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Effekte von elektromagnetischen Feldern auf humane chondrogene Zellen

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„Es ist nie zu spät,

das zu werden,

was man hätte sein können“

(George Eliot)

INHALTSVERZEICHNIS

1 Abkürzungsverzeichnis

ACAN	Gen für Aggrekan
cDNA	engl. complementary deoxyribonucleic acid
COL1A1	Gen für Kollagen Typ I alpha 1
COL2A1	Gen für Kollagen Typ II alpha 1
COLXA1	Gen für Kollgen Typ X alpha 1
EMF	Elektromagentisches Feld
ECM	Extrazelluläre Matrix
hMSCs	humane mesenchymale Stammzellen
IHC	Immunhistochemie
Knie-TEP	Totale Endoprothese des Knies
non-OA-Chondrozyten	nicht durch Arthrose veränderte Chondrozyten
OA	engl. Osteoarthritis entspricht dem deutschen Begriff Arthrose
OA-Chondrozyten	durch Arthrose veränderte Chondrozyten
PK	Pelletkultur
TGF-β3	Transforming growth factor beta-3
qRT-PCR	quantitative real-time polymerase chain reaction
SOX9	Gen für Transkriptionsfaktor SRY (sex-determining region Y)-box 9

2 Einleitung

2.1 Äthiologie der Arthrose

Die Arthrose auf Englisch Osteoarthritis (OA), ist eine degenerative, nicht entzündliche Erkrankung des Gelenkknorpels. Sie ist eine physiologische Alterserscheinung, wird aber auch durch starke Belastung, Gelenkfehlstellung oder durch Traumata mit resultierenden Knorpeldefekten verursacht. Charakteristisch für die OA ist ein fortschreitender Prozess aus einer Degeneration des Gelenkknorpels, der an der Oberfläche beginnt und bis in die Tiefe voranschreitet. Am Ende kommt es zum funktionellen Gelenkversagen und zur Behinderung. Die OA ist ein klinisches Syndrom, bestehend aus Gelenkschmerzen und begleitet von funktionaler Einschränkung sowie reduzierter Lebensqualität. Die Skizze in Abbildung 1 zeigt die Komplexität des OA-Prozesses als ein Zusammenspiel von schädigenden und reparativen Abläufen.

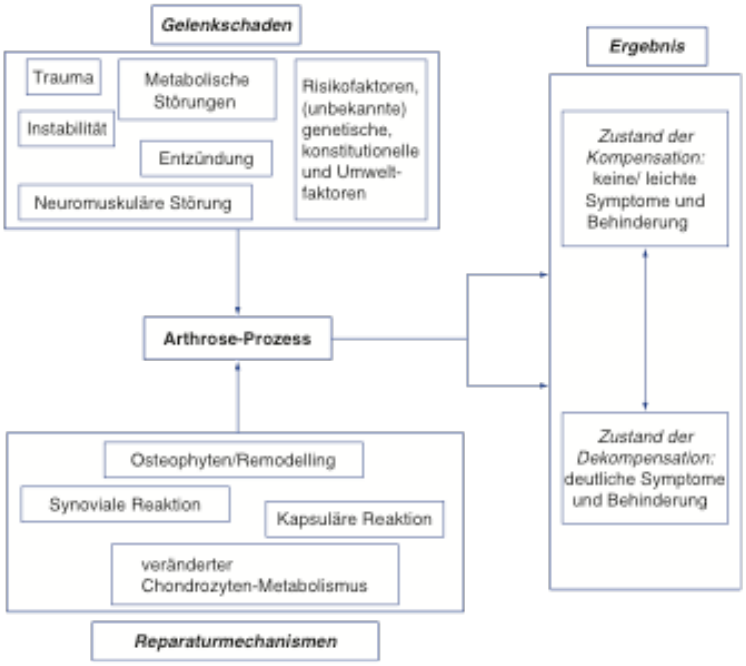

Abbildung 1: Skizze über das komplexe Zusammenspiel aus schädigenden und reparativen Abläufen im OA-Prozess [1].

Vor allem Knie- und Hüftgelenkarthrosen haben eine klinische und volkswirtschaftliche Bedeutung, da sie zu einer signifikanten Zunahme der

allgemeinen Mortalität durch Herz-Kreislauf-Erkrankungen aufgrund der Vermeidung von körperlichen Aktivitäten führen können [2, 3]. Die OA hat eine steigende Prävalenz aufgrund zunehmender Adipositas und der immer älter werdenden Bevölkerung. Mittlerweile sind über 40 Millionen Europäer und über 30 Millionen Nordamerikaner davon betroffen [4, 5].

2.2 Behandlungsansätze

Für die OA gibt es bisher keine Heilung, da keine Reparaturmechanismen existieren, welche die physiologische Gewebestruktur und Funktion vollständig wiederherstellen können [6]. Eine multimodale Therapie aus pharmakologischer und physikalischer Behandlung sowie Gewichtsreduktion wird in den meisten Richtlinien empfohlen. Allerdings führen konservative Maßnahmen oft nur zu kurzfristigen Verbesserungen oder ihre Wirkung fehlt insgesamt.

Einen weiteren Therapieansatz bietet die Regenerative Medizin mit Zelltherapien wie dem Tissue Engineering, welches Behandlungsmöglichkeiten bereitstellt. Neue Konzepte sollen mit autologen Chondrozyten, Stammzellen, Gerüsttechnologien oder adjuvanten Therapien bei der Regeneration beschädigter Gelenke helfen [7-9]. Abbildung 2 stellt das Zusammenspiel der einzelnen Komponenten der Regenerativen Medizin schematisch dar [7]. Ultima ratio beim Versagen konservativer Maßnahmen und Regenerativer Medizin ist schließlich der Gelenkersatz.

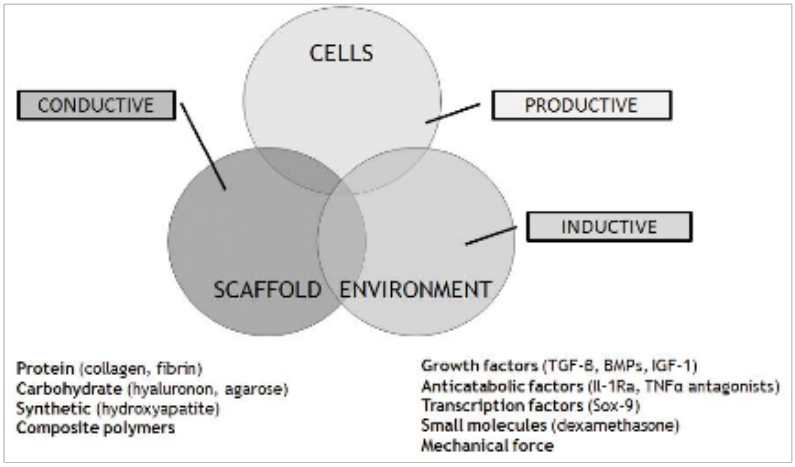

Abbildung 2: Zusammenspiel einzelner Komponenten der Regenerativen Medizin als Konzept für die OA-Therapie [7].

2.3 Elektromagnetische Felder als Therapie bei Arthrose

Die Therapie mit elektromagnetischen Feldern (EMFs) ist eine innovative, konservative Therapieoption der OA. Die EMF-Therapie basiert auf physikalischen Prinzipien, zu denen das Wolffsche Gesetz, der piezoelektrische Effekt und das Konzept der Strömungspotentiale gehören. Das Wolffsche Gesetz wurde im 19. Jahrhundert aufgestellt und besagt, dass sich der Knochen an eine bestehende Belastung anpasst [10]. So kommt es entweder zu einer Zunahme der Festigkeit bei Belastung oder einer Abnahme der Festigkeit bei Entlastung [10]. Es wird postuliert, dass EMFs in ähnlicher Weise auf Chondrozyten wirken können, indem sie diese zur Produktion von Extrazellulärer Matrix (ECM) anregen und somit der OA entgegenwirken können [11, 12]. Die Effekte von EMFs auf Gewebe wurden von Fukada und Yasuda schon im Jahre 1957 beschrieben, als sie das piezoelektrische Phänomen, die Änderung der elektrischen Polarisation im Knochen untersuchten [13]. Andere Studien beschreiben den Effekt der EMFs auf die Strömungspotentiale, welche direkten Einfluss auf die Proteoglykansynthese von Chondrozyten und damit auf die Synthese der ECM haben, indem sie die intrazelluläre Calciumkonzentration beeinflussen [14-18].

Es wurde gezeigt, dass eine EMF-Therapie Schmerzen reduzieren und die Mobilität von Patienten im Vergleich zu einer Placebo-Therapie verbessern kann [3, 19-21].

Studien zeigten auch, dass EMFs das chondrogene Potential humaner mesenchymaler Stammzellen (hMSCs) während der chondrogenen Differenzierung erhöhen [22, 23]. In einer Studie unserer Arbeitsgruppe zeigte sich bei hMSCs unter nicht optimalen Bedingungen eine Zunahme der Expression des knorpelspezifischen Gens der ECM Kollagen Typ II [22]. Die Zunahme war dabei abhängig vom Differenzierungszustand der hMSCs. Auch bei der OA geht man davon aus, dass die vorhandenen Chondrozyten in einem nicht optimalen Zustand vorliegen [24, 25]. Es ist nicht bekannt ob degenerativ veränderte Zellen anders auf EMFs reagieren als nicht durch Arthrose veränderte Chondrozyten (non-OA-Chondrozyten). Die Datenlage für die EMF-Therapie ist sehr inhomogen. Bei experimentellen Untersuchungen wurden viele unterschiedliche Feldstärken, Frequenzen und Laufzeiten der jeweiligen EMF verwendet. Ebenso wurden unterschiedliche Tiermodelle benutzt oder Chondrozyten *in vitro* einem EMF ausgesetzt. Genauso

variieren die Methoden um die Effekte von EMFs auf Knorpelgewebe oder Knorpelzellen nachzuweisen [5].

2.4 Ziel der Dissertation

Das Ziel der Arbeit war es die Auswirkungen von EMF auf humane chondrogene Zellen abhängig von deren Zustand und der Feldstärke des anliegenden EMF zu untersuchen. Hierzu wurden humane chondrogene Zellen verschiedener Ursprünge in einem in-vitro Modell bei unterschiedlichen Feldstärken untersucht.

In Veröffentlichung I [26] wurde zum einen das in-vitro Modell etabliert sowie ein Screening des gesamten Genexpressionsniveaus von chondrogen differenzierten hMSCs durchgeführt. Ziel war es, zu untersuchen ob sich während der Behandlung mit einem EMF Veränderungen bei der Chondrogenese von hMSCs ergeben.

Die Veröffentlichung II [27] analysierte mit Hilfe eines dreidimensionalen Modells, welches primäre humane Chondrozyten unterschiedlicher Differenzierungsgrade verwendete, die Wirkung von EMFs verschiedener magnetischer Feldstärken.

3 Veröffentlichungen

3.1 Veröffentlichung I

Mayer-Wagner S, Hammerschmid F, Blum H, Krebs S, **Redeker JI**, Holzapfel BM, et al. Effects of single and combined low frequency electromagnetic fields and simulated microgravity on gene expression of human mesenchymal stem cells during chondrogenesis. Epub 2016/05/16. doi: 10.5114/aoms.2016.59894.

(Impact-factor im Jahre 2016 von Arch Med Sci: 1.969)

Einleitung: Aus der Literatur ist bekannt, dass niederfrequente elektromagnetische Felder (LF-EMF) und simulierte Mikrogravitation (SMG) die Chondrogenese beeinflussen. Ziel unserer Studie war es, ein Bioreaktorsystem zu entwickeln um während der chondrogenen Differenzierung von humanen mesenchymalen Stammzellen (hMSCs) LF-EMF und SMG einzeln oder kombiniert anzuwenden.

Methoden: Es wurde ein rotierendes Bioreaktorgefäß mit einem extern gelegenem Motor mit einer Magnetfeldspule mit Magnetfeldgenerator und eigenen Kühlungssystem kombiniert und in einem Brutschrank installiert (Abbildung 3) [28].

