fibroblasts migration and proliferation and regulates angiogenesis and extracellular matrix homeostasis [16]. The platelet-derived growth factor (PDGF) promotes the mitogenesis of mesenchymal cells and fibroblasts [17], induces proliferation of satellite cells, and inhibits the end stages of myoblast differentiation [18]. Vascular endothelial growth factor (VEGF) promotes endothelial cells mitogenesis and migration [19] and stimulates myoblast migration [20]. The neoangiogenesis plays a critical role in the healing process of muscle injuries. The new vessels sprout from the health tissue surrounding the lesion and provide the supply of oxygen, growth factor, and blood stem cell to enhance the regeneration process [21]. Thus, the restoration of vascular pattern in the injured area represents an early and necessary phase for regeneration and morphological and functional recovery of muscle tissue.

Based on the multitude of their biological effects, the clinical application of growth factors is affected by considerable side effects. An overexpression of growth factors such as TGF-β and FGF has been related to inhibition of myoblasts differentiation and muscle fiber regeneration [15]. In addition, growth factors explain their stimulatory effect on both muscle cells and fibroblasts. Particularly, TGF-β is one of the most important growth factors related to scar formation during healing, and it seems to drive the differentiation of myogenic cells into myofibroblastic cells. For this reason, muscle fiber regeneration and scar-tissue production can be considered two concomitant and competitive processes.

Also, the expression of growth factors is closely regulated by a large number of extracellular matrix (ECM) proteins, namely, the heparin sulfate proteoglycans and the small leucine-rich proteoglycans (SLRPs) [22, 23]. Several growth factors need to bind the heparan sulphate proteoglycans and the SLRPs to provide their biological effects. Thus, the application of growth factors to promote healing of the damaged muscle tissue should include the administration of these specific ECM molecules.

To date, available data from experimental settings are contradictory. Some authors did not report any beneficial effects by the administration of FGF-2 [24] or overexpression of skeletal muscle specific isoform of IGF-1 (mIGF-1) at the injured region [25]. FGF-2, IGF-1, and nerve growth factors can promote muscle healing process, increasing resistance to tensile loading when compared to untreated muscles [26, 27]. Moreover, mouse myoblasts transduced with the IGF-I gene increase their growth rate and enhance the contractile force production of skeletal muscle substitutes consisting of hydrogel and IGF-I engineered myoblasts [28].

A combination of growth factors can be used to regulate the different process of regeneration of muscle tissue and scar tissue production. Thus, the application of IGF-I combined with TGF-β allows to induce muscle regeneration, preventing the formation of a fibrous scar [29].

3. Platelet-Rich Plasma (PRP)

Platelet-rich plasma (PRP) therapy represents an interesting biological technique to provide tissue repair by inducing chemotactic, proliferative, and anabolic host cellular responses [30]. PRP is an autologous product consisting of bioactive agents derived from patients' own platelets [31–33]. Usually, PRP is administered by local injection of the PRP solution or the application of a PRP gel at the time of surgery.

Given the large amount of biological agents required for tissue repair, PRP could be an ideal biological autologous product providing a balanced combination of mediators able to improve the healing process. In clinical practice, the blood clot at the site of injury is replaced with a smaller volume of PRP solution or gel. The increased concentration of platelet at the site of lesion provides a higher concentration of healing bioactive factors than in physiological conditions. To date, PRP has been proposed for management of tendon [34–36], ligament [37, 38], muscle [39], nerve [40, 41], bone [42, 43], and joint injuries [44, 45].

The effectiveness of autologous conditioned serum (ACS) has been compared with Traumeel/Actovegin in a nonrandomized nonblinded pilot study (level III) on muscle strain injuries in professional sportsmen [39]. The ACS

was obtained from whole blood, and it contained bioactive proteins including interleukin-1β (IL-1β), TNF-α, IL-7, fibroblast growth factor-2 (FGF-2), IL-1Ra, HGF, PDGF-AB, TGFβ1, and IGF-1. Traumeel is a homeopathic formulation containing both botanical and mineral ingredients in homeopathic concentrations. Actovegin is a deproteinized calf blood hemodialysate consisting of a physiological mix of amino acids. Although both treatments were safe, the ACS allowed to reduce the time to full recovery and the amount of edema and/or bleeding at MRI images.

These findings have been also confirmed in professional soccer players with muscle lesions varying for size and location [46]. Athletes were managed with activated pure PRP (P-PRP) injections. Full resumption of normal training activities was restored in half of the expected time compared to matched historical controls. The same leukocyte-free PRP preparation has been found effective to manage adductor longus strain in a professional bodybuilder [47].

ACS and PRP have been also evaluated in laboratory settings. ACS was compared with saline solution in a contusion injury model. ACS showed an earlier activation and/or recruitment of satellite cells, and an earlier fusion, with larger regenerating myofibers, compared with controls [48]. PRP increases proliferation of muscle cells, differentiation of satellite cells and synthesis of angiogenic factors in an *in vitro* setting [49]. PRP and leukocyte and platelet-rich plasma (L-PRP) have been also compared in a laboratory-controlled study using a muscle strain rat model. The authors demonstrated that PRP is more effective than L-PRP in terms of myogenesis enhancement and contractile function [50].

Although preliminary data are encouraging, there are some reasons of concern about PRP treatment. First of all, PRP could induce a fibrotic healing response in muscle tissues, by increasing local concentration of TGF. According to experimental data, TGF seems to be able to induce fibrosis in cultured muscle tissue [9]. Moreover, the effectiveness of PRP could be affected by leukocytes within the injected solution, because their enzymes (proteases and acid hydrolases) can damage muscle tissue [51].

Finally, several devices and systems are available for PRP preparation. Therefore, PRP products applied in several studies consist of a basic mixture of grow factors including different concentration of each single agent. Moreover, level I studies performed with adequate outcome measures and follow-up assessment are lacking.

To date, no PRP formulation has solid evidence of effectiveness to heal muscle injuries. Pilot clinical studies indicate that PRP therapies may enhance muscle repair after strain or contusion. Moreover, laboratory data indicate the ability of several growth factors to enhance myogenesis. However, at present, there is no evidence to recommend or discourage the adoption of PRP in clinical practice. Further research is required to standardize formulations (number of platelets and/or leukocytes) and administration regimens, including volume of injection and timing of treatment, to optimize PRP application for management of muscle injuries.

4. Cell Therapy

In the last decade, regenerative medicine and tissue engineering increased their role in management of musculoskeletal diseases. Transplantation of stem cells has been considered a new strategy to repair injured tissues [52–64]. Different areas of application have been explored, such as articular cartilage [65–68], bone [69–72], ligament, and tendon [73–77]. The expectations for future therapeutic strategies are great.

The idea of cell therapies for muscle regeneration has been developed from the observation that skeletal muscle has regenerative capacity [78–80]. Several studies have investigated the role of stem cells in muscle healing, showing their direct participation in tissue regeneration and their influence in healing modulation [12, 81, 82]. However, severe muscle injuries are characterized by concomitant activation of regenerative activities of the satellite cells and profibrotic activities of fibroblasts [83, 84].

The specific expression pattern of growth factors in the region of injury determines the dominant cell type in the wound healing process [12]. High levels of TGF-β3 are related to the activation of mesenchymal progenitor cells (MPCs) derived from traumatized muscle to promote wound healing after muscle injury [85]. On the other hand, high levels of TGF-β1 are related to activation of fibroblasts to produce disorganized extracellular matrix leading to fibrosis in the muscle tissue [81, 82]. The fibrotic tissue affects the ability of the satellite cells to repair the muscle tissue.

There are distinct subsets of myogenic cells. Muscle satellite cells (SCs) are localized under the basal lamina of muscle fibers [86]. They respond to regenerative stimuli by proliferating to form myoblasts which, in turn, differentiate and fuse in multinucleated myotubes [87, 88]. Their capability to renew and to produce differentiated progeny suggests that they are the adult stem-cell population of skeletal muscle [89]. They are also known as Pax7+ cells, based on their expression of the muscle-specific paired box (Pax) transcription factor Pax7 [90]. However, SCs consist of heterogeneous cell population, including Myf5+ cells (90%) and Myf5− cells (10%) [91]. The first of them are committed to the myogenic lineage because of expression of Myf5 which is an initiator of myogenic differentiation [92].

Other stem cells have been identified. They are both muscle specific, such as mesoangioblasts and pericytes, skeletal muscle precursors (SMPs), muscle stem cells (MuSCs), side-population (SP) cells, and PW1-cells, and nonspecific, such as embryonic stem (ES) cell, amniotic fluid stem (AFS) cells, mesenchymal stem cells (MSCs), and mesenchymal cells from bone marrow [93]. They are able to contribute to muscle regeneration with different myogenic potential, but their potential is still undefined. Satellite cells seem to be sufficient for the regenerative need of damaged adult skeletal muscle *in vivo* [94]. The MSCs present a great migration potential toward the areas of induced muscle degeneration and undergo myogenic differentiation, providing regeneration of muscle tissue. The MSC transduction with transcription factors, such as MyoD, has been also investigated to enhance their potential of myogenic differentiation [95].

The properties of the stem cells in the muscle have been analyzed using animal models of muscle dysfunctions and injuries. Improved muscular structure has been observed in mice used as Duchene Muscular Dystrophy models treated with stem cells [96–98]. Better muscle regeneration has been obtained by the use of muscle-derived stem cells (MDSCs) in models of induced skeletal muscle injury [99]. MDSCs provide an improvement of muscle healing because of their ability to recruit capillaries and nerves into the injured region [99]. They are also able to differentiate directly into endothelial cells and cell types with neuronal characteristics [100]. For these reasons, muscle regeneration seems to be more powerful with MDSCs application compared with satellite cells application.

A model of hindlimb ischemia, analogous to exercise-induced compartment syndrome, showed potential benefit of injections of marrow-derived stromal cells in term of perfusion, fibrosis development, and atrophy [101]. Results from ongoing studies on MDSCs implantation after musculoskeletal contusion are awaited [84].

The role of stem cells in musculoskeletal disease needs to be confirmed. Studies are still limited, and many questions are still unanswered. Several issues should be taken into account, such as safety and efficacy, immunogenicity, and biochemical factors involved in survival and differentiation of stem cells. Further research is needed to identify mechanisms involved in muscle regeneration to exactly understand the therapeutic potential of stem cells.

5. Scaffolds

Regenerative medicine is a multidisciplinary approach to produce living, functional substitutes for restoration, maintenance or improvement of the function of damaged tissue or organ. Tissue engineering is a specific approach included in regenerative medicine field. The tissue engineering consists of association of three main elements: cells, factors or stimuli, and biomaterials [102].

Musculoskeletal tissue engineering aims to obtain functional replacement of lost or damaged bone, cartilage, skeletal muscle, and tendon/ligament. In skeletal muscle injuries, tissue engineering represents a biological alternative for replacement of large tissue loss after severe damage.

Skeletal muscle tissue engineering could be performed by two different approaches: *in vitro* and *in vivo*. In *in vitro* tissue engineering, SCs from adult skeletal muscle are expanded and seeded on a 3D scaffold to produce a cell-biomaterial construct. After the differentiation of stem cells, the neotissue graft could be transplanted in the injured region. In *in vivo* tissue engineering, the isolated SCs are charged on a 3D scaffold carrier and promptly transplanted. Thus, the delivery of stem cells in the muscle lesion is obtained [93].

Efficient skeletal muscle regeneration is strongly related with features of biomaterials used to fabricate scaffold and with the regenerative potential of cells used for scaffold seeding. The source of cells used for scaffold seeding should be chosen based on the features of the damaged tissue. Cells can be autologous or allogeneic, including also stem cells where it is required.

The scaffold is a 3D-structure able to mimic the anatomical and biomechanical properties of the native tissue. The scaffold for muscle tissue engineering should be able to flex and stretch [103]. Moreover, they should be able to promote the alignment of myoblasts the assembly of myotubes. Nanostructured scaffolds are more efficient in promoting myotube assembly than microstructured scaffolds [104].

The biomaterials used to fabricate scaffold can be natural (like collagen) or synthetic (e.g., ceramics, polymers of lactic, and glycolic acid) and soluble or insoluble. Scaffold must have biocompatibility and biodegradability properties [105]. Biocompatibility is essential to prevent toxicity and immunogenicity biomaterial-related inducing the immune-response in the host muscle. Biodegradability allows gradual substitution of the scaffold by the newly formed muscle tissue. Moreover, the scaffold should integrate molecules or cells, providing a controlled delivery of growth factors, cytokines, plasmids, drugs, or other anabolic stimuli [106–109]. In skeletal muscle tissue engineering, biomaterials should support the myogenic process, providing a microenvironment which allows cell survival, proliferation and/or differentiation to repair, and/or regenerate the damaged tissue.

Both synthetic and natural scaffolds have been investigated for tissue engineering approaches to muscle regeneration. The polylactic-co-glycolic acid (PGA) is a synthetic biodegradable biomaterial showing appropriate rigidity and connection, appropriate for muscle tissue engineering. Constructs of myoblasts and polyglycolic acid meshes have been evaluated in a muscle regeneration rat model. Regenerate tissue-like structures have been found with aligned myoblasts along strands of polymer fibers. The PGA scaffold allowed the alignment of myoblasts and the assembly of myotubes, reproducing the organization of muscle fibres [110].

In the field of natural biodegradable biomaterials, different 3D scaffolds have been developed. Collagen scaffolds with parallel oriented pores have been used to reproduce the three-dimensional organization of skeletal muscle [111]. Permanent myogenic cells were infiltrated in these scaffolds and were cultured to induce their proliferation and differentiation [111]. The collagen scaffold with oriented pore structure showed the ability to induce skeletal muscle-like tissue regeneration with aligned multinucleated myotubes according to the orientation of pore structure [111]. In addition, cell-scaffold constructs were able to support mechanical forces generated in muscle tissue [111]. These results have been also found in an *in vitro* study in which a multilayered cultures of rat neonatal satellite cells in collagen 3D scaffolds were performed [112].

Fibrin is another natural biodegradable 3D scaffold used to obtain muscle regeneration. Three-dimensional fibrin matrix has been used as carrier to inject myoblasts in the injured muscle region of a rat model. The fibrin carrier induced no inflammatory reaction and allowed integration of myoblasts into host muscle fibers [113]. The fibrin matrix also allows to produce strained fibrin gel by applying continuous tensile strain to fibrin scaffold. The morphological features of strained fibrin gels induce the alignment of seeded myoblasts. Moreover, the aligned cells are parallel to

the direction of the strain reproducing the organization of skeletal muscle tissue [114]. The fibrin matrix also allows the differentiation of myoblasts, cultured in a three-dimensional pattern, under electrical stimulation [115]. Finally, fibrin scaffolds have been also combined with adult human cells to regenerate muscle after large tissue loss in a mouse model with large defect of tibialis anterior muscle. Constructs of fibrin microthreads and adult human cells were used, showing the role of constructs in host tissue regeneration by forming skeletal muscle fibers, connective tissue, and PAX7 positive cells [116].

Another type of natural scaffold is a hyaluronan-based hydrogel that has been used to perform the delivery of either SCs or MPCs in a mice model. The construct SC-hydrogel showed more enhancement of regeneration process with a higher number of new myofibers than MPC-hydrogel or hydrogel alone. In the muscle receiving the SCs, there was a functional SC niche associated with neural and vascular networks [117].

The acellular muscle ECM has been also investigated in muscle tissue-engineering field. The acellular muscle scaffold was derived from the extensor digitorum longus muscle, and was injected with myoblasts. The constructs allowed cell survival and proliferation and showed longitudinal contractile force on electrical stimulation [118].

Each type of scaffold shows specific proprieties and peculiar advantages. The final goal of scaffold fabrication consists of promoting the proliferation of muscle stem cells, their differentiation, and parallel alignment to obtain a new skeletal muscle-like tissue. The application of scaffold for regeneration of muscle tissue could represents an interesting approach particularly in the major trauma with large loss of tissue. In the majority of muscle injuries, the role of scaffold remains unclear and maybe not as important as in bone or cartilage regeneration. In fact, the skeletal muscle is characterized by different layer of connective tissue, such as endomysium, perimysium, and epimysium, which seems to be able to drive the regeneration of new muscle fibers without the need of scaffold. Further studies are required to identify the best scaffold for skeletal muscle tissue engineering. However, the combination of available techniques could represent the right way to fabricate the ideal scaffold.