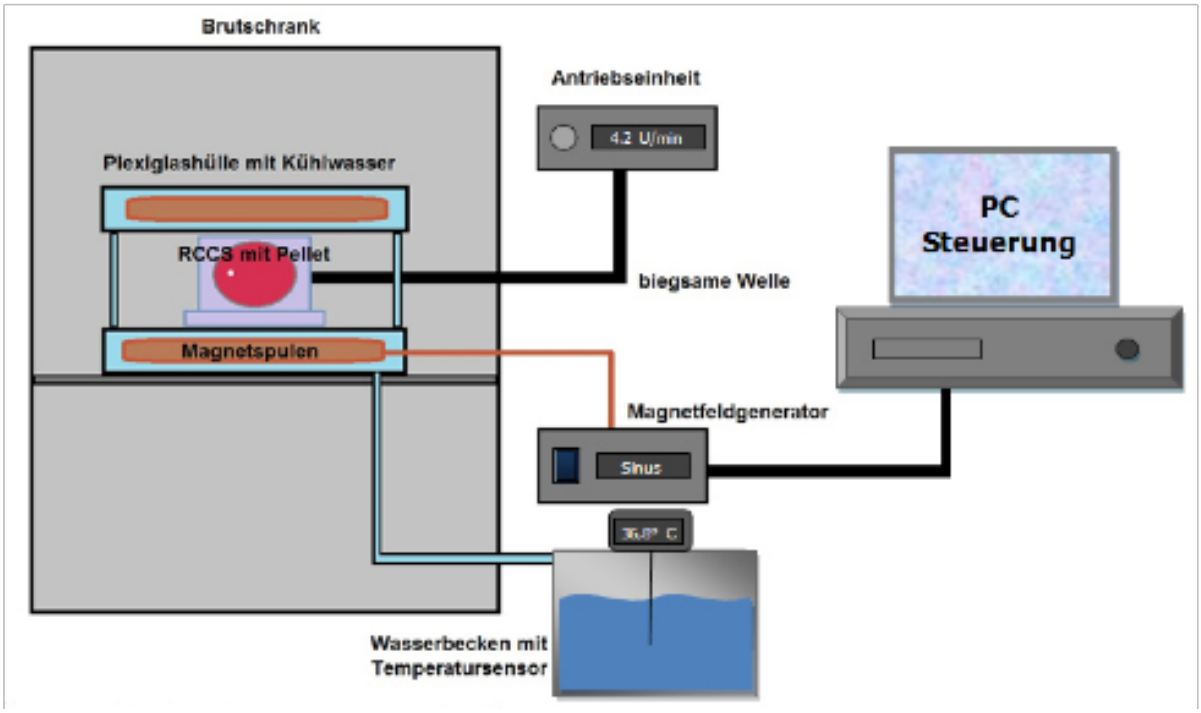

Abbildung 3: Schematische Darstellung der einzelnen Komponenten des Versuchsaufbaus [28].

Pelletkulturen aus hMSCs in Passage 5 wurden mit oder ohne dem Wachstumsfaktor Transforming growth factor beta-3 (± TGF-β3), der die Zelldifferenzierung beeinflusst, unter vier verschiedenen Bedingungen für 3 Wochen kultiviert: unter SMG (Gruppe 1), unter LF-EMF (Gruppe 2), unter SMG/LF-EMF (Gruppe 3) und als Kontrollgruppe bei 1 g (Gruppe 4). Für die histologische und immunhistochemische (IHC) Analyse wurden aus den Pelletkulturen (PKs) Gefrierschnitte angefertigt. Um Proteoglykane und Polysaccharide darzustellen, wurden Schnitte mit einer Safranin-O-Färbung angefertigt. Eine IHC-Färbung wurde für das ECM-Protein Kollagen Typ II erstellt. Zur molekularbiologischen Untersuchung wurden die PKs mit Hilfe eines Mikro-Dismembrators aufgeschlossen und RNA-Proben gewonnen. Nach einer reversen Transkription jeder RNA-Probe wurde mit der erhaltenen cDNA eine quantitative real-time PCR (qRT-PCR) auf knorpelspezifische Gene (COL2A1, COLXA1 und ACAN) sowie eine Microarray-Analyse durchgeführt.

Ergebnisse: Die chondrogene Differenzierung wurde durch eine qRT-PCR für COL2A und ACAN in allen PKs bestätigt, die mit TGF-β3 kultiviert worden waren.

Eine Behandlung mit SMG reduzierte die COL2A1-, ACAN- und COLXA1-Expression signifikant im Vergleich zu den Kontrollkulturen. Eine gleichzeitige Behandlung mit SMG und LF-EMF zeigte im Vergleich zur alleinigen SMG-Behandlung eine signifikant höhere COL2A1-Expression. Die alleinige SMG-Behandlung veränderte die Genexpression der PKs signifikant; Insbesondere wurden COLXA1, aber auch COL2A1, welches das chondrogene Potenzial darstellt, reduziert ($p < 0,05$). Eine alleinige LF-EMF-Behandlung zeigte keine signifikante Wirkung auf die Expression von COL2A1, ACAN und COLXA1 im Vergleich zur Kontrolle. Auch auf Microarray-Basis konnte keine Änderung in der Genexpression der PKs festgestellt werden. Die Anwendung einer Kombination aus SMG- und LF-EMF-Behandlung erzeugte einen signifikanten Anstieg von COL2A1 im Vergleich zu PKs, die nur unter SMG-Bedingungen kultiviert worden waren. Die histologischen und IHC-Färbungen der PKs stimmen mit den molekularbiologischen Ergebnissen überein: PKs mit alleiniger SMG-Behandlung zeigten eine schwächere Safranin-O- und Kollagen-Typ-II-Färbung. PKs mit alleiniger LF-EMF-Behandlung zeigten eine intensive Anfärbung von Safranin-O in der Mitte des Pellets sowie eine ähnliche lokalisierte Anfärbung für Kollagen Typ II. Die Kombination aus SMG- und LF-EMF-Behandlung zeigte im Zentrum der Pellets starke Safranin-O-Färbung und eine einheitliche Färbung für Kollagen Typ II.

Schlussfolgerung: Es wurden die Auswirkungen einer alleinigen und kombinierten Behandlung einer SMG und einem EMF auf die Genexpression von hMSCs untersucht. Die SMG senkte die Hypertrophie, aber auch das chondrogene Potenzial von hMSCs. Die Kombination aus SMG und LF-EMF lieferte einen Rettungseffekt des chondrogenen Potentials von hMSCs, obwohl unter optimalen Bedingungen kein LF-EMF-Effekt beobachtet wurde. Die Studie liefert neue Erkenntnisse darüber, wie LF-EMF und SMG die Chondrogenese von hMSCs beeinflussen und wie sie voneinander abhängige Wirkungen erzeugen.

3.2 Beitrag der Doktorandin zur Veröffentlichung I

Der Eigenanteil der Ko-Autorin an diesem Projekt war neben der Hilfe bei der ursprünglichen Planung und der Manuskriptbearbeitung die Unterstützung bei der Etablierung des EMFs im Bioreaktor, die Unterstützung der Zellkulturversuche, sowie

die Herstellung der RNA-Proben, die für das Screening des gesamten Expressionsprofils der hMSCs benötigt wurden.

3.3 Veröffentlichung II

Redeker JI, Schmitt B, Grigull NP, Braun C, Buttner A, Jansson V, Mayer-Wagner S. Effect of electromagnetic fields on human osteoarthritic and non-osteoarthritic chondrocytes. BMC Complement Altern Med. 2017;17:8.
(Impact-factor im Jahre 2017 von BMC: 2.288)

Hintergrund: Es gibt eine Vielzahl an Studien zum Effekt elektromagnetischer Felder auf Chondrozyten mit unterschiedlichen Ergebnissen. Deshalb gehört die Behandlung der OA mit EMFs bis heute noch nicht zum klinischen Standard. Das Ziel dieser Studie war es, die Auswirkungen von EMFs auf OA-Chondrozyten gegenüber non-OA-Chondrozyten zu vergleichen. Ein weiteres Ziel war es herauszufinden, ob der Effekt von EMFs nur an die Chondrozyten-Qualität oder auch an eine spezifische magnetischen Feldstärke (5 mT oder 8 mT) gekoppelt ist. Hierzu wurden humane Chondrozyten in einem 3D-in-vitro Modell, das den Goldstandard für chondrogene in-vitro Analysen darstellt, bei unterschiedlichen EMFs untersucht.
Methoden: Humane OA-Chondrozyten wurden aus Knorpelgewebe von Patienten gewonnen, die eine Knie-TEP erhielten. Non-OA-Chondrozyten wurden von jungen, teils verstorbenen Patienten aus nicht degenerativ verändertem Knorpel gewonnen. Die 3D-Pelletkulturen (PKs) aus humanen OA-Chondrozyten, und non-OA-Chondrozyten wurden für 7 Tage einem sinusförmigen 15 Hz-EMF (5 mT oder 8 mT) ausgesetzt. Das EMF wurde mit einer Magnetfeldspule simuliert, die mit einem Frequenzgenerator kombiniert und in einem Inkubator eingebaut war. Die Kontrollgruppen wurden unter Standardbedingungen ohne EMF (0 mT) kultiviert. Für die histologische und immunhistochemische Analyse wurden aus den PKs Gefrierschnitte angefertigt. Um Proteoglykane und Polysaccharide darzustellen, wurden von jedem Pellet Schnitte mit Safranin-O- und Alcianblau-Färbungen angefertigt. IHC-Färbungen wurden für die ECM-Proteine Kollagen Typ I und Kollagen Typ II erstellt. Zur molekularbiologischen Untersuchung wurden die PKs mit Hilfe eines Mikro-Dismembrators aufgeschlossen und RNA-Proben gewonnen. Nach

einer reversen Transkription jeder RNA-Probe wurde mit der erhaltenen cDNA eine qRT-PCR auf knorpelspezifische Gene (COL2A1, ACAN und SOX9) sowie COL1A1 durchgeführt.

Ergebnisse: Im Vergleich zur Kontrolle erhöhten alle OA-Chondrozyten der Patienten, die eine Knie-TEP erhielten mit einer 7-tägigen 5 mT EMF-Behandlung signifikant die Expression von COL2A1 und ACAN. Im Gegensatz dazu wurde keine Veränderung der Genexpression bei non-OA-Chondrozyten aus gesundem Knorpelgewebe beobachtet. Mit einer 7-tägigen 8 mT EMF-Behandlung zeigten weder OA-Chondrozyten noch non-OA-Chondrozyten eine signifikante Veränderung der Genexpression (Abbildung 4) [27].

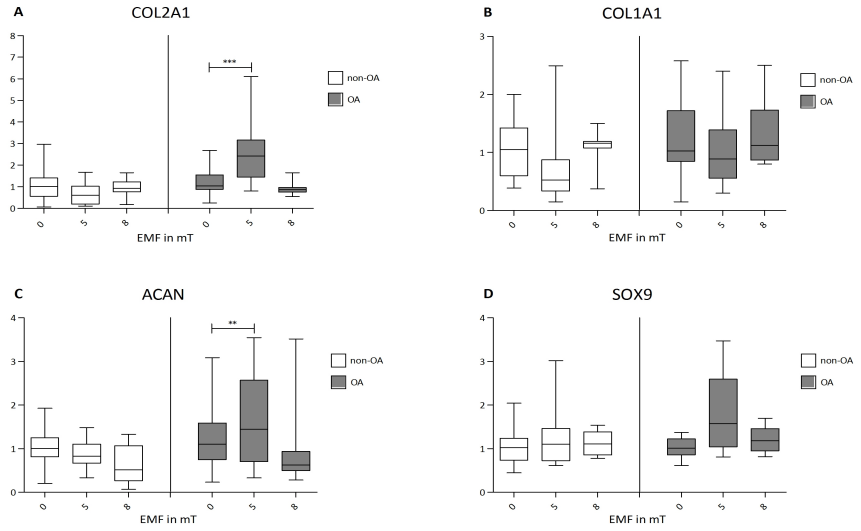

Abbildung 4: Veränderung der Genexpression in OA-PKs und non-OA-PKs von COL2A1 (a), COL1A1 (b), ACAN (c) und SOX9 (d) nach einer 7-tägigen EMF-Exposition mit 5 oder 8 mT, normalisiert auf die Kontrollgruppe, die ohne ein EMF kultiviert wurde; GAPDH wurde als Referenzgen verwendet [27].

Beim histologischen und immunhistochemischen Vergleich der PKs zeigten non-OA-PKs eine homogenere und kompaktere Struktur als OA-PKs. OA-PKs, die in einem 5 mT EMF behandelt wurden, zeigten eine ähnliche Färbung für Kollagen Typ II wie non-OA-PKs, während unbehandelte OA-PKs weniger Färbung für Kollagen Typ II zeigten (Abbildung 5) [27]. OA-PKs und non-OA-PKs mit einer 8 mT EMF-Behandlung zeigten keine Unterschiede in den Färbungsintensitäten für Kollagen Typ I und Typ II.

Schlussfolgerung: Es wurden die Auswirkungen eines 5 mT und eines 8 mT EMFs auf humane OA-Chondrozyten und non-OA-Chondrozyten in einem 3D-in-vitro-Modell untersucht. Eine 5 mT EMF-Behandlung erhöhte die Expression knorpelspezifischer Gene in OA-Chondrozyten, während für Chondrozyten, die nicht degenerativ verändert waren, keine Veränderungen in der Genexpression beobachtet wurden. Eine 8 mT EMF-Behandlung zeigte keinerlei Wirkung.

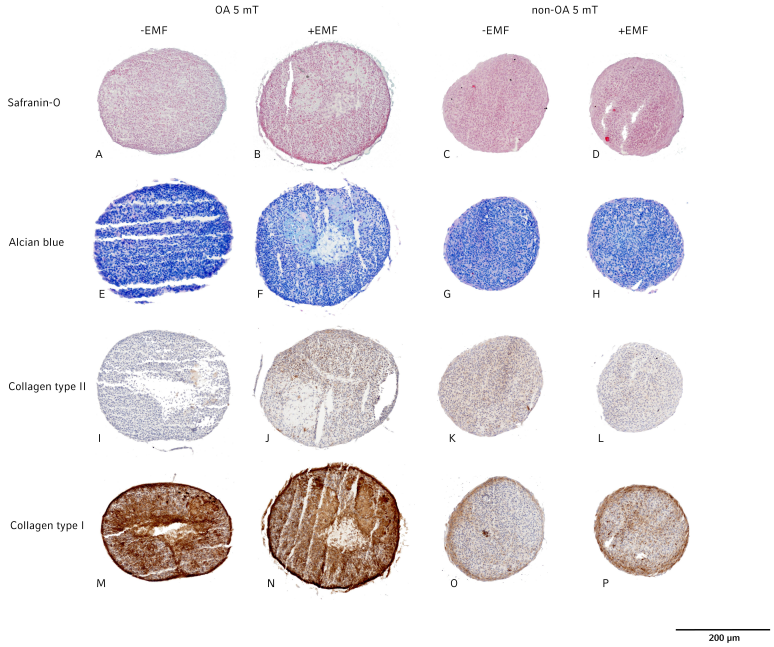

Abbildung 5: Histologische und immunhistochemische (IHC) Färbungen von Pelletkultur-Sektionen (PKs) eines OA-Patienten und eines non-OA-Patienten nach einer 7-tägigen EMF-Exposition mit 5 mT (B, F, J, N, D, H, I, P) oder ohne EMF-Exposition (A, E, I, M, C, G, K, O). Die Safranin-O-Färbungen (A, B, C, D) und die Alcianblau-Färbungen (E, F, G, H) waren für alle PKs positiv. Die IHC-Färbung für Kollagen Typ II für die OA-PK nach EMF-Exposition (J) und non-OA-PKs mit und ohne EMF-Exposition (K, L) war deutlicher als bei der A-PK ohne EMF-Exposition (I). Die IHC-Färbung für Kollagen Typ I von OA-PKs (M, N) zeigte eine stärkere Färbung als non-OA-PKs (O, P) [27].

3.4 Beitrag der Doktorandin zur Veröffentlichung II

Die Doktorandin war aktiv an der Planung und Organisation der Studie beteiligt. Neben der Gewinnung der Proben aus dem Operationssaal und der Rechtsmedizin, führte die Doktorandin die Zellversuche, Färbungen und die qRT-PCR eigenständig durch. Die Auswertung der Daten sowie die Erstellung des Manuskripts erfolgte in enger Zusammenarbeit mit Frau Prof. Dr. med. Susanne Mayer.

4 Zusammenfassung

Das Hauptziel der vorliegenden Dissertation ist die Untersuchung der Auswirkung elektromagnetischen Feldern (EMFs) auf humane chondrogene Zellen. Grundlage dieser kumulativen Dissertation sind die zwei aufgeführten Publikationen, die in renommierten orthopädischen Fachzeitschriften erschienen sind:

- **Veröffentlichung I:** Mayer-Wagner S, Hammerschmid F, Blum H, Krebs S, **Redeker JI**, Holzapfel BM, et al. Effects of single and combined low frequency electromagnetic fields and simulated microgravity on gene expression of human mesenchymal stem cells during chondrogenesis. Epub 2016/05/16. doi: 10.5114/aoms.2016.59894.
  (Impact-factor Arch Med Sci im Jahr 2016: 1.969)

- **Veröffentlichung II: Redeker JI**, Schmitt B, Grigull NP, Braun C, Buttner A, Jansson V, et al. Effect of electromagnetic fields on human osteoarthritic and non-osteoarthritic chondrocytes. BMC Complement Altern Med. 2017;17:8.
  (Impact-factor im Jahre 2017 von BMC: 2.288)

Beide Arbeiten hatten das Ziel weiteres Verständnis darüber zu erlangen ob und wie EMFs auf humane chondrogene Zellen wirken. Dafür wurden humane Chondrozyten in einer 3D-Pelletklutur einem EMF ausgesetzt, um anschließend ihr Genexpressionsprofil auf knorpelspezifische Gene zu analysieren. Zusätzlich sollte die Wirkung des EMFs auf die Pelletkulturen durch die Darstellung von Proteinen der extrazellulären Matrix histologisch und immunhistochemisch dargestellt werden [27]. Außerdem wurde untersucht wie sich eine Behandlung mit EMFs bei der chondrogenen Differenzierung von Stammzellen auswirkt [26]. Die Ergebnisse beider Arbeiten deuten darauf hin, dass EMFs die Genexpression und Produktion von Proteinen der extrazellulären Matrix nur bei solchen Zellen ändern, die in einem degenerativ veränderten Zustand vorliegen. EMFs zeigen in beiden Studien keinerlei Wirkungen auf Zellen des gesunden, hyalinen Knorpels und chondrogen differenziert Zellen in optimaler Differenzierungsqualität. Zusätzlich hängt der Effekt des EMFs auf humane chondrogene Zellen von der magnetischen Feldstärke des EMFs ab.

Weitere Studien zur klinischen Relevanz dieses Effekts sind notwendig, um für Patienten mit Arthrose innovative Therapieansätze zu entwickeln.

5 Summary

The main aim of the present thesis is to investigate how electromagnetic fields (EMFs) might have an impact on human chondrogenic cells. The basis of this thesis are the two listed puplications that appeared in renowned orthopaedic journals:

- **Paper I:** Mayer-Wagner S, Hammerschmid F, Blum H, Krebs S, **Redeker JI**, Holzapfel BM, et al. Effects of single and combined low frequency electromagnetic fields and simulated microgravity on gene expression of human mesenchymal stem cells during chondrogenesis. Epub 2016/05/16. doi: 10.5114/aoms.2016.59894.
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- **Paper II: Redeker JI**, Schmitt B, Grigull NP, Braun C, Buttner A, Jansson V, et al. Effect of electromagnetic fields on human osteoarthritic and non-osteoarthritic chondrocytes. BMC Complement Altern Med. 2017;17:8.
  (Impact-factor in 2017 by BMC: 2.288)

The aim of both studies was to gain further understanding of whether and how EMFs affect human chondrogenic cells. Human chondrocytes were exposed to an EMF in a 3D pellet culture to analyze their gene expression profile on cartilage-specific genes. In addition, we aimed to visualize the effect of EMF on chondrocytes histologically and immunohistochemically by visualizing extracellular matrix proteins [27]. We also investigated how treatment with EMFs affects the chondrogenic differentiation of stem cells [26]. The results of both studies suggest that EMFs alter gene expression and production of extracellular matrix proteins only in chondrogenic cells that are in a sort of degenerated condition. In both studies EMFs had no influence on cells from hyaline cartilage and chondrogenic cells with optimal differentiation quality. In addition, the effect of EMF on human cells depends on the field strength of the EMF.

Further studies on the clinical relevance of this effect are necessary to develop innovative therapeutic approaches for patients with osteoarthritis.

6 Originalveröffentlichung I

Effects of single and combined low frequency electromagnetic fields and simulated microgravity on gene expression of human mesenchymal stem cells during chondrogenesis

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Abstract

Introduction: Low frequency electromagnetic fields (LF-EMF) and simulated microgravity (SMG) have been observed to affect chondrogenesis. A controlled bioreactor system was developed to apply LF-EMF and SMG singly or combined during chondrogenic differentiation of human mesenchymal stem cells (hMSCs) in 3D culture.

Material and methods: An external motor gear SMG bioreactor was combined with magnetic Helmholtz coils for EMF (5 mT; 15 Hz). Pellets of hMSCs (±TGF-β3) were cultured (P5) under SMG, LF-EMF, LF-EMF/SMG and control (1g) conditions for 3 weeks. Sections were stained with safranin-O and collagen type II. Gene expression was evaluated by microarray and real-time polymerase chain reaction analysis.

Results: Simulated microgravity application significantly changed gene expression; specifically, COLXA1 but also COL2A1, which represents the chondrogenic potential, were reduced ($p < 0.05$). Low frequency electromagnetic fields application showed no gene expression changes on a microarray basis. LF-EMF/SMG application obtained significant different expression values from cultures obtained under SMG conditions with a re-increase of COL2A1, therefore rescuing the chondrogenic potential, which had been lowered by SMG.

Conclusions: Simulated microgravity lowered hypertrophy but also the chondrogenic potential of hMSCs. Combined LF-EMF/SMG provided a rescue effect of the chondrogenic potential of hMSCs although no LF-EMF effect was observed under optimal conditions. The study provides new insights into how LF-EMF and SMG affect chondrogenesis of hMSCs and how they generate interdependent effects.

Key words: bioreactor, electromagnetic fields, simulated microgravity, chondrogenesis.

Introduction

Untreated cartilage defects in adults lead to osteoarthritis as there is no spontaneous repair mechanism which is able to completely restore physiological tissue structure and function [1, 2]. Procedures such as matrix-associated autologous chondrocyte transplantation (MACT) are used to facilitate the regenerative capacity and improve clinical outcome. As the use of chondrocytes is associated with donor site morbidity and dedifferentiation, human mesenchymal stem cells (hMSCs) might provide an opportunity to obtain a sufficient amount of cartilaginous cells. Advanced cell therapies for articular cartilage regeneration are developed using various bioreactor systems [3]. The main challenge in chondrogenic differentiation of hMSCs is to prevent terminal ossification represented by hypertrophy and at the same time maintain the chondrogenic potential of cells [4].

Adjuvant therapies such as electromagnetic fields (EMF) and simulated microgravity (SMG) have been used to improve cartilaginous tissues, but the literature data are very diverse.

Microgravity has been reported to reduce osteogenesis [5–8], and SMG has been shown to exert various effects on cartilaginous cells. Simulated microgravity has been reported by our group to reduce hypertrophy during chondrogenic differentiation of hMSCs [9]. However, SMG was also shown to decrease the chondrogenic potential of hMSCs [9–12].

Electromagnetic fields were shown to improve the chondrogenic potential of cells under certain conditions [13] and have been reported to develop multiple effects on hMSCs [14]. Under Low frequency electromagnetic fields (LF-EMF), the chondrogenic differentiation of hMSCs in 3D culture showed a significant increase of collagen type II expression at higher cell passages, although no EMF effect was obtained under optimal differentiation conditions [15]. However, EMF have also been reported to cause adverse effects [16–18], and the level of concern about EMF in the general population is high. Among general practitioners, there is no association between correct knowledge and concern [19]. Nevertheless, there is a lack of studies examining general EMF effects on chondrogenic cells. We therefore examined in this study the influence of LF-EMF during chondrogenesis by a screen of the entire gene expression level to detect beneficial and adverse gene expression changes which might occur under EMF treatment of cartilage defects. To our knowledge there has been no similar approach in the literature.