6. Conclusion

Skeletal muscle injuries are the most common injury in sport, occurring with direct and indirect mechanisms. Their effective management is a challenging issue in orthopaedic sport medicine because of the residual effects, such as severe long-term pain and physical disability. Skeletal muscle injuries cause time loss of activity and increased risk of recurrent injury. For these reasons, they constitute a health problem for athletes and an economic problem for clubs and sponsors.

In most of the instances, current therapy consists of conservative management including RICE protocol and administration of NSAIDs or intramuscular corticosteroids. However, current management of muscle injuries does not often provide an optimal restore of preinjury status because of the fibrosis which occurs during the repair process of injured muscle. Experimental studies highlight the biological bases of muscle healing after contusion, strain, or laceration injury. This provides the rationale basis for new biological therapies, such as PRP and growth factors, cell-based therapy and tissue engineering. Biological strategies may well be more favourable to healing. Although PRP application is encouraged, reasons for concern persist in its use for muscle injury management, and its mechanism of action remains uncertain. Further research is required to allow a standardized and safety use of this product in clinical practice. Cell-based strategies have been investigated only in limited and inconsistent studies. The role of stem cells needs to be confirmed. Further research is required to identify mechanisms involved in muscle regeneration and in survival, proliferation, and differentiation of stem cells. Skeletal muscle tissue engineering represents a biological alternative for replacement of large tissue loss after severe damage, based on combination of adult or embryonic stem cells, factors or stimuli, and biomaterials. However, further studies are required to identify the best biomaterial to fabricate the ideal scaffold, the best cell source for scaffold seeding, and the role of growth factors and other stimuli used to functionalize the scaffold.

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Review Article

Tissue Engineering for Rotator Cuff Repair: An Evidence-Based Systematic Review

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The purpose of this systematic review was to address the treatment of rotator cuff tears by applying tissue engineering approaches to improve tendon healing, specifically platelet rich plasma (PRP) augmentation, stem cells, and scaffolds. Our systematic search was performed using the combination of the following terms: "rotator cuff", "shoulder", "PRP", "platelet rich plasma", "stemcells", "scaffold", "growth factors", and "tissue engineering". No level I or II studies were found on the use of scaffolds and stem cells for rotator cuff repair. Three studies compared rotator cuff repair with or without PRP augmentation. All authors performed arthroscopic rotator cuff repair with different techniques of suture anchor fixation and different PRP augmentation. The three studies found no difference in clinical rating scales and functional outcomes between PRP and control groups. Only one study showed clinical statistically significant difference between the two groups at the 3-month followup. Any statistically significant difference in the rates of tendon rerupture between the control group and the PRP group was found using the magnetic resonance imaging. The current literature on tissue engineering application for rotator cuff repair is scanty. Comparative studies included in this review suggest that PRP augmented repair of a rotator cuff does not yield improved functional and clinical outcome compared with non-augmented repair at a medium and long-term followup.

1. Introduction

Rotator cuff tears are an important cause of shoulder pain and disability [1–4]. Despite its frequency and great health care costs in industrialised countries, the best management options for rotator cuff tears are still debated [5, 6]. One of the reasons is that the pathogenesis of rotator cuff tears is still largely unknown [7–12]. Moreover, the cuff has a limited ability to heal back to its insertion on the humerus after the repair process is ended. Given this limited ability for healing, novel biomechanical strategies (double-row techniques [13–16]) and biological augmentations (such as growth factors and cytokines, platelet rich plasma (PRP) [17], tendon graft [18–20], and tissue engineering with mesenchymal stem cells [21]) have been proposed to enhance rotator cuff tendon healing. They hold the promise to yield more successful outcomes for the management of patients with tendon pathology.

The purpose of this systematic review was to address the treatment of rotator cuff tears by applying tissue engineering approaches to improve tendon healing.

2. Methods

We identified all published studies in the English language addressing tissue engineering for rotator cuff repair, using a methodology already validated in our setting. Two independent reviewers performed a search of the Medline database on PubMed, CINAHL (Cumulative Index to Nursing and Allied Health Literature), EMBASE, and the Cochrane Central Register of Controlled Trials from

inception of database to July 2011, using the combination of following terms: "rotator cuff", "shoulder", "PRP", "platelet rich plasma", "stem cells", "scaffold", "growth factors", and "tissue engineering". Before conducting the literature search, we established the study design and specific objectives. Studies were included in our systematic review if they met the following guidelines: (1) they provided level I-II evidence addressing the area of interest outlined above, (2) they included measures of functional and clinical outcome, (3) they had minimum 3 month followup, and (4) they were published in peer review journal. Citations from relevant studies, as well as from any review articles captured by the search, were also examined to determine if they were suitable for inclusion. Studies not meeting these guidelines were excluded. Patient demographic information, rotator cuff tear features, surgical techniques, objective and subjective outcome measurements, radiological examinations, and complications were extracted from the studies. The objectives were to evaluate the clinical and structural outcomes of patients receiving tissue engineering strategies compared to control group patients.

3. Data Abstraction

The data were independently extracted by three reviewers from each of the selected studies. The demographic data collected included the type of study, level of evidence, number of patients enrolled, age, gender, and mean followup. The collected features of rotator cuff tears included tear size according to the classification of DeOrio and Cofield [22] (small: <1 cm; medium: 1 to 3 cm; large: 3 to 5 cm; massive: >5 cm) or arthroscopic classification of tear retraction (grade 1: the tear edge is lying over the greater tuberosity; grade 2: the tear exposed the humeral head without retraction to the glenoid; grade 3: the tear is extended to the glenoid; grade 4: the tear is retracted medial to the glenoid).

Surgical technique data were also recorded, including the surgical repair procedure, number and type of anchors, type of arthroscopic knot, suture type, and concomitant procedures.

Preoperative and postoperative data included range of motion; strength, evaluated in terms of strength in external rotation (SER) and clinical outcome scales (Constant and Murley [23]; University of California, Los Angeles-UCLA [24]; American Shoulder and Elbow Surgeons-ASES [25]; Disabilities of the Arm, Shoulder and Hand-DASH; Shoulder Pain and Disability Index-SPADI; Simple Shoulder Test-SST; Visual Analog Score for Pain-VAS) [26–28].

Postoperative imaging modality and outcome (complete healing, partial healing, and no healing) were also analyzed. The complications related to the surgical procedures and the biological augmentations were also recorded.

4. Results

The search strategy identified 861 articles. Evaluation of title and abstract left 11 articles to be evaluated. Full text of all the eligible papers was screened for inclusion and exclusion criteria, leading to 3 studies on PRP augmentation included in the review [17, 29, 30]. No clinical studies on application of stem cells and scaffolds for rotator cuff repair were found. The study selection process and reasons for exclusions are summarized in Figure 1. Of the three included studies, one level I study evaluated patients with rotator cuff tear in whom the repair was augmented with membrane of platelet-rich fibrin matrix [17], one level I study with PRP and autologous thrombin [30] and one level II study with PRP gel [29].

5. Patient Demographics

There were 2 randomized controlled trials (Level I) [17, 30] and 1 prospective cohort study (Level II) [29] (Table 1). In 2 studies, the followup was completed by 100% of patients [17, 29], whereas in 1 study it was completed by 85% of patients [30]. The mean age of patients ranged between 55 and 60 years in both PRP and control group for all the studies. Each study compared the study groups. No statistically significant differences were found in terms of age, gender, and followup [17, 29, 30].

6. Surgical Technique

All the studies described the surgical procedure consisting of arthroscopic rotator cuff repair with suture anchor fixation (Table 2). In all the studies, the number of suture anchors was established according to the size of rotator cuff lesion in both control and PRP group. Suture anchors ranged from 1 to 3 in patients with small or medium tears and from 3 to 5 for large or massive tears. None of the studies performed statistical analysis comparing the mean number of anchors between the two groups. In two studies, the authors used bioabsorbable suture anchors [29, 30] and metallic suture anchors in the other study [17]. Rotator cuff repair was performed with different arthroscopic techniques. Castricini et al. [17] performed a double-row technique with metal suture anchors (Fastin RC Anchor; DePuy Mitek, Raynham, Massachusetts) in which medial row was secured using nonsliding knots in a mattress configuration, whereas lateral row used sliding knots with 3 alternating half hitches. Randelli et al. [30] performed a single-row technique with absorbable suture anchors (Bio-Corkscrew; Arthrex, Naples, FL, USA). Jo et al. [29] performed a suture bridge technique with absorbable suture anchors (Bio-Corkscrew; Arthrex, Naples, FL, USA) in which medial row was secured using a slippage proof knot, whereas lateral row was secured using PushLocks (Arthrex) or suture anchors.

In addition to rotator cuff repair, concomitant procedures were performed in both groups in all the studies. In the study by Castricini et al. [17], 25 patients in the control group (56%) underwent acromionplasty, 22 (49%) underwent a biceps tenodesis, and 5 underwent a biceps tenotomy (11%); 12 patients (28%) underwent at acromionplasty, 21 (49%) underwent a biceps tenodesis, and 3 (7%) underwent a biceps tenotomy in the PRP group. Randelli et al. [30] performed an acromionplasty in 27 patients (100%), biceps tenodesis in one patient (4%), and biceps tenotomy in

Table 1: Study and demographic data.

Study	Level of evidence		Patients	Mean age (Range or ± SD), y	Men/Women	Followup (Range or ± SD), months	Size of lesion			
Castricini et al., 2011 [17]	I	Control group	45	55.2 (37–69)	23/22	20.2 (16–30)	20 Small RCT	25 Medium RCT	0	0
		PRP group	43	55.5 (41–72)	17/26		18 Small RCT	25 Medium RCT	0	0
Randelli et al., 2011 [30]	I	Control group	27	59.5 (±10.7)	13/14	23	12 Minor	7 Moderate	4 Severe	4 Massive
		PRP group	26	61.6 (±8.3)	8/18		9 Minor	7 Moderate	3 Severe	7 Massive
Jo et al., 2011 [29]	II	Control group	23	59.80 (±8.84)	9/14	20.30 (±1.89)	2 Small RCT	15 Medium RCT	3 Large RCT	3 Massive RCT
		PRP group	19	61.80 (±8.86)	6/13	18.94 (±1.63)	1 Small RCT	7 Medium RCT	5 Large RCT	6 Massive RCT

RCT: rotator cuff tear.

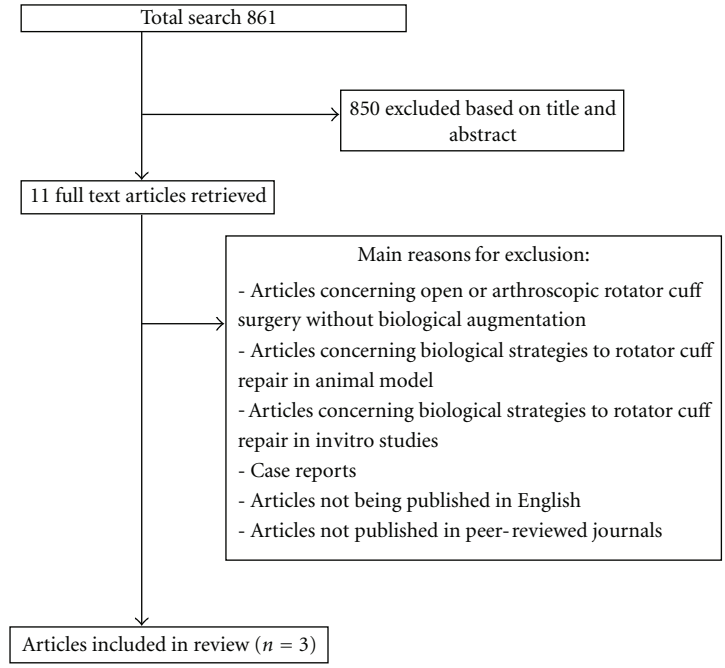

Figure 1: Flowchart of the search strategy and selection of articles.

18 patients (67%) in the control group. They performed acromionplasty in 26 patients (100%), biceps tenodesis in 4 patients (15%), and biceps tenotomy in 15 patients (58%) in the PRP group. Jo et al. [29] rarely performed an acromionplasty: 4 patients (17%) in the control group and 3 patients (16%) in the PRP group.

The PRP augmentation of the rotator cuff was performed with different techniques. Castricini et al. [17] used a platelet-rich fibrin matrix (PRFM) which was a flat membrane of antilogous suturable fibrin. It was applied under the supraspinatus tendon, above the bleeding surface of the greater tuberosity, by using one of the suture limbs of lateral anchors and by pulling the other end of the suture. Randelli et al. [30] used activated PRP combined with antilogous thrombin, which was loaded with syringes. They injected this product between the bone and the repaired rotator cuff and then performed a dry arthroscopic check of the clot formation. Jo et al. [29] used PRP gel. In each patient, three PRP gels were placed in the repair site at the tendon-bone interface during the arthroscopic repair procedure. When the PRP gels were in place, medial and lateral row sutures were tied, and PRP gels were snuggled between the repaired tendon and the bone insertion.

7. Rehabilitation Protocol

The postoperative rehabilitation was the same for the control group and the PRP group in each study, limiting performance bias. A rest period was performed in all the studies. Castricini et al. [17] performed 3 weeks of immobilization using a sling with an abduction pillow. Jo et al. [29] performed 4 weeks of immobilization for small to large tears, and 6 weeks for massive tears, using an abduction brace. Randelli et al. [30] performed a short rest period of 10 days wearing the sling. During the rest period, Castricini et al. [17] allowed only pendulum exercises, whereas Jo et al. [29] allowed shrugging, protraction, and retraction of shoulder girdles: mobilization of the elbow, wrist, and hand; and external rotation of the arm to neutral according to patient compliance. Passive range of motion (ROM) and active-assisted ROM exercises were allowed after 3 to 6 week rest period, according to author protocols [17, 29].

In the study by Randelli et al. [30], patients started passive assisted exercises after the rest period to obtain a complete passive ROM restoration. At 30 days from surgery, assisted active range-of-motion exercises were allowed.

Strengthening exercises of the rotator cuff and scapular stabilizers were performed after 6–8 weeks [17, 30] or 12 weeks [29], according to author protocols. Light sports activities were allowed 3 months after surgery, whereas full return to sports, overhead activities, and heavy manual work were allowed after a minimum of 6 months, based on patient recovery [17, 29].

8. Clinical Shoulder Scores

All the studies used the Constant score, and 2 used the UCLA and SST scores [29, 30]. In addition, Randelli et al. [30] used also SER and VAS scores, whereas Jo et al. [29] used ASES, DASH, and SPADI scores (Table 3).

Castricini et al. [17] found a statistically significant improvement from the preoperative to postoperative mean values in the Constant score for each group ($P = 0.001$), but no statistically significant differences when comparing the 2 groups.

Table 2: Surgical techniques and concomitant procedures.

Study		Surgical technique	Total N of anchors	Type of anchors	Type and size of suture	Type of knots	Concomitant procedures	Complications
Castricini et al., 2011 [17]	Control group	Arthroscopic RC repair with double-row technique	2 for each patients	Metal suture anchors (Fastin RC Anchor; DePuy Mitek)	No. 2 Ethibond Excel (Ethicon)	Medial sutures: nonsliding knot in a mattress configuration; lateral sutures: sliding knot	Acromionplasty 25; Tenodesis 22; Tenotomy 5	0
	PRP group	Arthroscopic RC repair with double-row technique with membrane of PRFM augmentation	2 for 41 patients and 3 for 2 patients				Acromionplasty 12; Tenodesis 21; Tenotomy 3	0
Randelli et al., 2011 [30]	Control group	Arthroscopic RC repair with single-row technique	1.6 ± 0.7	Absorbable suture anchors (Bio-Corkscrew; Arthrex)	—	—	Acromionplasty 27; Tenodesis 1; Tenotomy 18	0
	PRP group	Arthroscopic RC repair with single-row technique and injection of PRP and autologous thrombin	2 ± 0.9		—	—	Acromionplasty 26; Tenodesis 4; Tenotomy 15	0
Jo et al., 2011 [29]	Control group	Arthroscopic RC repair with suture bridge technique	2 or 3 for small/medium tears; 3 to 5 for large/massive tear	Absorbable suture anchors (Bio-Corkscrew; Arthrex); PushLocks (Arthrex)	No. 1 Polydioxanone II suture (Ethicon)	Medial sutures: slippage proof knot; knotless suture anchor repair	Acromionplasty 4	0
	PRP group	Arthroscopic RC repair with suture bridge technique and application of PRP gel					Acromionplasty 3	0

RC: rotator cuff; PRFM: platelet-rich fibrin matrix.