In a further step this study compared single and combined effects of LF-EMF and SMG on chondrogenesis of hMSCs in scaffold-free high-density 3D pellet constructs. In contrast to previously used SMG bioreactor systems, a controlled approach was used, which omitted any form of inadvertent EMF interference from power units of the SMG system by using solely an external motor gear. This approach made it possible to examine pure LF-EMF and pure SMG effects on chondrogenesis singly and combined for the first time.

The aim of this study was to show for the first time an entire gene expression screen, describe how LF-EMF and SMG affect chondrogenesis, and discuss whether they exert an influence on each other.

Material and methods

SMG

The LF-EMF/SMG bioreactor system consisted of an external motor gear and an internal vessel holder (Figure 1 A). Vessel rotation was transmitted by a flexible shaft to a 10 ml RCCS vessel (Rotary Cell Culture System; Synthecon Inc., Houston, USA) holder. The SMG bioreactor was combined with two magnetic Helmholtz coils (LF-EMF bioreactor) with an external cooling system and an external frequency generator. The vessel holder containing the pellet was situated within an incubator (Figure 1 A). To assure SMG conditions for each pellet, the rotation speed was adjusted to the velocity at which the pellet was maintained in free-fall condition at approximately 7 rpm. Previously, resulting shear forces were determined using dummy pellets. Simulated microgravity was applied between days 15 and 21 within the terminal chondrogenic differentiation phase, where hypertrophy is usually observed [20]. Pellets were transferred to the RCCS vessel during this phase.

LF-EMF

Two magnetic Helmholtz coils (D = 30 cm, 278 turns of enamelled copper wire, D = 1.5 mm, R = 2.5 Ω, L = 38.5 mH for one coil, distance between coils 15 cm, current = 3 A) placed in a cooling box (Neue Magnetodyn GmbH, Munich, Germany) connected to an external water bath (refrigerated circulator, DC50-F3 Haake, Vreden, Germany) produced a low frequency sinusoidal LF-EMF of 15 Hz and 5 mT magnetic flux density. The coils were driven by a frequency generator with power amplifier (M80, Neue Magnetodyn) (Figures 1 A, B). The field was applied three times a day (every 435 min) for 45 min throughout the differentiation period of 21 days. All stimulations were performed inside an incubator (CB150, Binder, Tuttlingen, Germany) under sterile conditions with a constant temperature level of 37 ±0.25°C.

Chondrogenic differentiation of hMSCs

Adult bone marrow derived hMSCs (Lonza, Walkersville, MD) were expanded in monolayer

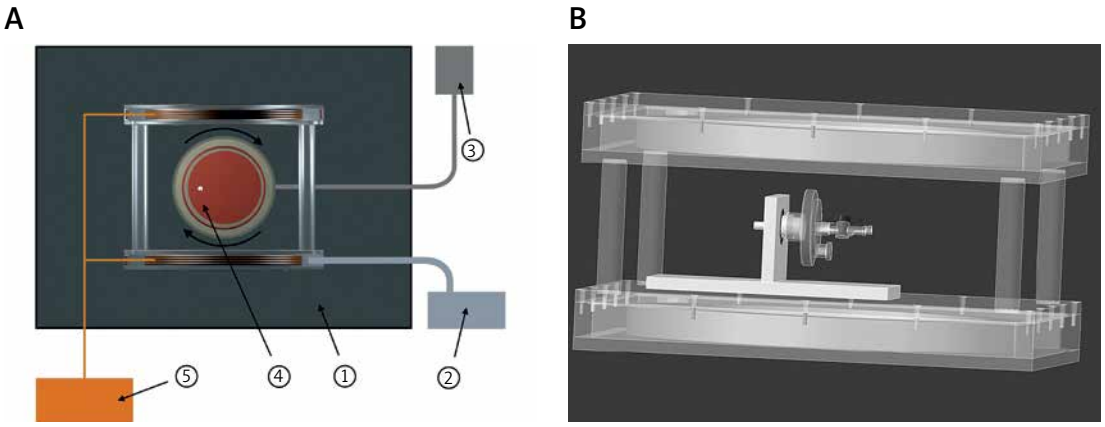

Figure 1. SMG/LF-EMF bioreactor system: **A** – Schematic drawing of setup compounds: incubator (1), LF-EMF solenoid with external cooling system (2), external motor gear of the SMG (3) connected to RCCS vessel by a flexible shaft, floating pellet (white) within the RCCS vessel (4), external frequency generator of the LF-EMF (5). **B** – Drawing of the position of the SMG bioreactor and RCCS vessel within the Helmholtz coils

triple flasks (Nunc, Roskilde, Denmark) in a humidified atmosphere, 5% CO_2, at 37°C, in growth medium (α-medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum (PAA, Pasching, Austria), 50 U/ml penicillin/streptomycin (Biochrom), 2 mM glutamine and 5 ng/ml recombinant human fibroblast growth factor 2 (FGF-basic; Pepro Tech, Rocky Hill, NJ)). At 70–80% confluency, cells were passaged using 0.05% trypsin containing 0.02% EDTA (Biochrom). To generate pellet cultures, 4×10^5 hMSCs were centrifuged (150 g, 5 min) at passage 4 in a 15 ml falcon tube (TPP, Switzerland). For differentiation, pellets were cultured over a period of 21 days in differentiation medium (high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Biochrom) supplemented with 10 mg/l insulin, 5.5 mg/l transferrin, 5 µg/l selenium, 0.5 mg/ml bovine serum albumin, 4.7 µg/ml linoleic acid, 0.1 µM dexamethasone, 0.2 mM L-ascorbic acid-2-phosphate, 0.35 mM L-proline (all Sigma-Aldrich, Steinheim, Germany), 30 U/ml penicillin/streptomycin (Biochrom) and 10 ng/ml recombinant human transforming growth factor-β3 (TGF-β_3; R&D Systems, Wiesbaden, Germany)).

Pellet cultures were distributed into four groups: 1) Controls cultured under 1g conditions (n = 14); 2) SMG (n = 4); 3) LF-EMF (n = 14); and 4) LF-EMF/SMG (n = 5). The pellet culture system is limited to one culture per tube/RCCS. Pellet cultures were also maintained in the absence of the growth factor TGF-β_3.

Histology and immunohistochemistry

Pellets were embedded in Tissue-Tek (Sakura, Zoeterwoude, the Netherlands) prior to cryo-sectioning. Serial sections were stained with safranin-O (Fluka, Buchs, Switzerland) and fast green (Chroma, Münster, Germany) as previously described [21] to estimate the content and distribution of proteoglycans. For immunohistochemistry, samples were treated with chondroitinase and incubated overnight at 4°C with a primary antibody for collagen type II (Department of Medical and Physiological Chemistry, Uppsala, Sweden) diluted 1 : 6. For negative controls, the first antibody was omitted. Then samples were treated with a goat-derived biotinylated mouse-specific antibody (Vector Laboratories, Burlingame, USA) 1 : 200 in TBS for 1 h at room temperature (RT). Bound antibodies were stained with the VECTASTAIN ABC-Kit (Vector Laboratories, Burlingame, USA) and AEC (Sigma-Aldrich, Steinheim, Germany).

RNA preparation

Pellets from every group were separately disrupted under frozen conditions at 300 rpm using a Micro-Dismembrator S (Sartorius, Goettingen, Germany). RNA was directly isolated from freeze-milled preparations using 1 ml of TRIzol (Invitrogen, Germany). After adding 0.2 ml of chloroform (Sigma-Aldrich, Steinheim, Germany) and vigorously shaking, samples were incubated at RT for 10 min. Then samples were centrifuged at 15,000 x g for 20 min at 4°C, and the aqueous phase was transferred to a fresh tube. RNA was directly isolated using RNeasy Mini Kit (Qiagen).

Microarray analysis

Isolated total RNA was checked for purity (NanoDrop ND-1000, ThermoFisher, Waltham, USA) and integrity (Bioanalyzer 2100, Agilent, Santa Clara, USA). Control (WF) cultures were analyzed versus LF-EMF cultures (WF + EMF). 150 ng of total RNA was used for cDNA synthesis, amplification, fragmentation, and labelling using the Nugen Applause WTA ST and Encore Biotin labelling kits (Nugen, San Antonio, USA). Labelled probes were hy-

bridised to Affymetrix HuGene 2.0 ST GeneChips, washed, stained in an Affymetrix FS450 station, and scanned on an Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, USA). The array CEL-files were RMA normalised with the appropriate program package from Affymetrix and analysed for differential expression in R using the packages limma and siggenes. The false discovery rate was set to 0.1 and no fold-change cutoff was applied. All arrays passed the quality control checks implemented in the R package arrayQualityMetrics [22].

Quantitative real-time PCR analysis

For cDNA synthesis, 0.3 µg of total RNA was reverse-transcribed using QuantiTect Rev. Transcription Kit (Qiagen, Hilden, Germany) with DNase treatment. Quantitative RT-PCR was performed with a LightCycler (Roche Applied Science, Mannheim, Germany) using 2 µl of SYBR Green Master Mix (Roche Applied Science, Mannheim, Germany) and 2.5 µl of 1 : 4 diluted cDNA in a 10 µl final volume. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward TGC ACC ACC AAC TGC TTA GC, reverse GGC ATG GAC TGT GGT CAT GAG [23]; collagen type II α_1 chain (COL2A1) forward GTT ATC GAG TAC CGG TCA CAG AAG, reverse AGT ACT TGG GTC CTT TGG GTT TG [24]; collagen type 10 α_1 chain (COLXA1) forward CAA GGC ACC ATC TCC AGG AA, reverse AAA GGG TAT TTG TGG CAG CAT ATT [25] and cartilage-specific proteoglycan core protein (aggrecan) forward CAG CAC CAG CAT CCC AGA, reverse CAG CAG TTG ATT CTG ATT CAC G [24] were used as primers. The following RT-PCR conditions were used for COL2A1 and aggrecan: polymerase activation 95°C for 10 min, 40 cycles at 95°C for 10 s, 65°C for 10 s and 72°C for 15 s. Conditions for GAPDH and COLXA1: 95°C for 10 min, 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 15 s. Reactions were performed in triplicate. Relative quantification of the target gene expression was generated normalizing to GAPDH. The LF-EMF, SMG and LF-EMF/SMG values were normalized to controls cultured with TGF-β3 under 1g conditions.

Statistical analysis

Analysis was conducted using Microsoft Excel 2010 and Prism 5.02 software for Windows. Values were reported as the mean, standard deviation and range. Statistical analysis was performed using the Mann-Whitney *U*-test as data were not normally distributed. The level of significance was set at $p \leq 0.05$.

Results

LF-EMF application

The magnetic flux density in the layer of the pellet cultures within the bioreactor has a homogeneous spatial distribution due to the Helmholtz configuration and is 5 mT ± 5%. The electric field at a certain distance from the coil centre was calculated with good approximation by $E_{max} = 2\ h\ \pi\ f\ B_x$ [25], where B_x is the peak value of the magnetic flux density, f its frequency and h is the height of the medium. With the given values the maximum induced electric field is below 1 mV/m in the region of the pellet. The current density was calculated by $J = \sigma * E_{max}$, where σ is the conductance of the medium. With the given values and a conductance of 1.6 S/m for the medium [26] the current density is below 0.9 mA/m².

The magnetic flux density of the Helmholtz-coils was simulated (Finite Element Method Magnetics (FEMM); Meeker, D. (2010), version 4.0.1) (Figure 2 A). The simulation was validated by measuring the B-field along the axis of the Helmholtz coils with a Gaussmeter (Bell 640, F.W. Bell, Orlando, FL) (Figure 2 B). The earth's static magnetic field perpendicular to the axis of the Helmholtz coils was measured at 45 mT.

SMG bioreactor

Bioreactors simulating microgravity are used to avoid the enormous costs of space flight experiments. The SMG condition often results in a certain amount of shear stress. Within this study a low resulting shear stress of 0.16 dyn/cm² ($(T_{max} = 3\mu * V_{pellet}/2\ r_{pellet})$ viscosity of the medium (µ): 1.05 kg/ms; sedimentation rate (V_{pellet}): 0.04 m/s; mean pellet radius (r_{pellet}): 0.4 mm) was obtained. Values were obtained by measuring caviar globes as dummy pellets (data not shown).