Table 3: Clinical outcomes.

Study	Outcome measures	Pre-op.	3 days	1 month	3 months	6 months	12 months	16 months	24 months	
Castricini et al., 2011 [17]	Constant score									*P* value
	Control group	42.9 (22–55)						88.4 (54–100)		<0.001
	PRP group	42 (30–53)						88.4 (72–99)		<0.001
	P value							0.44		
Randelli et al., 2011 [30]	Constant score									
	Control group	42.2 ± 15.2			57.8 ± 11	72.3 ± 12.6	75.7 ± 9.5		78.7 ± 10	
	PRP group	44 ± 16.5			65 ± 9	73.1 ± 8.7	78.3 ± 6.4		82.4 ± 6.3	
	P value	0.6			0.02	0.7	0.3		0.1	
	UCLA									
	Control group	14.5 ± 5.6			24.2 ± 4.9	29.2 ± 4.9	31 ± 4.1		31.3 ± 4.1	
	PRP group	15.3 ± 5.9			26.9 ± 3	30.6 ± 4.1	31.2 ± 5.2		33.3 ± 2.2	
	P value	0.6			0.03	0.3	0.7		0.06	
	SER (Kg)									
	Control group	2.3 ± 2			2.1 ± 1.3	3.3 ± 1.3	3.7 ± 1.5		4 ± 1.9	
	PRP group	1.9 ± 1.7			3 ± 1.6	3.9 ± 2.1	4.2 ± 2.8		4.3 ± 2.3	
	P value	0.4			0.04	0.2	0.5		0.5	
	SST									
	Control group	4.7 ± 2.8			7.1 ± 2.7	10.5 ± 2.3	10.6 ± 1.5		10.9 ± 1.4	
	PRP group	4.8 ± 3.1			8.9 ± 2.2	10.6 ± 1.4	11.1 ± 0.9		11.3 ± 0.9	
	P value	0.9			0.02	0.9	0.3		0.3	
	VAS									
	Control group	6.4 ± 2	6.3 ± 2.8	2.4 ± 2.6						
	PRP group	4.8 ± 2	4 ± 3.2	1.1 ± 2.2						
	P value	0.003	0.007	0.01						

Table 3: Continued.

Study	Outcome measures	Pre-op.	3 days	1 month	3 months	6 months	12 months	16 months	24 months
	Constant Score								
	Control group	50.78 ± 16.04			46.10 ± 17.75	64.56 ± 15.98	81.36 ± 11.97	82.00 ± 13.02	
	PRP group	46.47 ± 16.50			33.47 ± 14.39	63.36 ± 11.73	77.65 ± 13.02	79.12 ± 13.42	
	P value	0.397			0.036	0.910	0.493	0.476	
	UCLA								
	Control group	16.78 ± 4.73			22.00 ± 4.01	25.94 ± 4.84	29.77 ± 4.36	30.83 ± 4.96	
	PRP group	15.89 ± 4.98			18.00 ± 7.47	26.27 ± 4.43	30.12 ± 6.04	31.78 ± 6.15	
	P value	0.558			0.061	0.827	0.708	0.579	
	ASES								
	Control group	49.60 ± 17.74			60.58 ± 16.33	71.75 ± 18.21	89.19 ± 10.73	89.92 ± 17.03	
Jo et al., 2011 [29]	PRP group	43.95 ± 20.44			46.22 ± 20.06	72.63 ± 13.63	86.26 ± 19.95	87.61 ± 24.83	
	P value	0.343			0.031	0.530	0.712	0.744	
	DASH								
	Control group	45.69 ± 25.51			38.83 ± 20.14	23.80 ± 16.74	9.20 ± 9.87	8.48 ± 14.05	
	PRP group	52.85 ± 25.29			49.61 ± 23.12	24.85 ± 16.52	12.84 ± 18.89	13.19 ± 25.45	
	P value	0.369			0.166	0.703	0.588	0.473	
	SST								
	Control group	5.17 ± 2.99			5.40 ± 3.57	9.06 ± 5.45	10.64 ± 1.71	10.57 ± 1.73	
	PRP group	4.63 ± 3.29			4.40 ± 2.50	8.36 ± 2.25	9.59 ± 2.85	9.83 ± 3.31	
	P value	0.579			0.369	0.982	0.206	0.355	
	SPADI								
	Control group	46.25 ± 24.05			39.50 ± 23.02	25.71 ± 15.52	9.83 ± 10.59	10.08 ± 16.32	
	PRP group	54.47 ± 28.72			56.33 ± 23.97	28.69 ± 14.29	11.72 ± 18.22	12.03 ± 24.96	
	P value	0.318			0.045	0.745	0.869	0.673	

In the study by Randelli et al. [30], in both groups, postoperative values of Constant, UCLA, and SST scores significantly improved in comparison to the preoperative values at 3 months after surgery. There was a statistically significant difference between the PRP and control groups for all clinical outcomes at the 3-month followup (Constant, $P = 0.02$; UCLA, $P = 0.03$; SST, $P = 0.02$). However, no significant differences between two groups were found at 6, 12, and 24 month followup.

In the study by Jo et al. [29], preoperative values were similar between two groups for all functional scores. Postoperative values of all scores showed a progressive improvement in both groups. ASES, Constant, and SPADI scores were significantly higher in the control group compared with the PRP group at 3 months after surgery (Table 3). However, no significant differences between two groups were observed for any of these scoring systems at 6, 12, and 24 months of followup.

9. Strength and Range of Motion

Only one study provided strength measurements [30]. Authors measured the strength in external rotation (SER) in a sitting position with the arm at side (neutral position).

In the control group, SER score values started to increase at 6 months after surgery. Only at the last followup, there was a statistically significant difference between preoperative (2.3 kg ± 2 kg) and postoperative values (4 kg ± 1.9 kg) ($P = 0.01$). On the other hand, a statistically significant improvement of SER score was found at the first followup in the PRP group (from 1.9 kg ± 1.7 kg to 3 kg ± 1.6 kg; $P = 0.003$). The SER postoperative values increased until 6 months after surgery ($P < 0.001$), while at the last followup any significant improvement was recorded. However, there were no differences in strength measurements when comparing the results of control and PRP groups at 6, 12, and 24 months of followup.

Only in one study the evaluation of range of motion (ROM) was performed [29]. Before surgery, any difference of ROM between two groups was found. ROM decreased in the early postoperative period. Then, starting from 3 months after surgery, ROM increased gradually until final followup. At final followup, forward flexion, and abduction improved significantly in both groups ($P = 0.001$); internal rotation improved significantly only in the PRP group ($P = 0.033$); external rotation did not improve in either group ($P > 0.05$). No statistically significant difference in ROM was found at 3-, 6-, or 12-month followup.

10. Pain

Two studies performed an assessment of pain, expressed in terms of VAS score [29, 30]. In the study by Randelli et al. [30], the baseline values of VAS were significantly different between two groups. In the control group, postoperative values were significantly lower compared with preoperative values, starting from day 7 after surgery ($P = 0.003$). On the other hand, PRP group showed a statistically significant reduction of mean VAS score as soon as day 3 after surgery ($P = 0.04$).

The VAS score was significantly lower in the PRP group at 3, 7, 14, and 30 days of followup (Table 3). Moreover, a significant difference was found between the two groups at 24-month followup ($P = 0.002$).

In the study by Jo et al. [29], preoperative VAS scores were similar in the two groups. The reduction of postoperative values was significant and gradual over time until final followup in both groups (all $P = 0.001$). However, there were no significant differences between the two groups for any value at any time point of followup (all $P > 0.05$).

11. Radiological Assessment

All the studies included postoperative magnetic resonance imaging (MRI) to evaluate tendon integrity. Castricini et al. [17] performed MRI at a mean of 20.2 months from surgery for both the control group and the PRP group. Although, the authors reported a higher rate of tendon rerupture in the control group compared with the PRP group (10.5% versus 2.5%), the difference between arthroscopic repair with or without PRFM was not statistically significant ($P = 0.07$). Randelli et al. [30] performed MRI at a mean of 23 ± 5 months from surgery (25 ± 5 months for the control group and 21 ± 5 months for the PRP group). The mean radiological followup time was slightly longer in the control group ($P = 0.003$). Authors found a not statistically significant difference between the rates of tendon rerupture in the control group compared with the PRP group (52% versus 40%, resp.; $P = 0.4$). In the study by Jo et al. [29], the mean time between surgery and postoperative MRI was 13.93 ± 4.23 in the PRP group and 15.29 ± 5.6 in the conventional group ($P = 0.449$). Authors reported a higher overall retear rate in the control group (41.2%) than in the PRP group (26.7%), without any statistically significant difference ($P = 0.388$).

12. Complications

No complications related to the use of PRP were reported in the included studies.

13. Discussion

The current literature on tissue engineering application for rotator cuff repair is scanty. Although several authors advocate it, uncertainty still exists as to whether tissue engineering is able to yield improved results. Our review suggests that patients receiving PRP augmentation for rotator cuff repair do not show improved functional outcomes when compared with a nonaugmented repair at medium and long-term followup. At a short-term followup, patients managed with PRP augmented repair showed better control of post-operative pain [30]. On the other hand, the structural integrity of the rotator cuff seemed to be slightly better in the PRP augmented group, even though the small number of patients in the included studies did not allow definitive conclusions. Even though no results on the costs of PRP

surgery were available from the included studies, it is possible to speculate that PRP augmented rotator cuff repair yielded to increased economic costs, both for the duration of surgery and the cost for PRP preparation. However, these aspects need to be evaluated in future studies.

14. Selection Bias

Two of the studies included in this systematic review were randomized controlled trial [17, 30] and one was a cohort study [29] (Levels I to II). The random allocation of patients into two groups, receiving PRP treatment or not, should dramatically limit bias. In the study by Jo et al. [29], patients were informed about the use of PRP before surgery and decided themselves whether to have PRP placed at the time of surgery. Generally the 2 groups showed similar age, sex, dominance, symptom duration, and aggravation period before surgery, thus limiting the potential for selection bias.

The factors that have been shown to affect clinical outcome including age, gender, rotator cuff tear size, and acromioclavicular joint pathology were similar between groups in all the studies. In the study by Randelli et al. [30], 11 patients in the PRP group and 13 patients in the control group had only lesions of the supraspinatus, 6 patients in the PRP group and 4 patients in the control group had all three tendons involved. In the study by Jo et al. [29] there were no significant differences in anteroposterior and mediolateral tear sizes between the 2 groups, and rotator cuff muscle status evaluated using global fatty degeneration indices [6], modified tangent signs, and occupational ratios [12] were also not significantly different. Several studies in the open, mini-open, and arthroscopic literature showed that tear size is an important determinant of outcome and healing [3–5, 7, 11, 31].

Three studies reported no difference in clinical rating scales between groups. In the study by Jo et al. [29] the addition of PRP gel to arthroscopic rotator cuff repair was not found to accelerate the relief of pain; the recovery of ROM, strength, or function; or improve overall satisfaction as compared with conventional repair at any time point. Rather, the recovery of some measures in the PRP group, such as ASES, Constant, and SPADI functional scores, and abduction were slower than in the conventional group at 3 months after surgery [29]. The only significant improvement found in the PRP group was in internal rotation at final followup [29].

Randelli et al. [30] found statistically significant difference between the PRP and control groups for all the clinical outcomes (Constant, SER, UCLA, SST) at 3-month followup, but no significant differences between the PRP and control groups at 6, 12, and 24 months. Moreover, the pain score in the treatment group was lower than the control group at 3, 7, 14, and 30 days after surgery, but there was no difference between the 2 groups after 6, 12, and 24 months.

No studies showed significant difference in postoperative tendon healing. Castricini et al. [17] found no difference in tendon thickness and footprint size between the 2 groups. The only difference between the 2 groups was in tendon signal, whose significance was of difficult interpretation. Randelli et al. [30] found no significant difference in the MRI healing rate of the rotator cuff. The number of identified retears was 9 (40%) in the PRP group and 12 (52%) in the control group. This difference was not statistically significant. Retear rate was influenced by age, tear severity, and grade of retraction in the PRP group. Jo et al. [29] also found no significant improvement in structural integrity, and no significant difference in retear rates between the groups.

15. Performance Bias

Surgical technique was adequately described in all the studies [17, 29, 30]. Castricini et al. [17] used a double-row technique, Randelli et al. [30] used a single-row technique and Jo et al. [29] used a suture bridge technique. Performance bias may occur in studies where a disproportionate number of concomitant procedures are performed, but bias is largely limited because of homogeneity between groups. Rehabilitation protocol is another potential variable that may influence performance bias, but the same rehabilitation was implemented for each group in a single study. It was described in details in all the 3 studies [17, 29, 30].

16. Exclusion Bias

Castricini et al. [17] reported at last 16-month clinical results for all the patients (88) and radiological results for 78. In the study by Randelli et al. [30], of the 53 randomized participants, 45 completed clinical and radiological followup. Eight patients (4 for the treatment group and 4 for the control group) did not return at the final followup, and one patient in the PRP group died at about 1 year after the surgical intervention from cardiac arrest.

17. Detection Bias

All studies assessed clinical outcomes according to functional scores. The functional scoring systems used were Constant score, UCLA, ASES, SST, DASH, SPADI. All of these outcome scores have been validated as shoulder-specific outcome instruments [23–25]. All of the studies reported significant improvement between baseline and postoperative scores for each group.

Three studies detect no significant difference in clinical rating scales between the PRP group and the control group. However, Randelli et al. [30] detected a significant improvement in the Constant, SER, UCLA, SST between the PRP and control groups at the 3-month followup. The VAS score was found to be significantly lower in the PRP group at 3, 7, 14, and 30 days postoperative.

All the studies used postoperative MRI. Each study performed statistical analysis between the PRP group and control group.

Castricini et al. [17] reported the findings as tendon thickness, size of tendon footprint and intensity of the signal, grading each of these parameters on a scale from I to III. Randelli et al. [30] differentiated only between retear and

intact tendon. Jo et al. [29] used Sugaya's method [10] for evaluation of structural integrity: Types I, II, and III were considered healed, types IV and V were considered retears.

None of the studies reported a statistically significant improvement in the structural appearance with the PRP augmentation repair compared with the arthroscopic rotator cuff repair without augmentation. In the study by Randelli et al. [30], the number of identified retears was 9 (40%) in the PRP group and 12 (52%) in the control group, but this difference was not statistically significant. The repair integrity of the overall sample was significantly associated with age, shape, and tear retraction. The effect of prognostic factors was more evident in the PRP group. Also, in the study by Jo et al. [29] the overall retear rates between the 2 groups was not significantly different (8 cases (26.7%) in the PRP group and 14 (41.2%) in the conventional group).

These findings raise the debated question of PRP ability to improve tendon healing after rotator cuff repair. Experimental evidences indicates that PRP and growth factors aid tendon healing [8, 32]. This is the main concept behind the placement of PRP between bone and the torn end of a rotator cuff. However clinical studies failed to demonstrate significant improvement in the structural integrities of repaired tendons. Only the study by Randelli et al. [30] described accelerated healing in term of higher subjective scores (including daily living activities) at 3 months postoperative in the PRP group. Longer followup did not result in significant improvement of shoulder function or structural outcome. Reasons for this statistical insignificance were sought in nonoptimal concentration, activation status, or dose of PRP grow factors. Given the heterogeneity of PRP preparation products available on the market, it is possible that some preparations may be more effective than others. Future studies should be adequate in terms of standardization and characterization of the preparation of PRP to allow comparison of results. Tear severity has been advocated as another possible factor influencing studies results. However, preliminary results on this aspect are discordant. Randelli et al. [30] reported significant differences in some outcome measures at long-term followups in patients with stage 1 or 2 cuff tears.

A limitation of our review is the small number of available studies on the topic. Interest in PRP is increasing but researches are still ongoing. Only 3 studies have been recently published on PRP use for rotator cuff repair. Sample sizes are relatively small (53 [30], 88 [17], 42 [29] patients, resp.). PRP device was different between the included studies. However, it was always positioned at the bone to tendon interface.

18. Conclusions

In conclusion, the current literature on tissue engineering application for rotator cuff repair is scanty. Comparative studies included in this review suggest that PRP augmented repair of a rotator cuff does not yield improved functional and clinical outcome compared with nonaugmented repair at medium and long-term followup. At a short-term followup, patients managed with PRP augmented repair showed better control of postoperative pain. The structural integrity of the rotator cuff seemed to be slightly better in the PRP augmented group, even though the small number of patients in the included studies did not allow definitive conclusions. Relatively few studies, as well as small sample size, were the primary limitations of this systematic review. Randomized, prospective trials are needed for more definitive answers.

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Review Article

Growth Factors and Anticatabolic Substances for Prevention and Management of Intervertebral Disc Degeneration

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Intervertebral disc (IVD) degeneration is frequent, appearing from the second decade of life and progressing with age. Conservative management often fails, and patients with IVD degeneration may need surgical intervention. Several treatment strategies have been proposed, although only surgical discectomy and arthrodesis have been proved to be predictably effective. Biological strategies aim to prevent and manage IVD degeneration, improving the function and anabolic and reparative capabilities of the nucleus pulposus and annulus fibrosus cells and inhibiting matrix degradation. At present, clinical applications are still in their infancy. Further studies are required to clarify the role of growth factors and anticatabolic substances for prevention and management of intervertebral disc degeneration.

1. Introduction

The physiological homeostasis of the various tissues of the intervertebral disc (IVD) is regulated by the active maintenance of the balance between the anabolism and catabolism of IVD cells [1, 2]. This mechanism is maintained through a complex coordination of a variety of substances and molecules, including growth factors, enzymes, enzyme inhibitors and cytokines, that act in a paracrine or/and autocrine fashion [1, 2]. IVD degeneration usually begins from the second decade of life, and progresses with aging [3]. The lack of nutrients [4] and inappropriate mechanical loads [5] may result in loss, alteration, and dysfunction of cell viability and IVD properties [6]. Medical conditions associated with symptomatic IVD degeneration include IVD herniation, radiculopathy, myelopathy, spinal stenosis, instability- and low-back pain, and they represent the most common diagnoses facing spine specialists. The accepted treatment for IVD degeneration consists on discectomy with vertebral fusion. New biological strategies have not yet been proven to be effective [7, 8].

This review reports the state-of-the-art on the management of IVD degeneration using growth factors and anticatabolic molecules.

2. Biology of the Intervertebral Disc

The IVD is constituted by three parts: the annulus fibrosus (AF), the nucleus pulposus (NP), and the endplate (EP). The IVD matrix is composed of an ordered framework of macromolecules able to attract and hold water; the most represented structural components are collagens and proteoglycans [9]. Collagenous proteins are present in the AF while proteoglycans are present in the NP. The function of collagen is to provide form and tensile strength while proteoglycans are responsible for tissue viscoelasticity, stiffness, and resistance to compression through their peculiar

interaction with water. Only 20% of collagenous proteins are found in the central NP while a 50% of proteoglycans are located in AF and NP, respectively [9]. The integrity of the IVD is maintained by the balance between matrix synthesis/apposition and degradation. Integrity is maintained by a fine balance of the activity of cytokines, growth factors, enzymes, and enzyme inhibitors, in a paracrine or/and autocrine fashion. Morphological and molecular changes occur in the IVD with aging, determining the progressive degeneration and pathologic alteration of this particular tissue [10]. Morphological changes include dehydratation and tears of the AF, NP, and EPs [10]. Common molecular changes are decreased cell viability and diffusion of nutrient and proteoglycans synthesis, accumulation and increasing of degradative enzymes and degraded matrix macromolecules, and, finally, alteration in collagen distribution [10].

The anabolic function of various growth factors is explained with the accumulation and synthesis of matrix while cytokines demonstrated the opposite effect. They promote catabolism and inhibit synthesis of IVD matrix.

Several inflammatory mediators have been found in degenerated IVDs, but the real pathologic role of these mediators is unknown or not clearly defined. Nitric oxide (NO), interleukin-6 (IL-6), prostaglandin E2 (PGE2), TNF-alpha, fibronectin, and matrix metalloproteinases (MMPs) are some of the several mediators identified. IL-6, NO, and PGE2 have been proposed to be the inhibitory factors of proteoglycan synthesis. These factors are recruited into action by interleukin-1 (IL-1), which also plays a role in the direct degradation of the proteoglycan matrix. This process of direct breakdown by IL-1 is thought to be mediated by a family of enzymes known as MMPs. IL-1 likely plays a major role in the cascade of inflammatory mediators, but the nature of that role is not well defined [11], suggesting that the identification of all mediators that promote degradation of the IVD or accumulation of matrix should be investigated to explore new therapy strategies.

3. Biological Therapy Strategies

Therapeutic strategies under investigation for the biological treatment of IVD degeneration include the use of cellular components (mesenchymal stem cells, chondrocytes, disc allograft, culture expanded, disc cells, etc.), molecules influencing disc-cell metabolism and phenotype and matrix-derivatives. The rationale of the biological strategies for arresting and preventing IVD degeneration is linked with the possibility to improve the accumulation of ECM by promoting its synthesis and/or inhibiting its degradation.

This is also connected with IVD biological properties: cells of the AF and NP respond to a different number of cytokines. In fact, IVD degeneration is associated with reduced cellularity, and restoration may be aided by treatments that protect against cell death and apoptosis, or promote mitosis.

Several growth factors, including bone morphogenetic protein-2 (BMP-2), BMP-7 (also known as osteogenic protein-1 [OP-1; Stryker, Kalamazoo, Michigan]), growth and differentiation factor-5 (GDF-5), transforming growth factor-β (TGF-β), and insulin-like growth factor-1 (IGF-1) stimulate matrix production while interleukin-1 (IL-1) and tumor necrosis factor (TNF) inhibit the synthesis of matrix and enhance its catabolism.

3.1. Enzymatic and Nonenzymatic Mechanisms. Other target candidates of therapy include enzymatic and nonenzymatic mechanisms involved in reabsorbing and degrading the IVD matrix. Various types of proteinases, such as matrix metalloproteinases, cathepsins, and members of the ADAM (a disintegrin and metalloprotease) family, are implicated in IVD matrix degradation. Nonenzymic mechanisms involving molecules such as nitric oxide, peroxynitrite, and superoxide are also candidates, but the extent to which they are restrained by the anoxic environment of the IVD is unknown. Most of these experimental solutions have some *in vitro* data, but only few studies have been extended from *in vitro* observation to an animal model of IVD degeneration, especially to large animals models that can be compared to human IVD disease.

In addition, as several animals were used to study IVD degeneration, including chemical and nonchemical strategies to simulate IVD degeneration *in vitro* and *in vivo*, there is no agreement on which model best simulates human IVD degeneration.

3.2. Growth Factors. Growth factors (GFs) are fundamental components for the biological homeostasis of the IVD tissues, and are also fundamental for the development of the spine. Several GFs have been found in degenerated and normal IVD tissues, suggesting that IVD cells are capable of expressing and producing GF and to respond to GFs stimulation, changing their phenotype according to the GFs present at a given time. Indeed, stimulation of matrix synthesis by GFs alters the tissue homeostasis and changes cellular metabolism to the anabolic state [2].

The most studied GFs in IVD degeneration are TGF-b1-3 [12–14], IGF-1 [13, 15], basic fibroblast growth factor (bFGF) [16–18], growth differentiation factor-5 (GDF-5) [19], platelet derived growth factor (PDGF) [20], BMP-2 [21], and BMP-4 [19, 21].

TGF-b1 and -b2 and bFGF were found to be expressed in human degenerated IVDs [22]. TGF-b receptor II (TGF-bRII), FGF receptor 3, and BMPRII were also found to be present to a similar extent in both human diseased and normal tissues [23].

The rationale for the use of GFs for prevention and treatment of IVD degeneration lies in their capacity to regulate phenotype of IVD cells, promoting an anabolic state with enhanced matrix synthesis and accumulation.

3.3. In Vitro Studies on Growth Factors Application. Cell proliferation and matrix synthesis and metabolism can be upregulated when exogenous growth factors are applied to tissue or cell cultures.

In 1991, Thompson et al. [24] first described the effects of various growth factors, including TGF-b, epidermal growth

factor (EGF), and bFGF on proteoglycan (PG) synthesis by IVD cells. TGF-b1 was expressed frequently by NP cells and occasionally by AF cells when they were spatially associated with fibronectin-synthesizing cells [25]. Moreover, in human three-dimensional IVD cell cultures, TGFb stimulates cell proliferation of annulus cells after four days of exposure [26]. IGF-1 stimulated PG synthesis by bovine NP cells in serum-free conditions in a dose-dependent manner [15].

In addition, IGF-1 and PDGF significantly reduced the percentage of apoptotic AF cells induced by serum depletion in culture [27] while, in rabbit IVD cells, the responsiveness of IVD cells to IGF-1 and TGF-b decreases with increasing age [28]. Yoon et al. [29] showed that, in monolayer cultures of rat AF cells, recombinant human BMP-2 (10–1000 ng/mL) increased cell proliferation, mRNA expression of collagen type II, aggrecan, osteocalcin, and SOX9 and PG synthesis [29]. Another study reported that BMP-2 significantly increased the expression of aggrecan, collagen type II, TGFb1, and BMP-7 mRNA, and, on the other hand, downregulated versican gene expression [30]. BMP-2 increases and facilitates the expression of the chondrogenic phenotype by human IVD cells. rhBMP-2 increased PG synthesis and upregulated the expression of aggrecan, collagen type I, and collagen type II mRNA, compared to untreated control levels [31]. In rabbit IVD cells, BMP-7, also known as osteogenic protein-1 (OP-1) strongly stimulates the production and formation of PG and collagen and slightly affected cell proliferation [32].

In both NP and AF cells, recombinant human OP-1 (rhOP-1) stimulated the production and accumulation of PG and collagen in a dose-dependent manner (50–200 ng/mL) in the presence of 10% fetal bovine serum (FBS). Data from previous cadaveric studies indicated that the capacity of NP and AF cells to synthesize PGs decreases with age [33].

However, OP-1 significantly stimulated PG synthesis in all fetal, adult, and old bovine NP and AF cells [34], suggesting that the IVD cells in older animals are responsive to GFs, such as OP-1.

OP-1 (100–200 ng/mL) improved the *in vitro* production of PGs by human NP and AF cells cultured in alginate beads, especially in the presence of 10% FBS [32].

OP-1 also enhanced the accumulation of PGs in the matrix, and AF cells, which are more fibrochondrocytic, strongly responded to OP-1: OP-1 might be beneficial not only for nucleus repair but for anular repair too [32]. Takegami et al. treated the IVD cells cultured in alginate beads with IL-1 [35] or chondroitinase ABC (C-ABC) [36] to damage or to deplete the PG-rich matrices, simulating IVD degeneration. After depletion of the ECM following exposure of IVD cells to IL-1, OP-1 was found to be effective in the replenishment of a matrix rich in PG and collagen.

OP-1 treatment increased DNA content, and collagen and PG synthesis and accumulation in both early and advanced stages of IVD degeneration, but to a greater and successful extent in early stages of IVD degeneration [37]. The use of growth factors may be suitable in the early rather than in the late stages of IVD degeneration.

Growth and differentiation factor-5 (GDF-5), another member of the BMP family, was firstly described in the literature for its capacity to be a crucial factor responsible for skeletal alterations in brachypodism mice [38]. GDF-5-deficient mice showed IVD degradation and degeneration, and with a low T2-weighted signal intensity and loss of normal lamellar architecture of the AF at MRO, and disorganized NP with a decreased PG content [39]. GDF-5 stimulated collagen type II and aggrecan expression in mouse IVD cells [39].

Some studies also indicated that rhGDF-5 promoted matrix synthesis/accumulation and cell proliferation by both bovine NP and AF cells, with a better response by NP cells compared with AF cells [40].

Platelet-rich plasma (PRP) is a plasma fraction that contains multiple growth factors concentrated at high levels [30, 41, 42], and is produced by the centrifugation of the blood. PRP, administered in porcine cells cultured in alginate beads, is an effective stimulator of cell proliferation and PG and collagen synthesis, as well as PG production and accumulation, by both AF and NP cells [43].

The combination of autologous IL-1 receptor antagonist (IL-1ra)/IGF-1/PDGF proteins reduced the percentage of cellular apoptosis and the production of biochemical markers of IVD degeneration, such as IL-1 and IL-6. These results suggested the possibility of new strategies, using an autologous protein mixture, containing IL-1ra/IGF-1/PDGF, for the treatment of degenerative IVD disease [44].

3.4. In Vivo Studies on Growth Factors Application. The first study on GF injection into the IVD was reported by Walsh et al. [45] in the mouse caudal IVD degeneration model. In this study, IVD degeneration was induced by static compression [45]. A single injection of GDF-5, but not that of IGF-1, TGF-b, or bFGF, promoted IVD regeneration while multiple injections (four injections, one per week) of TGF-b showed a stimulatory effect. Finally, multiple injections of IGF-1, GDF-5, and bFGF did not show a significant enhancement of their primary effect [45]. A combined approach with a mechanical or cell-based device or a sustained delivery system was required to obtain a beneficial therapeutic effect.

An et al. [46] performed an *in vivo* experiment using the normal rabbit IVD and a single injection of OP-1 (2 lg per disc) to test the efficiency of OP-1 for the treatment of degenerative IVD disease. A single *in vivo* intradiscal injection of OP-1 resulted in increased PG content of the NP and disc height (15%) [46].

Additionally, the same authors conducted a study of radiographic and magnetic resonance imaging findings in the rabbit IVD after injection of OP-1 into the NP in the anular-puncture disc degeneration model. IVD degeneration was induced by anular puncture in two noncontiguous discs with an 18 G needle [47]. Four weeks after the anular puncture, either 5% lactose or OP-1 was injected into the center of the NP. Six weeks after the intradiscal administration of OP-1, an increase of signal intensity of NP in T2-MRI associated with restoration of disc height were observed and sustained along the entire experimental period, up to 24 weeks [48]. Histologically, the degeneration grades of the punctured discs in the OP-1-injected group were significantly lower than those of the lactose-injected

group. Biochemically, the PG content of the AF and NP was higher in the OP-1-injected discs than in the control group treated with lactose injection. This study demonstrated the feasibility of restoring degenerative rabbit discs by a single injection of OP-1 into the NP. In addition, the restoration of disc height, which usually began at 6 weeks, was sustained for up to 24 weeks.

Chondroitinase ABC (C-ABC) chemonucleolysis was used by several authors to simulate an animal model of disc degeneration in the goat disc [49, 50] and in the rat tail [34, 51], and was used also as an alternative degradation method to chymopapain treatment. The effect of OP-1 has been also validated in a chondroitinase-induced chemonucleolysis model in rabbits. These data suggest the feasibility of OP-1 administration in patients with IVD degeneration who had previously received chemonucleolysis that resulted in effective loss of disc height.

Moreover, regarding the stimulatory capacity on matrix accumulation and/or synthesis induced by OP-1, an injection of rhOP-1 performed following chemonucleolysis with C-ABC may be a successful strategy to oppose the degradative effects of the enzyme and to induce the healing of the IVD structure. In adolescent rabbits, C-ABC (10 mU) was first injected into IVDs to induce chemonucleolytic effects [52]. Four weeks after the injection of C-ABC, OP-1 (100 μg/disc) or vehicle was injected and the disc height was measured for up to 12 weeks after the OP-1 treatment. The disc height after the injection of C-ABC was significantly decreased (approximately 34%), and this finding was statistically significant. The injection of OP-1 induced a recovery of the disc height towards normal within 4 weeks after the rhOP-1 injection, gradually approaching the control level by 6 weeks. This change was sustained for up to 16 weeks. The results of an injection of rhGDF-5 in the rabbit anular-puncture IVD degeneration model also confirmed the efficacy of injection of growth ad differentiation factor. The IVDs were punctured by a needle as described in the OP-1 experiment to produce IVD degeneration. Four weeks after, the rabbits received a single injection of rhGDF-5 (10 ng, 1 μg, and 100 μg) or phosphate buffered saline (PBS) and were then followed up for 16 weeks for disc height with MRI findings, and histological grades with biopsy. The injection of rhGDF-5 resulted in a replacement of IVD height and improvements in MRI findings and histological grading scores with statistical significance ($P < 0.05$–0.001) [40].