A conventional commercially available Rotatory Cell Culture System (RCCS) developed by NASA to simulate microgravity generates low EMF in the layer of the pellets in the range of 0.1–0.5 mT with a sinusoidal modulation depending on the rotation speed of the motor. The controlled bioreactor used in this study excluded low inadvertent EMF by using an external motor gear.

Histology and immunohistochemistry

At the end of the differentiation period, positive staining for safranin-O and collagen type II was found in the centre of all pellet cultures treated with growth factors. Without growth factors, staining for safranin-O and collagen type II was negative. Control pellet cultures treated with growth factors showed more uniform staining for safranin-O and collagen type II. Cultures treated with LF-EMF alone revealed intense staining of safranin-O in the middle of the pellet and similar localized staining for collagen type II. In pellets cultured under single SMG there was weaker safranin-O and collagen type II staining. Pellets exposed to SMG and LF-EMF showed strong safr-

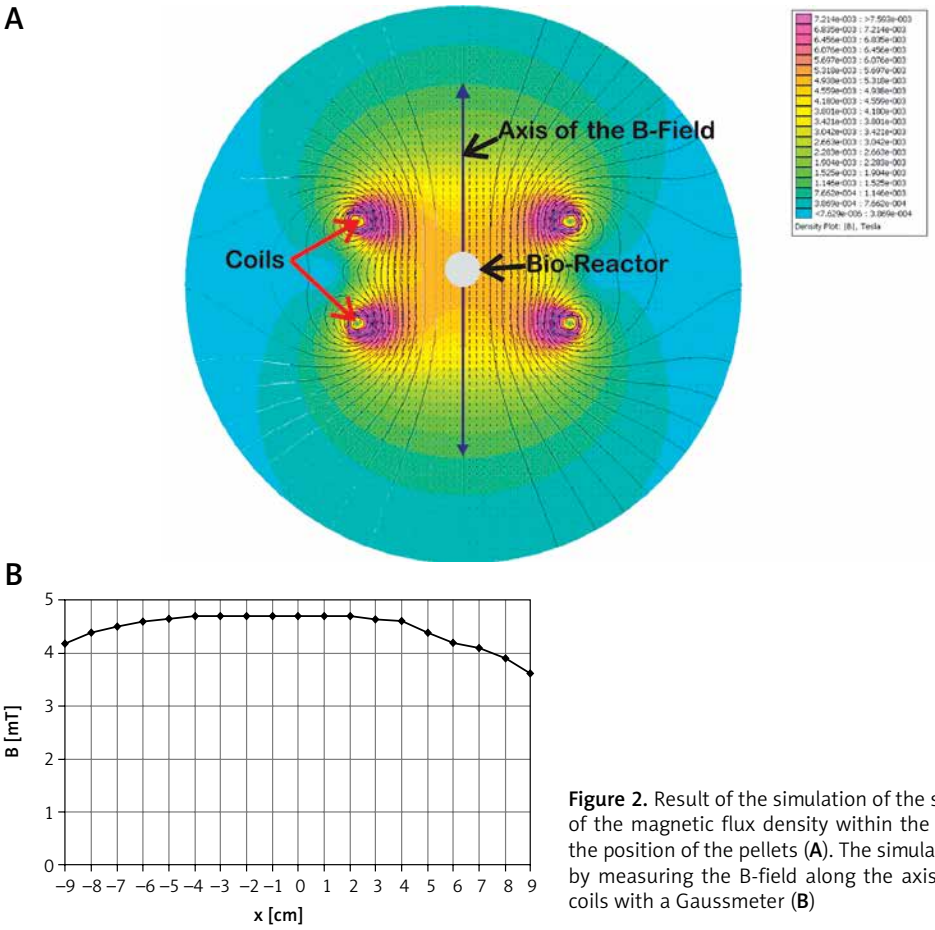

Figure 2. Result of the simulation of the spatial distribution of the magnetic flux density within the Helmholtz coils in the position of the pellets (**A**). The simulation was validated by measuring the B-field along the axis of the Helmholtz coils with a Gaussmeter (**B**)

anin-O staining in the centre and uniform staining for collagen type II (Figure 3).

Microarray analysis

Low-dose LF-EMF was applied to exert non-thermal cell stress on hMSCs during chondrogenic differentiation. Microarray analysis of hMSC cultures exposed to LF-EMF was used to analyse the gene expression pattern and to provide a better understanding of LF-EMF mechanisms. At passage 5 under optimal conditions, the microarray analysis provided no significant changes in gene expression caused by LF-EMF treatment. Rigorous control of input RNA and microarray array data showed optimal quality. The microarray findings were consistent with the findings obtained by RT-PCR and our results obtained from former studies using optimal chondrogenic differentiation conditions [14]. The following link was created to allow review of records: GSE57298:http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=srqhyuourzahzgp&acc=GSE57298.

Quantitative real-time PCR analysis

Chondrogenic differentiation was confirmed in cultures supplemented with TGF-β_3 by RT-PCR for COL2A1 and aggrecan. All results were normalized to control cultures treated with TGF-β_3. Untreated pellet cultures without growth factors did not express any of the chondrogenic markers (data not shown) and were therefore not used as normalizing controls.

The SMG significantly reduced COLXA1 expression compared to control cultures treated with TGF-β_3 (Figure 4). The application of SMG also significantly decreased the expression of COL2A1 and aggrecan. Pure LF-EMF showed no significant effect on the expression of COL2A1, aggrecan and COLXA1 compared to the control as described by gene expression analysis. LF-EMF/SMG showed significantly higher COL2A1 expression compared to SMG, but did not reach control levels. LF-EMF/SMG showed an insignificant increase of COLXA1 expression compared to SMG. The COL2A1/COLXA1 ratio reached comparable values for control conditions and LF-EMF application, followed by LF-EMF/SMG and then SMG (Figure 4).

Discussion

A bioreactor system was applied, which makes it possible to examine the effect of LF-EMF and SMG on *in vitro* chondrogenesis of hMSCs in 3D

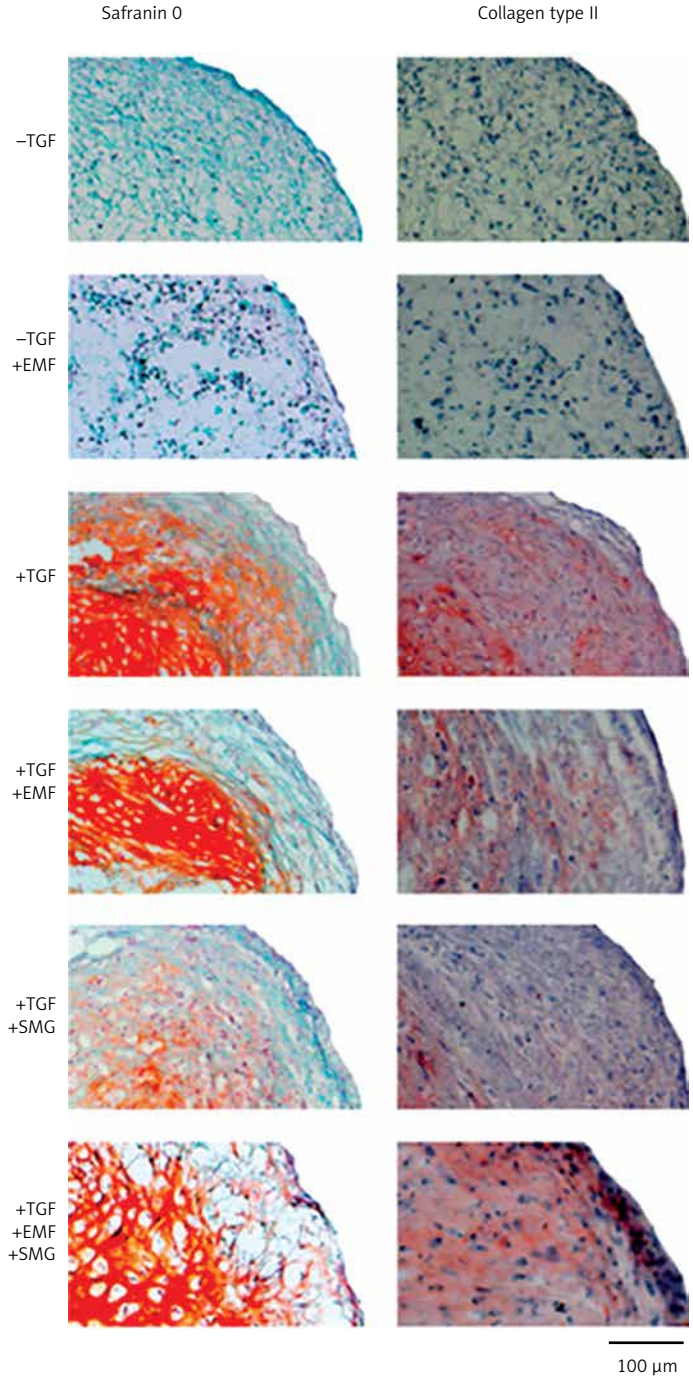

Figure 3. Safranin-0 staining and immunohistochemistry for collagen type II of pellet cultures treated with no additional growth factor (–TGF); no additional growth factor in the presence of electromagnetic fields (–TGF +LF-EMF), TGF-β_3 alone (+TGF); TGF-β_3 in the presence of electromagnetic fields (+TGF +LF-EMF); TGF-β_3 in combination with electromagnetic fields under simulated microgravity conditions (+LF-EMF +TGF +SMG) and TGF-β_3 under simulated microgravity conditions (+LF-EMF +SMG)

culture separately or combined under controlled conditions.

Single LF-EMF showed no effect on gene expression. Single SMG decreased the expression of COLXA1 and COL2A1. LF-EMF/SMG resulted in a significantly higher expression of COL2A1 when compared to SMG. LF-EMF/SMG insignificantly increased COLXA1 when compared to SMG. Altogether, the combination therapy LF-EMF/SMG was not significantly superior to control culture levels treated with growth factors. However, LF-EMF/SMG rescued the COL2A1 expression, which had been lowered by SMG.

Regarding the literature, space flights alter bone metabolism, resulting in osteoporosis [27]. Osteoblasts have been shown to change expres-

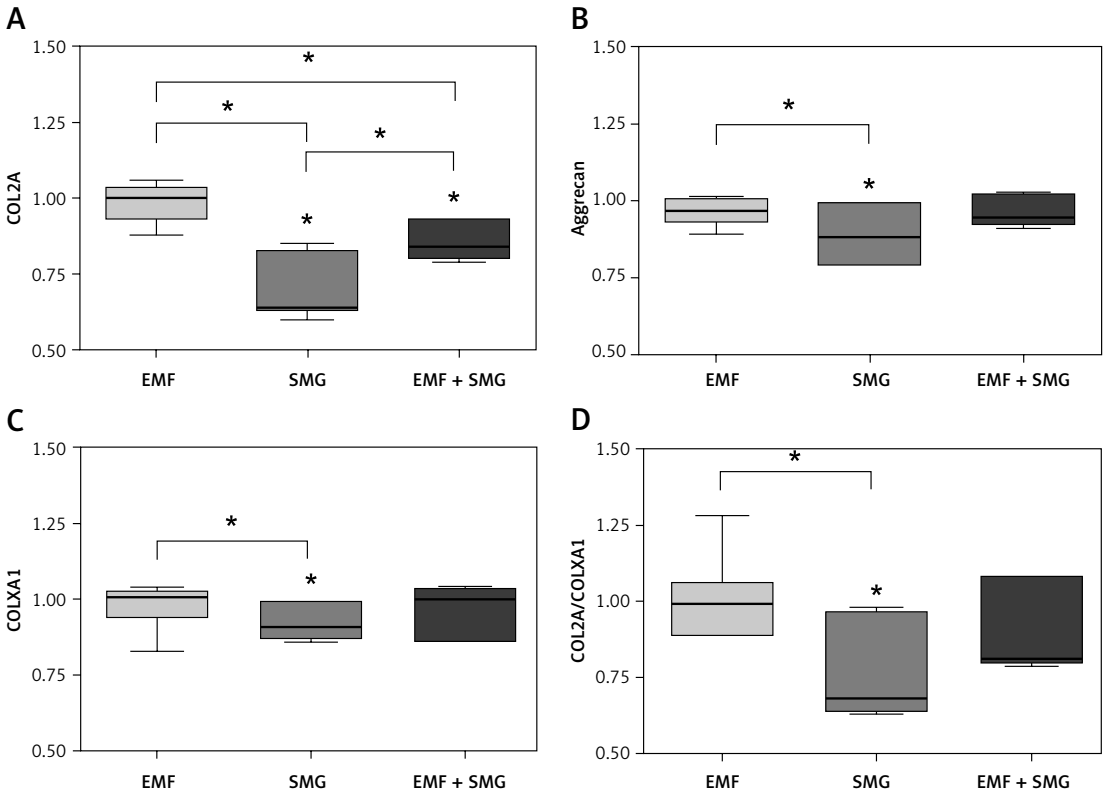

Figure 4. Expression of mRNA for COL2A1, COLXA1, aggrecan and the COL2A1 to COLXA1 ratio in pellet cultures (hMSCs from passage five) treated with TGF-β_3 and cultured under microgravity conditions (+SMG), under microgravity conditions treated with LF-EMF (+SMG +LF-EMF) or stimulated with pure LF-EMF (+LF-EMF). All levels were normalized to control cultures treated with TGF-β_3. Message levels of RNA preparations were analyzed by qRT-PCR and normalized to mRNA levels for GAPDH

sion levels under SMG [28]. Microarray experiments showed lower osteogenic gene expression of hMSCs seeded onto gelatin microcarriers under SMG compared to static culture after 1 week [12]. In our controlled 3D *in vitro* study, one week of SMG was shown to be effective in reducing hypertrophy, which is a first step during terminal ossification of hMSCs [9].