In 2-year-old rabbits, the intradiscal injection of rhGDF-5 induced a significant recovery of disc height during a 12-week observation period [40]. The response to the GDF-5 injection was faster than that seen in the adolescent rabbits. Two weeks after the injection, the percent disc height index (%DHI) in the rhGDF-5-injected discs was significantly higher than that in the PBS-injected discs. By 8 weeks afterinjection, the rhGDF-5-injected discs reached the DHI level of the nonpunctured control discs. At 12 weeks after the injection, at MRI the signal intensity in the NP of the rhGDF-5-injected discs was significantly higher than that of the PBS-injected discs. Biomechanically, the viscous and elastic moduli of the IVDs in the rhGDF-5-injected discs were significantly higher than those in the PBS-injected discs. These results indicated the effectiveness of an injection of rhGDF-5 in restoring the degenerative disc in the 2-year-old rabbit anular-puncture disc degeneration model, despite the concern that age-related changes in cell activity might affect the efficacy of growth factor injection therapy [40].

In addition, normal discs from the adolescent rabbits (5-6 months) used in these studies still have a large number of notochordal cells [31, 53]. Notochordal cells in the NP were substituted with fibrochondrocytes after the needle puncture, and this difference in cell populations needs to be taken in account for preclinical information in future human clinical trials.

An age-related decrease in PG and water content and increased degenerative signs in sagittal MRI scans was found in a cross-sectional study using 1–4-year-old rabbits [37]. One can speculate that mature or older rabbits also show less diffusion through the EP because of decreased vascularity, compared to adolescent animals. Therefore, repair of the degenerated IVD may be age-dependent, and mature rabbits may not be able to restore disc structure by growth factor injection as demonstrated in the young animals described above.

Several possible mechanisms for the long-term effects of growth factor injection warrant further investigation. First, the half-life of an injected protein in discs has been considered to be short, in the order of minutes [54]. Finally, the biological and metabolic changes found in the cells following a single injection of a growth factor might be sustained and thus could induce long-term changes in disc structure. However, the results from the use of other GFs have shown some discrepancies in the studies found in the literature.

Huang et al. injected saline (100 μL) or BMP-2 alone or BMP-2 with coral grafts, after a full-thickness anular tear, in rabbit discs [55]. Radiographs after the treatments revealed that degenerative changes were more frequent and severe in the animals treated with rhBMP-2 with or without the use of coral. At histology, rhBMP-2 promoted fibroblast proliferation and hypervascularity of the intervertebral disc after an anular tear. These discrepancies may result from the fact that the annular tear model simulates an acute condition, and the application of BMP-2 was performed during an acute phase.

Nagae et al. recently reported findings on the effects of PRP in the rabbit IVD following nucleotomy [56]. PRP was injected into the NP of degenerated IVDs after impregnation into gelatin hydrogel microspheres to produce a slow condition to release the biological factors found in PRP while other IVDs received PRP or PBS without impregnation into gelatine hydrogel microspheres. Gelatin hydrogel immobilize PRP growth factors physicochemically, allowing to release them in a sustained manner with the subsequent degradation of the microspheres. The progression of IVD degeneration was suppressed in the group injected with PRP-microspheres, compared to the PBS and PRP only groups. This finding confirmed Walsh's indication [45] about the necessity to find a sustained delivery system for growth factor injection, which must be specific and dependent on the kind of growth factor injected. In addition, when the

defect in the NP is associated with its pathogenesis, such as postdiscectomy, the use of such a scaffold or delivery system may provide a benefit to patents. Some authors found that the adenovirus is an effective vehicle for gene delivery with rapid and prolonged expression of target protein and resulting improvement in markers of disc degeneration. Ad-GDF5 or DMP-7 gene therapy could restore the functions of injured discs and has the potential to be an effective treatment [57, 58].

3.5. Limitations of Growth Factor Injection Therapy. There are several limitations of GF injection therapy for IVD degeneration. The cell number is substantially decreased in degenerated discs, especially in the advanced stages [24]. Therefore, a reduced number of cells are present in IVD degenerated IVDs, and only few of them are able to respond to GFs stimulation. Some authors reported specifics methods for prevention of this cellular deficit, such as the association of GFs injection and transplantation of healthy functional cells (i.e., autologous NP cells) [59–62]. Other authors utilized GFs and cells treated with transfection of a therapeutic gene, to obtain the combination effect on the implementation of cells number and the gene therapeutic effect [63].

Nutrition is another important factor linked with the pathogenesis of disc degenerative disease [64]. The IVD is the biggest avascular tissue in the human body, and its survival depends on nutrients diffusion from the vertebral bodies through the EPs. In the degenerated IVD, the sclerosis of the EP did not guarantee a correct diffusion of nutrients, and this condition worsens with a substantial alteration of the IVD tissues and with the worsening of IVD tissues condition and onset of an irreversible state. The increased demand for energy resulting from the stimulation by GFs or by cell supplementation may affect cell viability under those conditions where nutrient transport is compromised. Further investigations of the optimal environment for GF stimulation should be pursued.

Age-dependent limitations emerged in the therapy of IVD degeneration with GFs, especially concerning the possibility of the few cells present in the degenerated IVD, to respond to GFs stimulation [9]. Because of the emerging trend to treat disc degeneration in adults or older individuals by local injection of growth factors, degeneration stage-related characteristics and age-related findings, changes, and differences in the response to growth factors, are of particular interest for future studies [65].

Often, a delayed response in the recovery of disc height by IVD cells *in vivo* after administration of GFs is observed. The mechanism of this phenomenon remains undetermined. However, it is possible that the half-life of the injected GF may be longer than expected; the capacity to respond to a single administration of a growth factor can continue over a long period of time. Finally, the stimulation by growth factor may determine a cascade of events, eventually resulting in the healing and recovery of disc height.

The *in vitro* and *in vivo* evidence described above supports the hypothesis that the direct injection of GFs into the NP or the AF may be clinically effective as a new therapeutic strategy for the prevention and treatment of IVD degeneration.

The acceleration of the biological repair process by the stimulation of the cellular anabolic capacity will determine a new category of therapy, where no active treatment currently exists, between conservative therapies and more aggressive therapies such as surgical intervention of vertebral arthrodesis or disc replacement.

4. Anticatabolic Agents

The inhibition of degradative molecules can be important to prevent IVD degeneration. Anticatabolics prevent matrix degradation by inhibiting particular enzymes within the disc. Several families of enzymes are capable of breaking down the various molecular components of IVD matrix, including cathepsins, aggrecanases, and MMPs [66, 67]. MMPs play an important role in the normal turnover of matrix molecules, but they have also been linked with degradation of collagen, aggrecan, versican, and link proteins found in the degenerated disc [67]. The main members of the MMP family are collagenase (MMPs 1, 8, and 13), gelatinase (MMPs 2 and 9), and stromelysin (MMP 3). Collagenase and gelatinase cooperate in the breakdown and degradation of collagen contained in the AF. Stromelysin acts prevalently in the NP, and it is involved in the breakdown of the core protein of proteoglycans. Stromelysin is able to access the proteolytic cleavage sites, saving hyaluronate-binding regions, degraded proteoglycan aggregates, and glycosaminoglycan fragments as breakdown products [66–69]. Moreover, TNF-alpha-stimulated gene-6 (TSG-6), found in herniated disks, is another potential therapeutic molecule with anti-inflammatory properties which blocks destructive proteinases [70].

In addition, following the current information of synthesis and degradation of IVD tissues, the rate of disc-matrix biological metabolism may also be important. For example, the overall rate of disc metabolism in discs from younger individuals is higher than in discs from older individuals. Moreover, in discs from older individuals, aging promotes the accumulation of degraded products in comparison to the newly synthesized molecules, inducing a catabolic permanent state. This disparity of findings is evident for aggrecans when looking at aged IVDs. Aggrecan and versican degradation is usually performed by another family of metalloproteinases, the ADAMs. Two members of this family (ADAM-TS4 and TS5) show a particular affinity for aggrecan, and are also called aggrecanases [10].

Given the degradative properties of MMPs, more attention should be given to inhibiting MMPs methods, to try to slow or arrest IVD degeneration. MMP activity is physiologically inhibited by tissue inhibitors of MMPs (TIMPs) [69, 71]. Wallach et al. [71] successfully delivered an anticatabolic gene TIMP-I, using an adenoviral vector, into the cells from degenerated IVD. They also demonstrated an increased expression of TIMP-1 in disc cells and also an

increase in the proteoglycans content. In other studies, TIMP inhibited the breakdown of proteoglycans, such as aggrecan, and may be a potential molecular therapy strategy for the management of IVD degeneration [72, 73].

Finally, cytokines such as TNF-alpha and IL-1 may have critical roles in disc metabolism and the pathogenesis of IVD degeneration. Several inflammatory cytokine antagonists, including IL-1 antagonists and TNF-a antagonists, have also been studied with *in vitro* and *in vivo* [74].

IL-1Ra and Infliximab, which can reduce IL-1 and TNF-alpha blood levels, respectively, may be also useful as an alternative pharmacological therapy for the IVD degeneration [75–77]. In addition, recent studies demonstrated the possibility to reduce IVD degeneration by the inhibition of TNF-alpha molecules using etanercept. Perispinal etanercept injection may be useful for both acute and chronic disc-related pain, especially in patients with chronic refractory disc-related pain [78]. Recently, Horii et al. [79] conducted an interesting experiment using etanercept in the dorsal root ganglia (DRG) of rats. The neurotracer FluoroGold was applied to the surfaces of L4/5 IVD to label their innervating DRG neurons ($n = 30$). Of 30 rats, 10 were in a nonpunctured disc sham surgery control group whereas the other 20 were in experimental groups in which intervertebral IVDs were punctured with a 23G needle. Etanercept or saline was applied into the punctured discs ($n = 10$ each treatment). After 14 days of surgery, DRGs from L1 to L6 were harvested, sectioned, and immunostained for CGRP. The proportion of FluoroGold-labeled CGRP-immunoreactive DRG neurons was evaluated in all groups. CGRP was upregulated in DRG neurons innervating damaged discs. However, direct intradiscal application of etanercept immediately after disc puncture suppressed CGRP expression in DRG neurons innervating injured discs. In a double-blind, placebo-controlled, dose-response pilot study, Cohen et al. [80] reported that no serious side effects were observed after a single low-dose intradiscal injection of etanercept. The same conclusions were reached in a blind randomized controlled trial using etanercept injection for the treatment of sciatica [81]. The study [81] did not show benefits after the use of etanercept over placebo in the pharmacologic treatment of sciatica.

Finally, CPA-926 can be used in the IVD degeneration for its anti-inflammatory and anti-tumorigenic properties. Following oral administration of CPA-926, histological and radiographic findings on an annulotomy model of IVD degeneration in rabbits showed a delay of the onset of disc-height loss [82]. Further research into anticatabolic molecules should be performed.

4.1. Limitations of Anticatabolic Therapy. The most important limitation of this approach is linked to the chronic nature of the IVD degeneration disorder. IVD degeneration is a long-term problem, occurring gradually and worsening with aging. The normal *in vivo* half-life found for these therapeutic molecules is on the order of only few minutes, and this suggests that the effects of these molecules will not last for significant periods of time. Therefore, if anticatabolic therapy can be effective in the long term, repeat or continuous infusions need to be performed, limiting the clinical utility of direct molecular-based therapies.

5. Summary

The normal intervertebral disc is a complex structure able to dissipate loads and permit multiaxial motion of the spine. The homeostasis of the IVD tissues is maintained trough a fine balance between matrix synthesis and degradation, which determines biological changes and microstructural modification and consequent organization. IVD degeneration leads to the alteration and loss of function of the IVD tissues, disrupting the well-defined organization of IVD structures and also of the IVD biomechanical balance. The major problem of IVD degeneration is the progressive loss of proteoglycan and water in the NP, followed by matrix degeneration and presence of catabolic molecules in the IVD tissues. The restoration of the proteoglycans content in the NP, such as the inhibition of the molecules involved in the matrix degeneration including, can be a possible solution for management of IVD degeneration. Several biological molecules can be potentially useful and effective in IVD repair. GFs and anticatabolic molecules are possible fields of study and intervention. Many of these molecules have been investigated *in vitro*, and several have been used in animal model of IVD degeneration. The next step of research should be performed with animal model of IVD degeneration to better simulate the human condition. In addition, the use of GFs and of anticatabolic molecules demonstrated important limitations for future preclinical studies.

Major drawbacks in the field include the poor delivery methods, the rapid effect and formulation of therapeutic molecules, and the lower percentage of cells able to respond to GFs and anticatabolic stimulation found in degenerated IVD tissues. GFs injection can be useful in IVD degeneration if administered in the early phase of the disease while the reduced number of cells found in IVD degenerated tissues can be a major problem. Only few cells are present in IVD degenerated tissues and are able to respond to GFs stimulation. Anticatabolic molecules show a very short-term effect and the *in vivo* half-life found of these therapeutic molecules is on the order of only few minutes suggesting that the effects of these molecules will not last for significant periods of time. Future research should focus on biological therapy research for the treatment of IVD degeneration.

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Review Article

Tendon Regeneration and Repair with Stem Cells

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The use of stems cells in tendon repair is of particular interest given the frequency of tendon injuries worldwide together with the technical difficulty often encountered when repairing or augmenting tendons. Stems cells have the capability to differentiate into a variety of different cell types including osteocytes and tenocytes, and if normal architecture of damaged tendon (either macroscopic or microscopic) could be restored, this would significantly improve the management of patients with these injuries. There is already encouraging research on the use of stems cells clinically although considerable further work is required to improve knowledge and clinical applications of stem cells in tissue engineering.

1. Anatomy and Pathophysiology of Tendon Damage

Tendons attach muscle to bone and function to transmit tensile loads from muscle to bone and to enable the muscle belly to be at an optimal distance from the joint. The microstructural composition is approximately 20% cellular (fibroblasts secreting collagen) and 80% extracellular matrix. The extracellular matrix consists of mainly water, collagen, and ground substance [1].

More than 90% of collagen in tendons is type 1 with the remainder being type 3. These molecules are aligned in parallel to form microfibrils, which are further aggregated to form bundles [1]. This allows them to handle high unidirectional tensile loads. Ground substance consists mainly of proteoglycans, glycoproteins, and plasma proteins. These bind the extracellular water in the tendon, helping to stabilise the collagenous skeleton and contributing to the overall tendon strength. Elastin, secreted by fibroblasts, forms highly cross-linked sheets, allowing the tendon to stretch and coil, contributing to tissue recovery after loading.

The tendinous zone of insertion (enthesis) is a progressive structural change from tendon to bone, resulting in increased stiffness and decreased stress concentration. It is often the site of tendinopathic change and injury. It is divided into four zones; parallel collagen fibres at the end of tendon, unmineralised fibrocartilage, and mineralised fibrocartilage, which merges into cortical bone [1].

The blood supply to tendons is inferior to that of most other connective tissues. The blood supply is through a sparse supply of arterioles or a vincula (mesotenon) and subsequent diffusion through the tendon substance. They have a low metabolic rate. Both these factors have implications for the intrinsic healing potential of the tissue [1].

There are a number of factors affecting the biomechanical properties of tendons and subsequent tendinosis (degenerative tendon) or tendinopathy (an inflammatory reaction secondary to rupture or vascular damage) [2]. Ageing results in a decrease in collagen diameter and number. Endocrine factors play a part, and pregnancy is associated with a decreased stiffness of pelvic tendons. Pharmacological agents such as corticosteroids and anabolic steroids are associated with tendon rupture. Systemic disease and genetics affect the intrinsic healing potential. Repetitive microtrauma and fatigue failure often leads to calcification and an inflammatory reaction. Macrotrauma results in the acute rupture of a tendon due to a force above the ultimate tensile strength, either at the tendinous insertion onto bone or in the tendon substance itself.