Studies investigating the effects of SMG on chondrogenesis do not provide consistent results. Results range from increased collagen type II expression in rabbit MSCs after 4 weeks of SMG in a conventional direct motor gear RCCS [29] to reduced collagen type II expression of porcine chondrocytes when using a random positioning machine [11]. Comparing SMG to real microgravity (RM), lower collagen type II expression in chondrocytes was observed under RM conditions [11, 30]. It therefore cannot be excluded that EMF effects reported in the literature were induced by direct power units, which might have influenced the SMG results. To obtain controlled study conditions, it is necessary to exclude inadvertent EMF effects by a direct motor gear in SMG studies. In this study, under controlled conditions, one week of SMG induced a significant reduction of COL2A1 expression.

Electromagnetic fields applications result in divergent data regarding cartilaginous cells [13, 16]. Electromagnetic fields were shown to limit progression of OA in animal models [31–33]. It was reported that EMF counteracted IL-1β activity during chondrogenesis [34]. Electromagnetic fields have also been reported to stimulate growth factor synthesis of TGF-β_1 [35]. In this study LF-EMF had no effect on chondrogenesis under optimal differentiation conditions, which has been reported by our group previously [15]. Furthermore, we could show that LF-EMF application did not result in any gene expression changes of hMSCs during chondrogenic differentiation as examined by microarray analysis. This is of major importance as previous reports have linked EMF with an increased risk of carcinogenesis [16–18] and there is a growing concern in public health regarding EMF [36]. In this study, three weeks of LF-EMF did not cause any inadvertent gene expression changes. However, we cannot exclude that gene expression

changes might occur during EMF treatment under modified conditions.

The combined LF-EMF/SMG application resulted in a COL2A1/COLXA1 ratio that was not significantly different from control cultures treated with TGF-β_3. This implies that the combination therapy was not more effective than plain growth factor application. All parameters (time, shear stress, electromagnetic field) were chosen according to optimal results obtained for hypertrophy (SMG) and chondrogenesis (LF-EMF) regarding single applications [9, 15]. We therefore cannot recommend LF-EMF/SMG treatment in regenerative medicine for improvement of cartilaginous cells. Nevertheless, further studies are necessary to optimize the LF-EMF/SMG parameters and find a combination therapy which might render LF-EMF/SMG useful for tissue engineering.

However, LF-EMF/SMG treatment significantly increased the chondrogenic potential of hMSCs compared to SMG conditions. There was a rescue-LF-EMF effect observed, which was dependent on the effects resulting from SMG treatment. We had demonstrated similar findings of LF-EMF effects under suboptimal cellular conditions where LF-EMF effects were only observed in hMSCs with a lowered differentiation potential [15]. As LF-EMF alone did not show any effect on chondrogenesis, the LF-EMF/SMG effect is probably linked to the altered cellular situation induced by SMG. Increased growth factor synthesis [35] or anti-inflammatory effects of A_{2A} and A_3 adenosine receptors [37], as described for EMF, might be a reason for a re-improvement of the chondrogenic potential. EMF have also been proven to reduce the expression of pro-apoptotic and increase the expression of anti-apoptotic proteins [38]. Moreover, it has been reported that EMF are able to delay cellular senescence mediated by heat shock proteins [39]. Recent data show that EMF might even be able to mediate cell reprogramming into a pluripotent state [40]. Therefore, there are various possible explanations for the influence of EMF on hMSCs during chondrogenic differentiation. Altogether, EMF seems to act on hMSCs regarding differentiation in the form of a rescue effect, which is only observed under suboptimal cellular conditions.

In conclusion, LF-EMF did not cause any adverse effects during chondrogenic differentiation. The applied combination therapy of LF-EMF/SMG was not significantly superior to control conditions and therefore does not appear to be suitable for cartilage tissue engineering of hMSCs. However, under LF-EMF/SMG there was a rescue effect of LF-EMF observed regarding the chondrogenic potential of hMSCs. Further studies are necessary to improve LF-EMF/SMG combination therapies for hMSCs and evaluate the exact mechanism of LF-EMF rescue.

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Conflict of interest

The authors declared no conflicts of interest.

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RESEARCH ARTICLE Open Access

Effect of electromagnetic fields on human osteoarthritic and non-osteoarthritic chondrocytes

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Background: Studies of the effects of electromagnetic fields (EMFs) on cartilaginous cells show a broad range of outcomes. However EMFs are not yet clinically applied as standard treatment of osteoarthritis, as EMF effects are showing varying outcomes in the literature. The aim of this study was to examine effects of EMFs (5 mT or 8 mT) on osteoarthritic (OA) and non-OA chondrocytes in order to investigate whether EMF effects are related to chondrocyte and EMF quality.

Methods: Pellets of human OA and non-OA chondrocytes were exposed to a sinusoidal 15 Hz EMF produced by a solenoid. Control groups were cultivated without EMF under standard conditions for 7 days. Cultures were examined by staining, immunohistochemistry and quantitative real-time PCR for RNA corresponding to cartilage specific proteins (COL2A1, ACAN, SOX9).

Results: OA chondrocytes increased the expression of COL2A1 and ACAN under 5 mT EMF compared to control. In contrast no changes in gene expression were observed in non-OA chondrocytes. OA and non-OA chondrocytes showed no significant changes in gene expression under 8 mT EMF.

Conclusion: A 5 mT EMF increased the expression of cartilage specific genes in OA chondrocytes whereas in non-OA chondrocytes no changes in gene expression were observed. An 8 mT EMF however showed no effect altogether. This suggests that EMF effects are related to EMF but also to chondrocyte quality. Further studies about the clinical relevance of this effect are necessary.

Keywords: Electromagnetic field, Human chondrocytes, Osteoarthritis, Real-time PCR, Cartilage specific genes

Background

Osteoarthritis (OA) refers to a syndrome of joint pain accompanied by functional limitation and reduced quality of life. OA has a high prevalence, which is expected to increase due to the aging population and currently affects over 40 million Europeans [1]. It is one of the leading causes of pain and disability in everyday life worldwide. The severity of knee and hip OA disability has been associated with a significant increase in all-cause mortality and serious cardiovascular disease events due to the avoidance of physical activities that exacerbate symptoms [2]. The socioeconomic impact is very high due to the enormous and dramatically escalating burden of this disease [3]. As there is no cure for OA, a multimodal pharmacologic and non-pharmacologic approach followed by joint replacement is recommended by most guidelines. However, non-invasive multimodal approaches often only result in short term improvement or fail altogether. Additional strategies are needed to treat pain and improve joint function while minimizing side effects. Biological effects of electromagnetic fields (EMFs) in tissues were first described by Fukada and Yasuda [4], who investigated the piezoelectric phenomenon in bone. Electric and electromagnetic potentials were found to not only affect osteogenesis but also have chondroprotective properties [5]. In animal studies EMFs reduced knee OA lesions [6] and have the ability to antagonize the catabolic activity of cytokines in cartilage explants [5, 7, 8]. Other studies indicate that EMFs may affect intracellular calcium concentration triggering proteoglycan synthesis

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[9, 10]. EMF therapy has been shown to partially reduce pain and improve mobility for the treatment of OA as compared to placebo [2, 4, 11]. However, the question whether EMFs should be used as standard therapy for the treatment of OA remains open.

Experimental studies on EMFs are inconsistent with regard to electromagnetic fields, effector cells or tissues and effector responses. Two current reviews follow the same search approach and analyzed the effect of EMF for the treatment of OA as compared to placebo [2, 4]. The question remains whether EMF is generally a form of therapy suitable for everybody, or whether there are specific patients who might profit from a specific form of EMF therapy. Previous studies have shown that EMFs increase the chondrogenic potential of human mesenchymal stem cells (hMSCs) during chondrogenic differentiation [12]. Most astonishing was the fact that positive EMF effects occurred here only under suboptimal and not under optimal cellular conditions [12]. It is not known whether the suboptimal chondrocytes seen in OA respond differently to EMF than healthy chondrocytes. The aim of this study was to compare the effects of EMFs on OA versus non-OA chondrocytes. To our knowledge this is the first approach to specifically address this question. We further investigated whether the effect could be indeed by any EMF or whether a specific EMF is necessary. We hypothesized that specific EMFs would show more effects on degenerated osteoarthritic than on non-osteoarthritic chondrocytes.

Methods

Cell culture

Human OA articular cartilage was obtained from adult patients (patient 1-7) during total knee replacement (n = 7) (1 male, 6 female; mean age 67; range 58 - 85). Non-OA articular cartilage was obtained from young patients (patient 8-13) during triple arthrodesis performed as salvage procedure for refactory clubfoot (n = 3) or from human knee condyles *of deceased patients after trauma devoid of involvement* of the knee harvested within 12 h of death (n = 3) (together 5 male, 1 female; mean age 15; range 13 - 20) (Fig. 1). The study was approved by the Ludwig-Maximilians-University medical center ethics committee. Chondrocytes were isolated by enzymatic treatment using pronase (Roche Diagnostics, Mannheim, Germany) and collagenase (Sigma-Aldrich, St. Louis, MO) and cultured at 37 °C in a humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM)/Hams F-12 (1:1; Biochrom, Berlin, Germany) containing 10% fetal calf serum (PAA, Pasching, Austria), 1% MEM–amino acids (Biochrom, Berlin, Germany), 25 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), 50 IU/ml penicillin–streptomycin and 0.25 μg/ml Amphotericin B (Biochrom, Berlin, Germany). The medium was changed every second day. At 100% confluence, cells were passaged using 0.05% Trypsin containing 0.02% EDTA (Biochrom, Berlin, Germany) and were expanded in monolayer triple flasks (Nunc, Rosklide, Denmark). Experiments were performed with chondrocytes at passage 2. At day zero, 4×10^5 chondrocytes were lysed in 1 ml TRIzol Reagent (Life Technologies, Carlsbad, CA) and kept at −80 °C for further RNA-analysis. To form pellet cultures (n = 10 for each patient), 4×10^5 cells were centrifuged at 150 g for 5 min in a 15 ml polypropylene tube (TPP, Trasadingen, Switzerland). All pellet cultures were cultured for 48 h under the same conditions as mentioned above to allow pellet formation. Experiments were started by transferring pellets into 1.5 ml reaction tubes (Eppendorf, Hamburg,

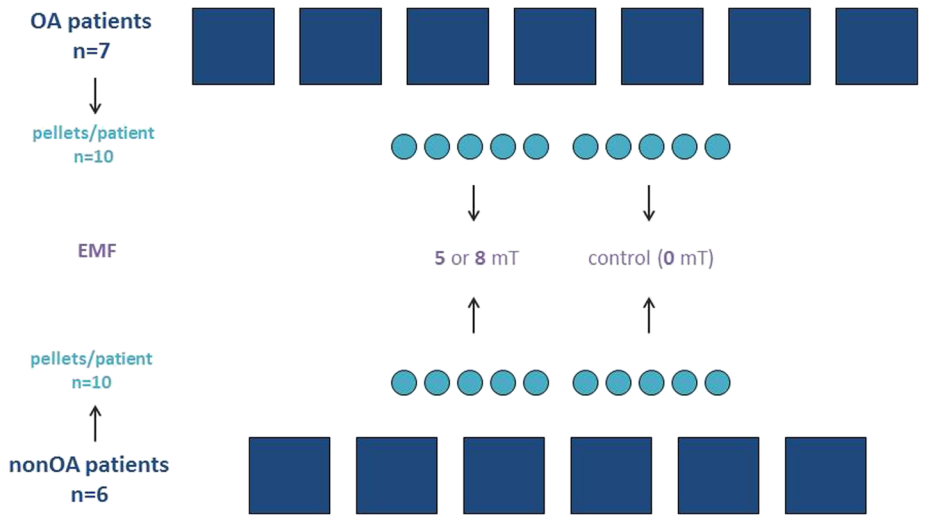

Fig. 1 Chart of the experiment with 7 OA patients and 6 nonOA patients. During the experiment 5 pellet cultures per patient were exposed to an EMF (5 mT or 8 mT). As control group 5 pellet cultures from the same patient were cultivated in a second incubator without a solenoid for 7 days

Germany), containing 1 ml of medium and sealed by breathe easy sealing membranes (Sigma-Aldrich, Steinheim, Germany). During the experiment 5 pellet cultures per patient (3 pellets for RNA-analyses and 2 pellets for histology and immunohistochemistry (IHC)) were exposed to EMF (5 mT or 8 mT) respectively within a solenoid. The respective control pellet cultures (n = 5) from the same patient were cultivated in a second incubator without a solenoid for 7 days, established as control [12] (Fig. 1). All experiments were performed for 7 days, with media being changed every second day.

Low frequency sinusoidal EMF

EMFs were generated with a solenoid (FA-P6-K, Neue Magnetodyn, Munich, Germany) combined with a frequency generator (M80, Neue Magnetodyn, Munich, Germany) as described by Mayer-Wagner et al. [12]. For experiments, pellets were cultured in 1.5 ml Eppendorf-Safe-Lock tube (Eppendorf, Hamburg, Germany) fixed in a tube-rack inside the coil. To allow gas-exchange tubes were closed with a permeable sealing membrane (Sigma-Aldrich, Steinheim, Germany). The EMF waveform was measured with a teslameter (Bell 640, F.W. Bell, Orlando, FL) inside the coil at the position of the pellet cultures.

In this configuration, the magnetic field is in parallel to the long axis of the base of the tube-rack. The electric field induced by the magnetic field in the plane of the pellets can be calculated with good approximation by the following equation: $E_{ymax} = 2h\pi fB_x\pi f$ [13], where B is the peak value of the magnetic flux density, fis its frequency and h is the height of the pellets. With the given values the maximum induced electric field is below 2 mV/m.

The particular waveform, amplitude and sinusoidal frequency of 15 Hz have been established previously in vivo [14, 15] and in vitro [12, 16, 17]. The homogeneity of the magnetic field within the solenoid was assured by measuring the spatial distribution of the magnetic flux density with a teslameter. The geomagnetic field in the incubator was determined to be 45 μT parallel to the rotation axis of the solenoid. The field (either 5 mT or 8 mT) was applied every 8 h for 45 min during the course of the 7 day experiment. The waveform was a pure sinusoidal wave with a total harmonic distortion <1%. The 8 mT field was applied in order to use an unspecific field, which has so far not been shown in the literature to induce effects but is still close to the 5 mT field described to produce an effect.

Histology

Pellet cultures (2 with EMF treatment and 2 without treatment for each patient) were washed in phosphate buffered saline (PBS) pH 7.4, incubated in 5% sucrose in PBS (21 °C, 15 min), dry embedded in Tissue-Tek (Sakura, Zoeterwoude, Netherlands) and frozen at −20 °C. Serial cryosections (8 μm) were prepared by mounting pellet cultures on SuperFrost glass slides (Menzel-Gläser, Braunschweig, Germany). Cryosections were fixed in acetone (AppliChem, Darmstadt, Germany) and dried at room temperature (RT). Serial sections were stained in triplicate with 0.75% safranin-O (Fluka, Buchs, Switzerland) and 0.02% fast green (Chroma, Münster, Germany).

Immunohistochemistry

Pellet sections were fixed in acetone (AppliChem, Darmstadt, Germany) (10 min, RT) and washed in washing buffer (PBS with 0.2% 5 Brij L23 Solution (Sigma-Aldrich, Steinheim, Germany)). After blocking endogenous tissue peroxidase activity with 0.3% H_2O_2 (Merck, Darmstadt, Germany) in aqua dest (4 min, RT) pellet sections were treated with 0.25 U/ml chondroitinase AC from *Flavobacterium Heparium* (Sigma-Aldrich, Steinheim, Germany) in PBS (30 min, 37 °C) followed by washing in washing buffer. Afterwards pellet sections were incubated with monoclonal mouse antibodies (mABs) against collagen type I (Sigma-Aldrich, Saint Louis, MO) diluted in 1:2000 in mAB-dilution-soluion (DCS, Hamburg, Germany) or collagen type II (DSHB, University of Iowa, IA) 1:6 in mAB-dilution-solution (30 min, RT). For negative controls the mABs were omitted. Pellet sections were washed in washing buffer and then incubated with a biotinylated horse-anti-mouse IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in mAB-dilution-solution (30 min, RT). Afterwards pellet sections were incubated with avidin-biotin-peroxidase-complex (ABC) (Vector Laboratories, Burlingame, CA). After repeated washing, the bound mABs were visualized with 3.3 diamino benzidine tetrahydrochlorhide (DAB) (Vector Laboratories, Burlingame, CA) for approximately 4 min without light. The reaction was stopped with aqua dest. And the sections were counterstained with haematoxylin (AppliChem, Maryland, CA) and embedded in Eukitt (O-Kindler, Freiburg, Germany). Representative images were obtained using a PreciPoint M8 microscope (PreciPoint, Freising, Germany) (Fig. 2).

Visual histological grading system

The morphology of pellet cultures was graded according to a visual histological grading system for generated neocartilage [18]. Two independent and blinded observers evaluated each pellet in 3 categories described below and assigned scores ranging from 0 to 3. Categories were added with equal weight to give a total score (Table 1).

Category A: Intensity of safranin-O staining. Each sample was observed with a 10× objective.

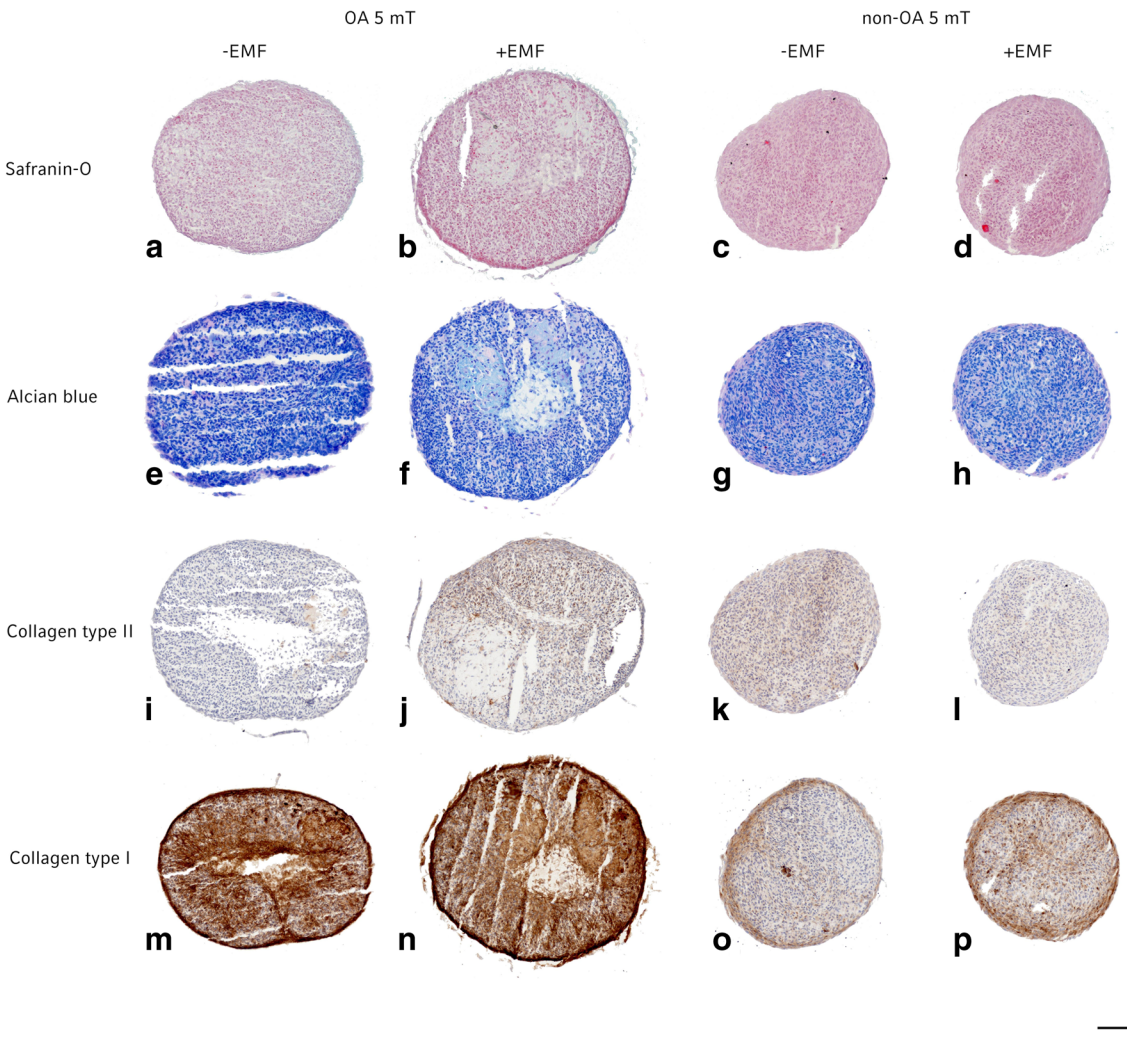

Fig. 2 Histology and Immunohistochemistry (IHC) staining of one OA-patient and one non-OApatient pellet cultures after 5 mT EMF exposition (**b, f, j, n, d, h, l, p**) or without EMF exposition (**a, e, i, m, c, g, k, o**). Safranin-O staining (**a, b, c, d**) and alcian blue staining (**e, f, g, h**) was positive for all pellet cultures. IHC for collagen type II was much more obvious for OA pellet culture after EMF exposition (**j**) and non-OA pellet cultures with and without EMF exposition (**k, l**). IHC for collagen type I of OA pellet cultures (**m, n**) showed a weaker staining than non-OA pellet cultures (**o, p**)

Table 1 Visual histological grading system

	A	B	C	total
OA				
EMF 0 mT	1	1	1	3
EMF 5 mT	2	2	1	5
EMF 8 mT	1	1	1	3
Non-OA				
EMF 0 mT	2	2	3	7
EMF 5 mT	2	2	3	7
EMF 8 mT	2	2	3	7

Three categories were evaluated: **A** intensity of safranin-O staining, **B** distance between cells/amount of matrix that was accumulated and **C** cell morphologies represented

Category B: Distance between cells and amount of matrix produced by chondrocytes were assessed with a 20× objective.

Category C: Cell morphology was examined using a 40× objective. A rounded morphology was expected for chondrocytes and the presence of pyknotic or fibroblast morphology was scored as poor cell quality.

RNA isolation

Cell pellets were disrupted under frozen conditions with 3000 rpm for 1 min, using a Micro-Dismembrator S (Sartorius, Göttingen, Germany). Total RNA was isolated directly from freeze-milled preparations, using 1 ml QIAzol Lysis Reagent (Qiagen, Hilden, Germany).

After addition of 0.2 ml chloroform (Sigma-Aldrich, Steinheim, Germany), samples were shaken and incubated at RT for 10 min. For phase separation, samples were centrifuged at 15000 g for 20 min at 4 °C and the aqueous phase was transferred to a fresh tube.