The healing response is variable and usually poor. There is an initial rapid haemorrhagic and inflammatory phase. This is followed by a proliferative phase, with fibroblast production of new matrix. Remodelling occurs several weeks

after injury consisting of maturation and orientation of collagen fibres. Although there are external influences affecting tendon repair which can be controlled, there are intrinsic metabolic limitations to healing. Often a surgical solution is necessary to repair or reconstruct tendon. Rotator cuff tears and subsequent repair illustrate the magnitude of the problem. Rotator cuff injuries accounted for 4.4 million outpatient appointments in the US in 2003 [3]. It is estimated that at least 13% of individuals between the ages of 50 and 59 and 51% of people over the age of 80 experience rotator cuff injuries [4], and over 50,000 patients in the US require direct repair each year [5]. Despite this, repair can fail up to 40% of the time, leading to impaired shoulder biomechanics and subsequent weakness and degenerative osteoarthritic changes [6]. Tissue engineering and the use of stem cells has sought to provide a solution to this common cause of musculoskeletal morbidity.

2. Stem-Cell Potential

Stem cells may be totipotent, pluripotent, or multipotent, depending on tissue type. Totipotent cells form all the cells and tissues that contribute to the formation of an organism. Only the embryo itself is totipotent. Pluripotent stem cells (PSCs) can form most cells of an organism from all three germ cell layers. Embryonic stem cells present in the fertilised oocyte, zygote, and morula [7]. Pluripotent cells have the ability to expand in vitro almost indefinitely and form tissues from ectoderm, mesoderm, or endoderm. There are concerns about tumour formation in vivo and major ethical concerns, however, which have thus far restricted their use.

Multipotent cells form a number of cells or tissues that are usually restricted to a particular germ layer. Multipotent cells are derived from specific tissue compartments in the adult. The two main types of multipotent stem cell are haemopoietic and mesenchymal type, and both are usually derived from adult bone marrow, but occasionally from fat, skin, periosteum, and muscle. Mesenchymal stem cells (MSCs) are multipotent, capable of differentiating into several connective tissue types including osteocytes, chondrocytes, adipocytes, tenocytes, and myoblasts [8]. Mesenchymal stem cells have the advantage of being easily obtainable in adult tissue and, with the appropriate microenvironment can differentiate into various target tissue types.

Research on tendon healing and the use of stem cells has thus far been limited to animal studies, with the majority using mesenchymal stem cells.

2.1. Mesenchymal Stem Cells. MSCs can arise from a number of sources as already highlighted. Kryger et al. [9] isolated tenocytes, sheath fibroblasts, bone-marrow-derived stem cells, and adipose stem cells from adult rabbits and used them in a flexor tendon model. Although adipose-derived stem cells proliferated faster in culture, at six weeks, there was no difference with regards to cell viability, senescence, or collagen expression.

Tempfer et al. [10] examined biopsies of intact human supraspinatus tendons and showed that stem cell tendon precursors (tenocytes) were present in the tissue. Pryce et al. [11] showed that TGF beta signalling may play an important role in the recruitment of tenocytes. Mazzocca et al. [12] aspirated bone marrow from the bone anchor tunnel in the humeral head during arthroscopic rotator cuff repair in 23 patients. Using a novel device, in the operating room, stem cells were isolated from this aspirate and their presence and osteogenic potential confirmed. This study showed that stem cell-rich bone marrow is exposed following arthroscopic drilling of the humeral head. These stem cells harbour potential to differentiate into osteoblasts and tenocytes to regenerate the bone-tendon interface. Novel solutions for the recruitment and activation of these cells in combination with growth factors, gene therapy and an appropriate scaffold may provide improved strength of the rotator cuff following surgical repair.

Awad et al. [13] showed that there was a significant improvement in tendon repair when MSCs were injected into patellar tendon defects in rabbits. Compared to a cell-free collagen control at four weeks, MSC-mediated repair tissue demonstrated significant increases in stress, modulus, and strain energy density of 26%, 18%, and 33%, respectively. Chong et al. [14] examined the histology and modulus of Achilles tendon defects in rabbits over a 12-week period. Compared to controls, it was shown that at three weeks, there was an improvement in collagen organisation and modulus in the MSC group. By 12 weeks, however, this difference was insignificant, suggesting that MSCs may improve tendon healing in the early stages only.

The tendon-bone interface is a common site of rupture, especially at repetitive low-loading forces, for example, in rotator cuff tendinopathy. Chang et al. [15] examined healing potential of infraspinatus tendon in rabbits at the tendinous insertion using a periosteal graft containing autologous MSCs. Histological examination from 4 to 12 weeks showed gradual progression in healing from fibrotic tissue to mineralised fibrocartilage. There was an associated significant increase in failure load with time compared to controls. Ju et al. [16] used Achilles tendon grafts in a rat anterior cruciate ligament model (ACL). He undertook an ACL reconstruction and then injected the tibiofemoral bone tunnel with MSCs. Tendon-bone analysis at 2 weeks showed the proportion of collagen fibres at the interface tissue was significantly higher in the MSC group compared to controls. At 4 weeks in both groups, the implanted tendon appeared to attach directly to bone. The benefit of injecting MSCs, therefore, may give early benefit in this model. A study by Nourissat et al. [17] evaluated healing at the tendon-bone interface at the Achilles tendon in a rat model. After the tendon-bone interface was destroyed, the tendon was either left to heal, or an injection was given of chondrocytes or MSCs. At 45 days, it was found that cell injection of either chondrocytes or MSCs significantly improved healing compared to controls left to heal without an injection. A new enthesis was produced in the injection groups but not in controls, and in only the MSC group was this organised as in normal enthesis tissue.

The treatment of tendonitis by stem cells was studied by Lacitignola et al. [18] in horses. Tendonitis was induced by an injection of collagenase into the superficial flexor tendons.

Three weeks later, bone marrow mesenchymal cells, bone marrow mononucleated cells, or controls of fibrin were injected into the tendons. In the stem cell-treated groups, there was significantly improved healing histologically with a higher collagen type 1 to type 3 ratio and improved fibre orientation compared to controls. Another equine flexor tendon model for tendonitis compared MSCs, MSCs with insulin-like growth factor-1 (IGF-1) gene-enhanced MSCs, and controls. Both IGF-1 MSCs and MSC groups showed significantly improved tendon histology at 8 weeks compared to controls [19].

2.2. Pluripotent Stem Cells. Watts et al. [20] reported on the use of an injection of foetal-derived embryonic-like stem cells for superficial flexor tendon injuries in horses. Compared to controls at eight weeks, there was no significant difference in tendon matrix gene expression, proteoglycan, collagen, or DNA content between the tendons. There was, however, improved tissue architecture, tendon size, lesion size, and linear fibre pattern in the lesions treated with stem cells. The tensile strengths of the healing tendons were not tested, however.

Turner et al. [21] reviewed the use of amniotic stem cells for the engineering of a diaphragmatic tendon graft in newborn lambs. Failure rate was higher in the control group (acellular prosthetic graft). Tensile strength testing and collagen levels were significantly higher in the grafts containing stem cells.

The use of stem cell-coated sutures could have obvious theoretical benefits in surgical repair of tendons. Yao et al. [22] evaluated the fats of pluripotential embryonic stem cells seeded to a suture carrier in acellularised, sectioned rabbit Achilles tendon. At day 5, fluorescence under microscopy showed live metabolically active pluripotential cells at the tendon repair site. The same author showed that cell adherence at seven days was greater in FibreWire sutures when first coated with poly-1-lysine or fibronectin [23].

Guest et al. [24] examined the difference between MSCs and embryo-derived PSCs injected into damaged superficial digital flexor tendons in horses. At 90 days following injection, there had no signs of immune reaction to the allogenic PSCs and no sign of tumour formation. Survival rate was greater, with PSCs maintaining a constant level over 90 days in contrast to MSCs which showed less than 5% survival over ten days and a subsequent decline thereafter. PSCs also showed an ability to migrate to other areas of damaged tendon in contrast to MSCs.

2.3. Tissue Engineering for Tendon Regeneration. There are now several studies illustrating the potential for the use of stem cells not only in tendon repair, but also other their use in other tissue engineering applications [25–29]. Several studies have shown that a mechanical stimulus improves tendon healing. It has been shown in patellar tendon defects in rabbits that two weeks of in vitro mechanical stimulation significantly increased collagen type 1 and collagen type 3 gene expression of stem cell-collagen sponge constructs. These constructs exhibited 2.5 times increased linear stiffness and 4 times the linear modulus of controls [30]. The degree of mechanical loading has been shown to affect cell differentiation. One study showed that low mechanical in vitro stretching of MSCs into tenocytes, where as larger stretching at 8% induced differentiation into adipocytes, chondrocytes, and osteocytes [31]. In clinical practise, lipid accumulation and calcification in a healing tendon may lead to pain and a detrimental functional outcome.

In vivo, the extracellular matrix of tendon provides fibroblasts with the architecture to support development and function. During tissue engineering, therefore, a scaffold is needed to mimic this matrix. The optimal cell: matrix ratio to support tendon function is debated. Juncosa-Melvin et al. [32] examined cell: collagen ratios in Achilles tendon defects in rabbits. It was shown that constructs with a lower cell density at 12 weeks achieved higher stiffness and modulus values. Nirmalanandhan et al. [33] showed that above a threshold value of cell density, percentage reductions in collagen concentration influence contraction kinetics more than equivalent percentage increases in cell seeding density. The alignment of stem cells on scaffolds may be important. Yin et al. [34] showed that foetal stem cells placed in randomly oriented scaffolds in vitro led to osteogenic differentiation. In contrast, aligned nanofibres induced the formation of spindle-shaped cells and tendon-like tissue. Obviously, controlling scaffold conditions is vital to the effective differentiation of these cells and the ultimate mechanical properties of the healing tissue.

Butler et al. [35] found that in rabbits with patellar tendon defects, there appeared to be 4 important factors which improved the biomechanical properties of the healing tendon. Replacing the suture with end posts in culture and lowering the MSC concentration in cell-scaffold constructs resulted in failure forces greater than peak in vivo forces that were measured for all activities and tangential stiffness similar to normal tendon. Augmenting the scaffold gel with a type 1 collagen sponge increased repair stiffness, and mechanically stimulating these constructs further improved biomechanics in the healed tendon.

2.3.1. Use of Growth Factors in Tissue Engineering. Recently, Gulotta et al. [36] has highlighted the importance of gene expression in stem cells for tendon healing. In a rat supraspinatus model, MSCs after injection were present and metabolically active, but no difference in the biomechanical strength of the repairs, the cross-sectional area, peak stress to failure, or stiffness compared to controls could be found. A further study compared an MSC group and a group who had received adenoviral MT1 matrix metalloproteinase-transduced MSCs (Ad-MT1-MMP). Although no difference was found at 2 weeks, at 4 weeks, the Ad-MT1-MMP group had significantly more fibrocartilage, higher load to failure, stress to failure, and stiffness values as compared to MSCs [37]. It has also been shown that MSCs expressing BMP-2 and the transcription factor Smad8ca lead to differentiation into a tenocyte lineage [38]. It has been proposed that expression of Smad8ca lead to the production of MMPs. Shahab-Osterloh et al. [39] showed that MSCs with adenoviral-induced Smad8ca and BMP-2 exhibit both tendinous and osteogenic properties in mice and can aid formation,

therefore, of bone-tendon interface. Numerous other studies highlight the beneficial effect of BMP in tendon-bone interface healing [40].

The quality of tendon that forms from bioengineering may be a concern still, with ectopic bone formation being a problem in the healing tissue. Harris et al. [41] showed that this is likely related to alkaline phosphatase activity and may be higher in 3D in vitro constructs compared to tissue engineering on a 2D monolayer.

3. Summary

Tendon healing is limited by numerous intrinsic and extrinsic factors [42–47]. These have implications for the athlete in a "macrotrauma" acute rupture setting or in repetitive microtrauma leading to tendonitis. Like all connective tissues, tendon is vulnerable to the effects of ageing, inevitably leading to cell senescence of tenocytes, resulting in an extracellular matrix devoid of collagen and weakened tissue. The principles of tissue engineering involve a complex interplay of factors [48–52]. Local delivery of growth factors, the use of plasmids, and scaffolds are several. These in combination with stem cells or genetically modified stem cells have been shown to contribute to tendon healing in numerous animal studies [53–56]. Concerns arise as to tumour formation and immune reactions to allogenic sources, and there are obvious ethical considerations. The use of stem cells is a promising treatment in the armamentarium of the physician or surgeon, but further research is needed to decide on the optimal strategy in humans.

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Review Article

Advances in Meniscal Tissue Engineering

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Meniscal tears are the most common knee injuries and have a poor ability of healing. In the last few decades, several techniques have been increasingly used to optimize meniscal healing. Current research efforts of tissue engineering try to combine cell-based therapy, growth factors, gene therapy, and reabsorbable scaffolds to promote healing of meniscal defects. Preliminary studies did not allow to draw definitive conclusions on the use of these techniques for routine management of meniscal lesions. We performed a review of the available literature on current techniques of tissue engineering for the management of meniscal tears.

1. Introduction

The menisci of the knee are two fibrocartilaginous C-shaped discs interposed between the femoral and tibial joint surfaces. They provide shock absorption, stabilization, lubrication, load distribution, and joint filler supplying femoral-tibial incongruity [1, 2]. Traumatic lesions of the menisci are common and induce changes in biomechanical behaviour of the joint affecting the load distribution and contact stresses [3]. The healing process of torn menisci depends on their morphologic features. Each meniscus consists of outer vascular part and inner avascular part. The vascular supply is an important factor to determine the potential healing of meniscal tears [4]. Therefore, lesions of the outer one-third of the meniscus are believed to have the greatest capacity for repair. Meniscal tears are usually located in the inner avascular part of the meniscus and are not able to heal spontaneously. Several strategies to repair and replace meniscus have been proposed, but only few of them have been shown to be effective [5–9].

Depending on the type of lesion, surgical approaches include total or subtotal meniscectomy, transplantation, and repair [10]. As the fibrocartilaginous tissue of the meniscus presents a limited regenerative capacity, new approaches are required to improve meniscal healing. In the last few decades, several emerging strategies, including growth factors, gene therapy, and application of mesenchymal stem cells (MSCs), have been proposed to increase healing of a damaged meniscus by tissue-engineered constructs. Tissue engineering is based on a combination of cells, growth factors, and scaffolds able to stimulate the meniscal healing [11, 12].

We performed a review of the available literature on current techniques of tissue engineering for the management of meniscal tears.

2. Cells Transplantation

Human menisci are populated by different cell types that might respond differently to various stimuli released from

the matrix [13, 14]. Cell-based therapy has significantly contributed to develop tissue-engineering strategies consisting of cells-scaffold constructs able to promote healing in an avascular environment [15]. Autologous fibrochondrocytes are one of the cell types used in meniscal repair. Fibrochondrocytes are able to proliferate and produce new extracellular matrix (ECM) [16]. The amount of glycosaminoglycans (GAGs) produced by fibrochondrocytes from the inner avascular part is more than the amount produced from a peripheral fibrous location when seeded into a porous collagen scaffold [17, 18]. Although these findings are encouraging, the application of autologous fibrochondrocytes in meniscal tissue engineering is limited by the difficulty to harvest a sufficient number of cells.

An alternative cell type used to promote the healing of meniscal lesions is the articular chondrocyte [19, 20]. Peretti et al. [19] described a porcine chondrocyte model where implantation of such cells was performed in the avascular part of the meniscus, using an allogenic scaffold seeded with autologous chondrocytes, showing that these chondrocytes were able to heal a meniscal tear [19]. Another potential cell therapy approach is represented by MSCs. These pluripotent cells are able to differentiate into specific therapeutic cell types (developmental plasticity) [21–23].

The effects of extrinsic stimuli (biochemical, physical, and mechanical) from the microenvironment, within a cell/scaffold combination, are a promising alternative for repairing large meniscal defects [24]. Several studies confirm production of abundant extracellular matrix around the cells, restoring a meniscal-like tissue in the avascular zone [25–28]. In particular, the combination of growth factors and mesenchymal stem cells within scaffold implants increased proteoglycan and/or collagen synthesis [26, 28, 29].

The effect of load on all these different cell types becomes an interesting field for future research. Moreover, their stimulation with the application of growth factors in combination with a mechanically loadable scaffold has been proposed as the focus of future studies.

3. Growth Factors

Growth factors typically act on target cells as signalling molecules, promoting cell differentiation and chondrocytic proliferation [30]. They also stimulate the synthesis and inhibit degradation of (extracellular matrix) ECM by a mechanism of downregulation of proteases [31]. Several growth factors have been demonstrated to have an effect on the healing of tears and on ECM synthesis in tissue and cell culture. In particular, transforming growth factor-β1 (TGF-β1) seems to have several regulatory activities to stimulate the production of extracellular matrix and collagen type II by meniscus cells [30, 32]. Application of this growth factor has resulted in the synthesis of specific proteoglycans to enhance both collagen and GAGs production and their biomechanical properties [33, 34]. Pangborn and Athanasiou [35] used TGF-β1 to have consistent effects on collagen and proteoglycan production by meniscal cells. TGF-β1 was applied to monolayer cultures for 3 weeks and generally showed a higher production of each ECM component.

Fibroblast growth factor-2 (FGF-2) is another important factor found in the cartilaginous matrix. It enhances proliferation of the joint chondrocytes, mesenchymal stem cells, osteoblast, and adipocytes. In addition, FGF can also maintain the ability of any cell types to differentiate [36, 37]. Recently, FGF-2 has been vectored with recombinant adenoassociated virus (rAAV) [38]. Histology demonstrated enhanced cell proliferation and expression of the α-smooth muscle actin (α-SMA) contractile marker, but it did not significantly enhance the synthesis of major extracellular matrix components or DNA contents.

Other authors have identified basic fibroblast growth factor (bFGF) as effective at stimulating extracellular matrix production in cell and tissue development. The ovine experimental model showed the presence of meniscal fibrochondrocytes responding to bFGF by proliferating and producing new extracellular matrix [16].

The insulin growth factors (IGFs), particularly IGF-1, are considered the main anabolic growth factor of articular cartilage [39, 40]. IGF-1 stimulates the synthesis of proteoglycans, collagen II, and integrins. In a recent study, the effects of three growth factors regimens was examined: basic fibroblast growth factor (bFGF) alone, bFGF plus transforming growth factor (TGF-β1), and IGF-1 [41]. The mixture of growth factors showed an upregulation of collagen II and aggrecans under the effects of TGF-β1 and IGF-1 that may be an important cellular response to mediate avascular meniscal healing.

The induction of angiogenesis is an important factor to stimulate the poor potential healing of meniscal tears. The vascular endothelial growth factor (VEGF) may promote better healing, stimulating angiogenesis to improve the healing capacities of meniscus tissue. However, a study by Petersen et al. did not lead to satisfactory results, and the local application of VEGF did not promote meniscus healing [42].

Bone morphogenetic proteins (BMPs) are members of the TGF-β superfamily and play an important role during embryogenesis and tissue repair by their osteoinductive properties [43, 44]. BMP-2 acts as a stimulus in the differentiation of mesenchymal cell. It also presents a migratory effect in endothelial cells or smooth muscle cells, but rarely in chondrocytes. Alternatively, BMP-7 can have a function in regulating matrix homeostasis and can inhibit the degradation processes. BMP-7 acts with different chondrogenic agents and is more effective than BMP-2 for chondrogenic differentiation of MSCs [45]. Minehara et al. [46] developed a new technique for seeding chondrocytes onto solvent-preserved human meniscus using the chemokinetic effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on chondrocytes seeded into solvent-preserved human meniscus. After a 3-week incubation, a natural chemokinetic effect of rhBMP-2 promoted migration and proliferation of chondrocytes. These findings demonstrate that BMPs induce a marked cellular response to improve meniscal repair.

4. Preparations Rich in Growth Factors

The application of growth factors has been proven to be effective for meniscal healing. Recently, platelet-rich plasma (PRP) may be better than the use of isolated growth factors. PRP is an autologous substance rich in platelets. It is easily prepared by spinning autologous blood in a centrifuge to form a dense fibrin matrix that can be placed directly at the meniscal repair site [47, 48]. Ishida et al. reported the regenerative effects of platelet-rich plasma in a rabbit model [49]. Cultured meniscal cells were prepared to assess proliferative pattern under the exposure to PRP. Histological findings showed the healing properties of PRP in extracellular matrix synthesis and cell proliferation.

5. Biomaterial Used in Tissue Engineering

Tissue engineering techniques using novel scaffold materials offer potential alternatives for managing meniscal tears. An ideal scaffold should have the basic structure of the meniscus, and it should be biodegradable and bio-reabsorbable in the long term. Probably, the most important functions are the induction of cell proliferation and production of extracellular matrix, using it as a carrier for stimulatory and inhibitory growth. The structure should be strong enough to withstand the load in the joint and maintain its structural integrity without damaging the articular cartilage [15, 31, 50–52].

Several materials used to fabricate scaffolds (natural or synthetic) may be considered for application in tissue engineering of the meniscal healing. The first to be developed are natural scaffolds as periosteal tissue, perichondral tissue, collagen, small intestine submucosa, silk, and meniscus tissue itself [53].

A multilayered (tribiological), multiporous silk scaffold system to mimic native meniscus architecture and shape was described [54]. Silk constructs showed a good biocompatibility with a florid chondrogenesis as well as other tissues [54–57]. The cells (human articular chondrocytes and dermal fibroblast cells) were seeded onto the silk scaffold in association with human chondrocytes for 28 days. Histological analysis showed an increase production of GAGs and proteoglycans and a colonization of ECM similar to native tissue from fibroblasts and chondrocytes.

Minehara et al. developed a cell-seeding technique using a solvent-preserved human meniscus as a scaffold [46]. The chondral cells were treated with recombinant human bone morphogenetic protein-2 (rhBMP-2) and cultured for 3 weeks. The histological and immunohistochemical analyses indicated that this repair tissue was mainly fibrous. Moreover, results suggest a potential application of rhBMP-2 as a natural chemokinetic factor into a scaffold for tissue engineering.

Collagen scaffolds have been also examined for tissue engineering of the meniscus. Meniscus cells seeded in these scaffolds may express alpha-smooth muscle actin (α-SMA) that has contractile capacities. This demonstrates the potential healing in wound contraction, but other physiological and pathological processes are still unknown [58, 59]. Mueller et al. studied collagen type I and II scaffolds seeded with canine fibrochondrocytes for 21 days [60]. Type II scaffold contained up to 50% more GAGs than type I scaffold. A limit of the collagen scaffolds may be their poor mechanical properties, as the shape of the construct cannot be varied.

The use of synthetic polymer-based scaffolds is a novel option offering the potential of earlier healing. Stewart et al. [61] used polyglycolic acid (PGA) scaffolds seeded with ovine meniscal chondrocytes. The cells were seeded onto the PGA scaffold in the presence of platelet-derived growth factor- (PDGF-) AB, PDGF-BB, insulin-like growth factor- (IGF-) I, transforming growth factor-beta1 (TGF-β1), and basic fibroblast growth factor (bFGF) and evaluated after 39 days. Histological analysis of sections from ovine meniscal chondrocytes PGA scaffolds did not show any difference in GAG or collagen production between the treatment groups. However, immunohistochemical analysis demonstrated a different expression of collagen production: the production of collagen type I was increased, whereas the collagen type II was decrease at day 39 in all constructs functionalized with growth factors. A concomitant high infiltration of cells was also found.

Another tissue-engineered strategy consists in a poly-L-lactic acid (PLLA) scaffold used in association with culture of meniscus cells and bFGF under hypoxic conditions [62]. After 4 weeks, histological evaluation demonstrated the presence of collagen and GAG, probably due to synergic effects of hypoxia and bFGF. An earlier study by Ionescu tested the effects of TGF-β1 as a function of age, on proliferation of bovine meniscus fibrochondrocytes (MFCs) in a polycaprolactone (PCL) cylindrical scaffold [63]. Even though the results indicated a loss of proliferation and migration capacity with aging, the addition of TGF-b showed better maintenance of overall explant properties.

6. Gene Therapy

Gene therapy is considered an alternative strategy to develop future protocols for tissue engineering of meniscus tissue, using viral or nonviral vectors or direct gene transfer [64, 65]. In this way, the transfer of genes used to encode healing factors is a valid technique to apply growth factors to the site of injury for extended period. The vectors most frequently used in meniscal lesion are adenovirus, adenoassociated virus (AAV), and retrovirus. Nonviral vectors are not indicated because of being less efficient, although they are less pathogenic. Viral vectors allow the insertion of genes into death cells and the production of growth factors.

Previously, we mentioned a study where FGF-2 in association with recombinant adenoassociated virus (rAAV) vectors were used [38].

Goto et al. [66] developed a gene therapy strategy based on monolayer cultures of human and canine meniscal cells infected with retroviruses carrying human TGF-β1 cDNA or marker genes. There was an increased synthesis of collagen and proteoglycan in response to the addition of TGF-β1.

Another possible technique for gene transfection is the injection of adenovirus vector encoding the hepatocyte growth factor gene (AdHGF) in cell-seeded bovine PGA scaffolds [67]. This strategy showed the formation of vascularised fibrous tissue by 2 weeks and vascularized meniscus-like tissue in 8 weeks. The authors concluded that gene transfer techniques could be used to induce blood vessel formation in tissue engineering meniscus samples.

7. Discussions

Application of tissue engineering is a promising alternative approach for the management of meniscus injuries. Advances in meniscal tissue engineering focus on the use of different cell sources, scaffolds, growth factors, or a combination thereof. The potential effect of cell-based therapy for meniscal tears could improve healing of lesion in the avascular zone and expand the indication for repair rather than removal. A variety of cell types such as autologous fibrochondrocytes, articular chondrocytes, and MSCs are available in large quantities into the body and can be used in tissue engineering [17–19, 28]. Of these, progenitor cells such as mesenchymal stem cells have the advantage to be easily expandable without the loss of their differentiation potential into a variety of mesenchymal tissues [68–80] including bone, tendon, cartilage, muscle, ligament, fat, and marrow strom [13, 21, 22]. Probably, the application of MSCs might be a better cell source than fibrochondrocytes for meniscus repair.

The long-term biochemical and biomechanical features of tissue engineering techniques are determined by a combination of a well-integrated cell population with a scaffold. The development of carrier scaffolds should provide mechanical stability of the meniscus, maintaining its structural integrity without damaging the articular cartilage [15, 51]. Several scaffold implants have been investigated in the management of meniscal tears.

The use of growth factors has been demonstrated to have an effect on the healing of tears and on ECM synthesis in tissue and cell culture [30, 61]. Direct introduction of growth factors, such as TGF-β, BMPs, IGF-1, FGF, and VEGF, has positively influenced the clinical outcome of the meniscal repair procedures [33, 59, 81]. Previous studies have demonstrated that the effects of TGF-β1 and BMPs have a better potential to help healing in tissue engineering [34, 45, 46]. The focus in this future research should be on the assessment of a mechanically loadable scaffold that retains growth factors and at the same time is degraded to allow revascularisation.

Alternatively, gene transfer techniques represent a favorable strategy for growth factor delivery, inducing vascularisation of tissue-engineered constructs [66, 67, 81]. Several viral vectors expressing therapeutic proteins such as growth factors have been investigated to assess their potential to improve remodelling and healing of meniscus. Although gene therapy is a relatively new field in tissue-engineered menisci, it is one of the treatment options in the future.

8. Conclusion

The importance of the meniscus in safeguarding joint function has gained a considerable interest in the recent years. The current therapeutic strategy to treat meniscal defects is partial or total meniscectomy, but this may predispose patients, especially younger individual, to early osteoarthritis changes. The management of meniscal pathology to promote a healing response is considered essential in dealing with these injuries. When possible, meniscal repair should be performed to try to maintain meniscal integrity and prevent long-term degenerative changes.

New therapeutic strategies of meniscal replacement and tissue engineering need to be developed, but they are still at their infancies [11, 12]. The first step, the need to develop autologous grafting procedure, consists in finding the best cell source for meniscus repair, which to date seems to be the MSCs. The second step consists in fabricating an opportune biological scaffold which is able to carry cells into the meniscal lesion and to allow their differentiation, proliferation, and ECM synthesis to produce a meniscal native-like tissue. The biological activity of scaffold should be implemented through its functionalization with growth factors, such as TGF-β1 and BMPs.

Further research is necessary to successfully address the difficult problem of meniscal regeneration. Advancements in this field should be strongly encouraged, because of autologous grafting through either tissue engineering for repair or that complete replacement following excision represents a suitable alternative to partial or total meniscectomy or cadaveric implants.

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Review Article

An Osteoconductive, Osteoinductive, and Osteogenic Tissue-Engineered Product for Trauma and Orthopaedic Surgery: How Far Are We?

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The management of large bone defects due to trauma, degenerative disease, congenital deformities, and tumor resection remains a complex issue for the orthopaedic reconstructive surgeons. The requirement is for an ideal bone replacement which is osteoconductive, osteoinductive, and osteogenic. Autologous bone grafts are still considered the gold standard for reconstruction of bone defects, but donor site morbidity and size limitations are major concern. The use of bioartificial bone tissues may help to overcome these problems. The reconstruction of large volume defects remains a challenge despite the success of reconstruction of small-to-moderate-sized bone defects using engineered bone tissues. The aim of this paper is to understand the principles of tissue engineering of bone and its clinical applications in reconstructive surgery.

1. Introduction

Bone is a highly vascularised tissue that constantly undergoes remodelling as a result of the balance between the activities of the osteoclasts and the osteoblasts, which allows adaptation to mechanical stresses, maintenance of bone health, and repair of small injuries. A recent study demonstrated that the coupling between osteoclastic bone resorption and osteoblastic bone formation is needed for bone homeostasis [1]. Because of the potential of bone to spontaneously regenerate, most small bone lesions, such as fractures, heal well with conventional therapy or surgery. During bone repair, the osteogenic process, under the influence of bone-derived bioactive factors, commences after the inflammatory phase and is initiated by precursor cells from the periosteum adjacent to the fracture site. This generates hard callus by intramembranous bone formation. An autologous bone graft or bone substitute is often required to assist in the healing of an extensive traumatic or postsurgical bone defect and of osseous congenital deformities. The majority of bone formation, however, is by enchondral ossification of the soft callus that appears after infiltrated mesenchymal cells are induced to chondrogenesis. This improved understanding of repair, and regeneration has helped with the development of orthopaedic tissue engineering [2].

Historically, a variety of substitutes like celluloid, aluminium, gold, vitallium, tantalum, stainless steel, titanium, methyl methacrylate resins, polyethylene, silicone elastomers, and hydroxyapatite ceramics have been tried [3]. The main concerns with the use of these synthetic materials for bone reconstruction were their inability to vascularise, integrate, and undergo remodelling. This may result in structural failure of the implant under load or pathological changes in the surrounding bone, as seen in stress shielding [4]. The other issues are inflammatory scarring, neoproliferative reaction in the adjacent tissues and infection [5]. Because of their high osteoinductive potential and remodelling characteristics, bioactive substitutes such as demineralized bone matrix (allogeneic or xenogeneic) have shown promise, despite risk of disease transmission, as well as cost and availability [6]. This led to the evolution of tissue engineering techniques (biologically enhanced allografts, cell-based therapies, and gene-based therapies) to treat bone defects.

Tissue engineering has been defined as the application of scientific principles to the design, construction, modification, and growth of living tissue using biomaterials, cells, and factors alone and in combination [7]. It involves the use of osteoconductive biomaterial scaffolds, with osteogenic cell populations and osteoinductive bioactive factors. The three components for tissue regeneration are (1) a degradable support or scaffold material; (2) bioactive factors, such as growth factors; (3) cells. The potential for bone tissue engineering therapies in clinical applications is exemplified by the clinical success of recombinant human bone morphogenetic protein-2 for the treatment of fractures [8].

The most promising primary tissue engineering strategies are (1) isolation of mesenchymal stems cells (MSCs), their ex vivo expansion, and seeding onto a scaffold to produce extracellular matrix (ECM) on the scaffold; (2) implantation of an acellular scaffold into the osseous defect [7]. Translation of this technology into practice requires an additional surgical procedure and the time lag for the bone graft to develop in vitro. A variety of novel ex vivo culture techniques have been designed to speed up the cellular production of ECM. Three principal ex vivo culture techniques utilized in bone tissue engineering are growth factor delivery, bioreactor systems, and gene therapy.

2. Stem Cells

A stem cell is a cell from the embryo, fetus, or adult that, under certain conditions, can reproduce for long periods. It can also give rise to specialized cells of body tissues and organs. The use of stem cells from the embryo or fetus has many ethical considerations, whereas the use of adult stem cells is generally well accepted by society. An adult stem cell is an undifferentiated or unspecialized cell present in differentiated tissue, which renews itself and becomes specialized to yield all of the cell types of the tissue from which it originated. Their progeny includes both new stem cells and committed progenitors with a more restricted differentiation potential. These progenitor cells in turn give rise to more differentiated cell types. The advantages of using stem cells rather than differentiated cells are a higher proliferative capacity, a higher regenerative potential over time, and the ability to allow revascularization of the avascular scaffold. Cells with osteoprogenitor features have been isolated from several tissues including periosteum, bone marrow, adipose tissue, and retina. The choice of source depends on accessibility, frequency of cells, and information of a particular cell system.

The sources of osteogenic human cells are primary cells, MSCs, embryonic stem cells, and induced pluripotent stem cells. We use cells after considering various factors like proliferation potential, osteogenicity, vasculogenicity, the homogeneity, and the phenotype stability, as well as cell safety after implantation. Differentiation of these cells can be obtained in vitro by changing the culture conditions after their expansion or by providing a new physiological microenvironment in the transplant area in vivo. The process involves the isolation of cells, with expansion in vitro culture and enrichment of appropriate cell type for enhanced bone formation, integration of the cells with host tissues, and expression of stable osteogenic phenotype.

Primary osteogenic cells can be derived from adult bone tissue and periosteum [9–14]. Due to donor site morbidity [15–17] and limited proliferation of primary cells, preparation of large autologous grafts from primary bone or periosteum-derived cells would be difficult [18–20]. Research suggests that stem cells derived from bone marrow (BMSC) can be expanded for a significant number of cell doublings without cell senescence. The bone marrow is a reservoir of multipotent stem cells for mesenchymal tissues that can differentiate into fibroblastic, osteogenic, adipogenic, and reticular cells [21]. Expansion of stem cells using bone marrow aspirates depends on the donor age, volume, and technique. Although, it has been demonstrated that BMSCs can be culture expanded to large numbers [21], the osteogenic potential of BMSCs is maintained in older individuals [22], and appropriate conditions in vitro (e.g., culture on collagen substrate and growth factor supplementation of culture media) [23, 24] can help maintain cell differentiation potential [25, 26].

Adipose tissue stem cells (ASCs), due to their accessibility and potential for differentiation into osteogenic cells, represent another attractive source for bone tissue engineering. The number of cells produced by expansion is influenced by the tissue harvesting procedure, as well as the site of tissue harvesting, for example, arm, thigh, abdomen, and breast [27, 28]. ASCs undergo similar mesenchymal lineage specific differentiation as BMSC. They also display similar surface antigen. The principle advantages of ASCs use are that they exist in abundant numbers, can be obtained with minimal donor morbidity, their proliferative capacity is unaffected by age, and they have the ability to regenerate bone in critical sized bone defects [29, 30].

Takahashi and Yamanaka [31] described the use of induced pluripotent stem (IPS) cells. The main concern in their conceptualisation was the risk of viral integration into the recipient genome. However, this was allayed by Okita et al. [32] producing virus-free IPS cells from embryonic fibroblasts. Another concern is the time frame taken for extended ex vivo culture to produce sufficient number of IPS cells from fibroblasts. Due to the availability in larger numbers, ASCs-derived IPS cells have the potential to address this issue.

Human embryonic stem cells (ESCs), isolated by Thomson et al. [33], have unlimited potential for proliferation in vitro and they can form any tissue in the body [34]. ESCs are commonly derived from the inner cell mass (ICM) of preimplantation stage blastocysts [34]. They can be either feeder dependent or independent. Due to the exposure of ESCs to animal components, they pose a serious risk of transmitting serious pathogens, and thus extensive screening is warranted prior to therapeutic applications.

Bone formation is further controlled by engineering adult stem cells to express genes like bone morphogenetic proteins (BMP2, BMP4, and BMP7), core binding factor $\alpha 1$ (Cbfa1), vascular endothelial growth factor (VEGF), and noggin [35]. In addition, human bone marrow osteoprogenitor cells can be isolated and enriched using monoclonal antibodies as selective markers, such as STRO-1, from a CD34+

fraction, SB-10 (reacting with ALCAM), SH-2 (reacting with CD105), and HOP-26 (reacting with CD63) [12, 13]. Fibroblast growth factor-2 (FGF-2) supplementation to the culture medium promotes cell proliferation and maintains their multilineage potential during expansion [14]. These cells can be combined with a suitable scaffold and used as an alternative to conventional bone autograft. The transplanted osteogenic stem cells can immediately begin to proliferate and lay down new bone matrix without removing the old matrix present in the autograft. The development of these cell-based technologies may result in decreased use of dead bone from conventional bone banks to induce new bone formation.

3. Scaffold

A key component in tissue engineering for bone regeneration is the scaffold that serves as a template for cell interactions and the formation of bone extra cellular matrix to provide structural support to the newly formed tissue [6, 7]. Mesenchymal stem cells alone are unlikely to be sufficient for bone regeneration. Although marrow injections are simple and provide a reduced risk of morbidity, for large skeletal defects, a scaffold of appropriate shape, size, and mechanical competence is required for bone reconstruction [2]. The use of the scaffold or matrix is not only in controlling growth factor and cell delivery but also to provide a structural template to fill the tissue lesion [22]. Ideally, the scaffold should facilitate cell infiltration, matrix deposition, and cell attachment and consist of osteoconductive materials such as bone protein and hydroxyapatite. They should be able to allow load bearing and stimulate osteogenesis. The scaffolds could be naturally occurring, synthetic polymers, or bioceramics. Biodegradable scaffolds provide the initial structure and stability for tissue formation but degrade as tissue forms, providing background for matrix deposition and tissue growth [15–18]. They can be used alone or in combination with growth factors or osteoconductive materials [7].

The scaffold aims to mimic the extracellular matrix in a regenerating bone environment. It has to be informative to the cells as well as provide mechanical support [7]. A biomaterial should easily integrate with the adjacent bone and favour new tissue ingrowth (osteoconduction). It should allow colonization by the host blood vessels, be biocompatible and resorbable.

Various synthetic biomaterials like inorganic ceramics (e.g., hydroxyapatite, coralline-derived hydroxyapatite, tricalcium phosphate, calcium sulphates, glass ceramics, calcium phosphate-based cements, and bioglass), metals, and synthetic biodegradable polymer composites have been investigated for their potential as bone scaffold materials. Calcium-phosphate ceramics were introduced more than 40 years ago as bone substitutes. The most common types of calcium-phosphate materials investigated for synthetic bone scaffold development are hydroxyapatite (HA), tricalcium phosphate (TCP), biphasic calcium phosphates (BCP), and bioglasses. From a functional perspective, you can divide these into rapidly resorbing, slowly resorbing, and injectable ceramics. TCP is a classic example of rapidly resorbing ceramic; it has got greater solubility than HA. Due to their porosity, TCP granules are a better option than the bulk form [35], while HA resorbs slowly, which is clinically a disadvantage. Composite modification of HA matrices has been tried to increase the resorption, for example, composite of HA and calcium carbonate and BCP. Injectable calcium phosphate cements were also in vogue. They are mainly composed of α-TCP, dibasic dicalcium phosphate and tetra calcium phosphate. Clinically, they have been used in treatment of distal radius fractures [35–37]. The main disadvantage of their clinical relevance as synthetic bone scaffolds is due to their inherent brittleness [38].

However, due to their physiochemical properties, biocompatibility, and controllable biodegradability, polymers have emerged as the principal material in bone tissue engineering. The most frequently investigated polymers are polylactic acid (PLA) and polyglycolic acid (PGA) [39–41]. Numerous polymers were used as scaffold materials in the past decade for bone regeneration like poly(a-hydroxy esters), poly(ethylene glycol), polydioxanone, poly(orthoesters), polyanhydrides, polyurethanes, and poly(propylene fumarate) [42]. To gain more control over the degradation rate, hydrophobicity, crystallinity, and biological functionality, researchers designed composite polymers in a chemical process called copolymerization where multiple constituents are combined resulting in a new material with desirable properties from each constituent [43]. Undoubtedly, the most commonly utilized copolymer for bioactive molecule encapsulation and release for bone tissue engineering is the copolymer poly(lactic acid-co-glycolic acid) (PLGA) [43]. The inherent deficiency of the compressive modulus in polymers may be reduced through integration of high modulus micro- and/or nanoscale constituents within the polymer matrix [43]. The most commonly researched constituent in polymer composites for bone scaffolds is micro- or nanoscale HA particles [44, 45]. Tensile strength, modulus, and crack resistance of polymers are improved by dispersing high modulus micro- or nanoscale constituents [15–17, 46]. Whereas drawbacks for utilizing natural polymers like collagen, glycosaminoglycan, fibrin, and silk include infection, fixed degradation rates, and immunogenicity. Gel-like matrices such as fibrin have been used for cell immobilization in combination with other scaffolds [47]. Currently, computer-assisted design/computer-assisted manufacturing (CAD/CAM) and rapid prototyping techniques allow the generation of custom-made scaffolds for cell delivery that fit into certain bone defects [22, 48].

There is a large number of osteogenic proteins that stimulate proliferation and differentiation of osteogenic cells in vitro and in vivo. Some osteogenic factors have been cloned and are commercially available as recombinant proteins. The most potent osteoinductive factors are bone morphogenetic protein (BMP). BMPs belong to the TGF-β family [49]. BMP-2 and BMP-7 are being clinically applied for fractures and nonunions [8, 50]. They have a short half-life, so local BMP delivery systems either require a high concentration bolus dose or sustained delivery for bone tissue engineering [51]. However, high BMP concentrations are associated

with increased osteoclastic activity and bone resorption [23, 24]. Other options are the direct implantation of a carrier that allows slow release or gene-based therapies, where a transgene for BMP expression is delivered to progenitor cells [52, 53]. Collagen carriers have historically been and remain the primary delivery system for BMPs to clinical defects. Because collagen has got poor BMP retention, higher BMP drug concentrations are required. Another concern is the potential for an immunogenic response or disease transfer from animal-derived collagen (e.g., variants of Creutzfeldt-Jacob disease or other prion-related diseases) [54]. A number of synthetic biomaterials have been proposed for BMP, such as inorganic ceramics, metals, and synthetic biodegradable polymers. Many of these materials are poorly biodegradable and radiopaque, whereas synthetic biodegradable polymers are mouldable and radiolucent. These characteristics make it easier to assess radiographic growth [55, 56].

Tissue engineering strategies aim at controlling the behaviour of individual cells to stimulate tissue formation. Currently, tissue-engineered bone is constructed using a perfusion bioreactor in vitro. Several different bioreactors have been investigated for tissue-engineering applications. Among these bioreactors are the spinner flask rotating wall vessel reactors and the flow perfusion culture bioreactors. Flow perfusion culture offers several advantages, notably the ability to mitigate both external and internal diffusional limitations as well as to apply mechanical stress to the cultured cells. In the perfusion culture, fluid flow can exert shear stress on the cells seeded on scaffold, improving the mass transport of the cells. Bioreactor systems of a variety of designs have also been utilized to enhance the in vitro performance of osteogenic cells before implantation. Bioreactors simulate the 3D dynamic and mechanical in vivo environment and are designed to provide cells seeded deep within a scaffold with all necessary nutrients and biological cues to survive, proliferate, differentiate, and produce ECM [57, 58]. Sikavitsas et al. [59] demonstrated proof of this concept by showing that after 16 days of culture, MSC-produced ECM was uniformly distributed in 3D scaffolds cultured in a flow profusion bioreactor, whereas the ECM was limited to the periphery in the case of standard static culture condition. Janssen et al. [60] demonstrated that direct perfusion bioreactor system is capable of producing clinically relevant volumes of tissue-engineered bone in a bioreactor system, which can be monitored on line during cultivation.

In summary, many factors can influence the osteoblastic differentiation of marrow stromal cells when cultivated on three-dimensional tissue engineering scaffolds. In creating ideal bone tissue engineering constructs consisting of a combination of a scaffold, cells, and bioactive factors, a flow perfusion bioreactor is a much more suitable culture environment than static culture in well plates. The bioreactor eliminates mass transport limitations to the scaffold interior and provides mechanical stimulation to the seeded cells through fluid shear [61]. Scaffold properties such as pore size impact cell differentiation, especially in flow perfusion culture. In addition, the bone-like ECM created by the in vitro culture of marrow stromal cells on porous scaffolds creates an osteoinductive environment for the differentiation of other marrow stromal cell populations. Therefore, bone tissue engineering constructs created by in vitro culture have excellent potential for bone regeneration applications in the clinical setting.

4. Clinical Outcomes

In literature, there are numerous studies demonstrating the effectiveness of bone tissue engineering techniques in the rodent model; however, little has been produced demonstrating its role in reconstructing osseous defects in larger animals. Petite et al. [62] investigated the role of in vitro expanded MSCs on a coral scaffold in large segmental bone defects in sheep. The study compared this technique with using the scaffold alone and the use of scaffolds with fresh bone marrow. With the tissue-engineered technique, clinical union was demonstrated in three out of seven bone defects, compared with no evidence of clinical union in any of the defects that were left empty or filled with scaffold alone.

With regards to the use of tissue engineering strategies in human bone reconstruction, published literature is sparse. Schimming and Schmelzeisen [63] reported the use of periosteum-derived tissue-engineered bone for the augmentation of the posterior maxilla. At three-month followup, eighteen out of twenty-seven patients demonstrated an excellent clinical, radiological, and histological outcome. Marcacci et al. [64] reported the use of ex vivo expanded bone marrow-derived MSCs implanted on a macroporous hydroxyapatite scaffold in four patients with large bone defects. One patient had a four-centimetre bone defect of the mid-diaphysis of the tibia following unsuccessful bone lengthening, another had traumatic loss of four centimetres of bone from the distal diaphysis of the ulna, the third patient had a seven centimetres bone defect of the humerus following a fracture and the final patient had six centimetres of traumatic bone loss from the ulna. The scaffolds were of the shape and size to fit each defect when implanted. External fixation was used for mechanical stability and removed after 6.5 months for the first patient, at 6 months for the second patient, at 13 months in the fourth patient, and 7 months for the final patient. Abundant callus formation along the implants and good integration with the host bones were evident on radiography and computed tomography after 1-2 months. At a minimum of 1-year followup, good integration of the implant to host bone was evident. All the patients reported favourable limb function outcome [64].

5. Conclusion

The use of tissue engineering for the reconstruction of bone defects has exciting potential; however, there is much work to be done before this strategy can be considered a serious clinical option [65–71]. The majority of research in MSC-based bone reconstruction has looked at isolation and expansion in vitro of MSCs, their delivery to defect sites and techniques to improve proliferation potential, and direct the MSCs towards osteogenesis using the appropriate factors [72–86].

Whilst animal studies have proven to show some success, the use of tissue engineering to repair bone defects in humans remains a challenge with limited clinical data. The reason for the perceived failure of these strategies in humans is thought to lie with an inadequate vascular supply, leading to cell death of implanted cells. There have also been concerns raised by the poor resorbability of the scaffolds and instability of the scaffold fixation. Whilst much work has been done on the factors involved in tissue engineering, more study is required to improve the key factor of cell survival in human models, such as improving nutrient and oxygen supply. Eventually, randomised controlled trials will be required to determine the effectiveness of tissue engineering approaches to bone reconstruction in humans before clinical use can be considered a viable option.

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