Total RNA precipitation was performed by mixing 0.5 ml Isopropanol (Sigma-Aldrich, Steinheim, Germany) with the aqueous phase. After incubation at RT for 10 min samples were centrifuged at 15000 g over night at 4 °C. RNA pellets were washed twice with 1 ml 75% Ethanol (Merck, Darmstadt, Germany) and centrifuged at 15000 g for 20 min at RT. After drying pellets, total RNA was dissolved in 32 μl RNAse free water (Gibco, Darmstadt, Germany). Concentration and purity was determined by Nanodrop (ND-1000, Thermo Fisher, Waltham, MA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

For cDNA synthesis, 0.5 μg total RNA from each pellet culture (3 with EMF treatment and 3 without treatment for each patient) was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Quiagen, Hilden, Germany). QRT-PCR was performed using a LightCycler 96 System (Roche Diagnostics, Mannheim, Germany). Each reaction contained 5 μL of FastStart Essential DNA Green Master Mix (Roche Diagnostics, Mannheim, Germany), 2.5 μl of 1:3 diluted cDNA and 0.3 μl (300 nM), for test genes or 0.5 μl (500 nM), for reference gens of primer in a 10 μl final volume.

The following primers were used: Glycerinaldehyd-3-phosphat-dehydrogenase (*GAPDH*) [19], collagen type II α_1 chain (*COL2A1*) [20], collagen type 1 α_1 chain (*COL1A1*) [21], cartilage-specific proteoglycan core protein (*ACAN*) [20] and SRY (sex determining region Y)-box 9 (*SOX9*) [20] (Table 2).

Thermal cycling parameters included a denaturation step for 10 min,40 cycles of 95 °C for 10 s, 60 °C /65 °C for 10 s and 72 °C for 15 s. Reactions for each pellet were performed in triplicate and the mean of relative quantification values was calculated by using the delta-delta Ct method [22] with *GAPDH* as the reference gene. Box-plots (Fig. 3) of cartilage specific genes and *COL1A1* were established by normalizing qRT-PCR data of pellets with 5 and 8 mT EMF treatment to the mean of control pellets without EMF treatment.

Table 2 Gene sequences of primer pairs and annealing temperatures

Gene	Sequence	Annealing temperature
GAPDH forward	TGC ACC ACC AAC TGC TTA GC	60 °C
GAPDH reverse	GGC ATG GAC TGT GGT CAT GAG	60 °C
COL2A1 forward	GTT ATC GAG TAC CGG TCA CAG AAG	65 °C
COL2A1 reverse	AGT ACT TGG GTC CTT TGG GTT TG	65 °C
ACAN forward	CAG CAC CAG CAT CCC AGA	65 °C
ACAN reverse	CAG CAG TTG ATT CTG ATT CAC G	65 °C
COL1A1 forward	TGA CCT CAA GAT GTG CCA CT	65 °C
COL1A1 reverse	ACC AGA CAT GCC TCT TGT CC	65 °C
SOX9 forward	AGA CCT TTG GGC TGC CTT AT	60 °C
SOX9 reverse	TAG CCT CCC TCA CTC CAA GA	60 °C

Statistical analysis

All RNA variables were described graphically by box-and-whisker plots and numerically using appropriate measures of location and dispersion. Due to the positive skew of their distributions of our outcome variables, we used the decadic logarithms instead of the original values as dependent variables in our models. The appropriateness of the log transformation was checked graphically using the residual plots for the resulting models. The effects of diagnosis, electromagnetic field strength, and their interaction with the log-transformed outcome variables were explored by means of mixed effects model with a random intercept per patient using the MIXED procedure of the Statistical Analysis System SAS, version 9.4 for Windows (SAS Institute, Cary, NC, USA). $P < 0.05$ were regarded as statistically significant.

Results

Histology and immunohistochemistry

Positive staining for safranin-O and alcian blue were found in all pellet cultures. All pellet cultures showed signs of collagen type II and type I with differences in density. Representative images of pellet cultures are shown in Fig. 2 with pellet sections from one OA patient cultured without EMF (Fig. 2a, e, i, m) and one cultured with 5 mT EMF (Fig. 2b, f, j, n). Figure 2 also shows pellet sections from one non-OA patient cultured without EMF (Fig. 2c, g, k, o) and one with EMF 5 mT (Fig. 2d, h, l, p).

Comparing pellet cultures of non-OA to OA chondrocytes, non-OA cultures showed a more homogenous and compact structure than pellets from OA chondrocytes. A stronger staining for collagen type I was observed in OA cultures (Fig. 2m, n). OA cultures treated with specific 5 mT EMF showed a similar staining for collagen type II to non-OA cultures (Fig. 2j, k, l) whereas untreated OA pellets showed less staining for collagen type II (Fig. 2i).

OA pellets treated with 8 mT showed no differences in staining compared to the control OA group without EMF (data not shown).

No differences in staining intensity for collagen type II and collagen type I were found for pellet cultures of non-OA chondrocytes which had been treated with

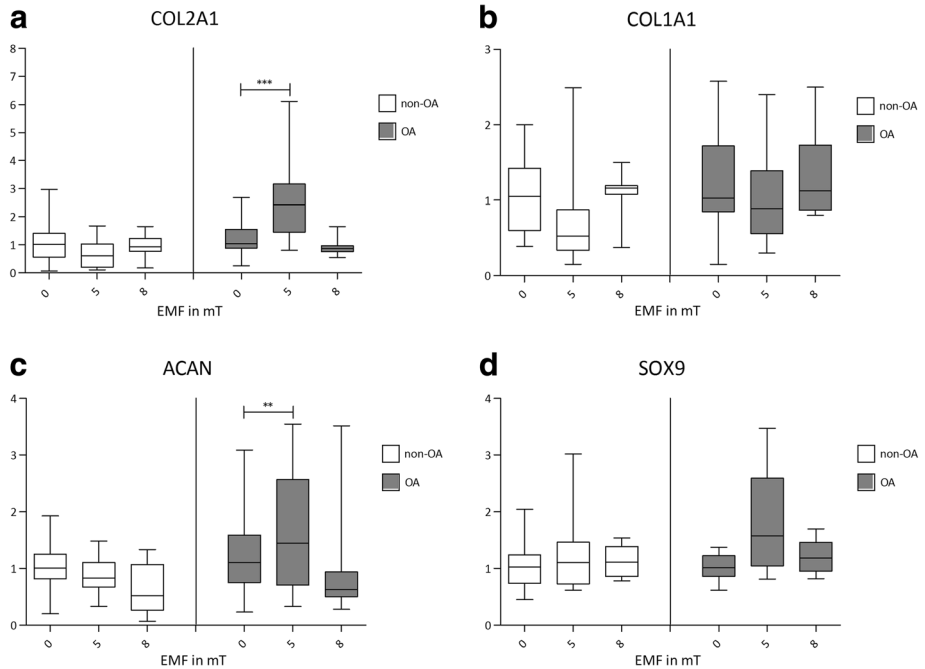

Fig. 3 Alteration of normalized gene expression of COL2A1 (**a**), COL1A1 (**b**), ACAN (**c**) and SOX9 (**d**) to GADPH expressed by pellets of OA (n = 12 with 5 mT and n = 9 with 8 mT) and non-OA patients (n = 9 with 5 mT and 8 mT) after 7 days in a 5 mT or 8 mT EMF normalized to the respective control group without (0 mT) EMF exposition

EMF (5 mT/8 mT) versus the control non-OA group (5 mT data in Fig. 2; 8 mT data not shown).

Visual histological grading

Table 1 shows results of the visual histological grading system. Pellets from OA patients with 5 mT EMF treatments had a higher score in category A and B than in the OA control group without EMF treatment. The pellets from OA patients treated with 5 mT EMF did not reach the scores from pellets of non-OA patients. OA pellets treated with 8 mT showed the same score as the untreated controls. Pellets from non-OA patients showed no difference in all three categories (0 mT/5 mT/8 mT) and presented the best cartilage-like cell morphology with strong staining for collagen type II, formation possessed the rounded morphology typical of chondrogenic cells and are surrounded by an extracellular matrix (Table 1).

Quantitative real-time PCR

All pellet cultures from OA patients and non-OA patients expressed the cartilage specific markers *COL2A1*, *ACAN* and *SOX9* mRNA and the unspecific *COL1A1* mRNA (Fig. 3a-d).

OA cultures from the 5 mT EMF treatment group presented with significantly higher expression ($p < 0.001$) of COL2A1 and ACAN ($p < 0.01$) than the OA control group with 0 mT EMF treatment (Fig. 3a, c). In contrast, no changes in gene expression were observed in pellets from non-OA patients.

OA cultures treated with 8 mT EMF showed also no significant differences in gene expression compared to OA control cultures with 0 mT EMF treatments (Fig. 3a-d).

No significant changes in gene expression were observed for pellets from non-OA patients treated with or without 8 mT EMF (Fig. 3a-d).

Discussion

Effects of 5 mT and 8 mT EMF on OA and non-OA chondrocytes were examined in a 3D culture system. The 5 mT EMF increased *COL2A1* and *ACAN* gene expression of OA chondrocytes. The 8 mT EMF did not show any effects on OA and non-OA chondrocytes gene expression. The effects of EMFs on chondrocytes are inconsistent in the literature [4, 23]. A specifically beneficial EMF is difficult to characterize as in the literature electromagnetic fields with a broad range of intensity have been used in similar settings. EMFs in the range from 0.5 up to 5 mT seem to generate positive results on cartilage [11]. Within this study a 5 mT EMF, which had previously been shown to exert beneficial effects on the chondrogenic differentiation of hMSCs [12], was examined versus an EMF of 8 mT. No effects of the 8 mT EMF on gene expression was observed in OA nor in non-OA chondrocytes. It can therefore be postulated that although there is a field spectrum for beneficial effects of EMF on cartilaginous

cells, it is possible to reduce EMF effects by slight adjustments to electromagnetic field.

As EMF effects depend very much on multiple parameters including frequency, magnetic field peak amplitude as well as exposure length [24] we were interested whether EMF effects also vary with on cell quality. To our knowledge, studies comparing EMF effects on human OA to non-OA cartilage are lacking. In previous published studies EMFs were applied to OA cartilage explants, where they were shown to increase proteoglycan synthesis after 7 days [7]. EMF effects were also examined in bovine articular cartilage explants, where an EMF (2.3 mT, 60 Hz), increased proteoglycan synthesis both under basal conditions and in the presence of interleukin-1 [24]. Positive effects of 1, 2 and 3 mT 60 Hz EMFs on the expression of cartilage specific genes and glycosaminoglycan synthesis were also observed in non-OA bovine chondrocytes [8]. When particular parameters were used, EMFs were even able to prevent degenerative changes in cartilage explants with a potency almost reaching low-intensity pulsed ultrasound research [25].

During this study EMF effects on human OA and non-OA chondrocytes were cleary divergent. The application of 5 mT EMF resulted in an upregulation of *COL2A1* and *ACAN* gene expression in OA chondrocytes. In contrast to OA chondrocytes, non-OA chondrocytes did not seem to significantly profit from EMF.

Previoussimilar results were shown in a study of hMSCs where EMF effects were only observed under suboptimal cell culture conditions and EMF effects were dependent on the cellular quality of the hMSCs [12]. Why EMF stimulation is mainly effective in degenerated or suboptimal chondrogenic cells remains to be answered. Variable reasons are possible to explain the stronger influence of EMF on degenerated chondrocytes.

Regarding the pathogenesis of OA adenosine receptors (ARs), A_{2A} and A_3 play an important role in the anti-inflammatory pathway by decreasing expression of nuclear factor κB (NF-κB) a transcriptional factor of inflammatory cytokines such as interleukin-1 beta (IL-1ß), interleukin-6 (IL-6) and tumornecrosis factor alpha (TNF-α) [26]. EMFs have been described to increase anti-inflammatory effects by inducing up-regulation of A_{2A} and A_3 ARs [27, 28].

An other mechanism for a re-improvement of chondrogenic potential through EMF may be increased growth factor synthesis [29]. EMFs act on transforming growth factor beta 1 (TGF-β_1) -growth factor release and may thereby restore the chondrogenic potential of degenerated or suboptimal chondrogenic cells. In addition, EMFs may have anti-apoptotic effects by decreasing the expression of proteins involved in the pro-apoptotic pathway and simultaneously increasing expression of anti-apoptotic proteins [30]. EMF treatment seems to improve suboptimal or degenerate cellular conditions for chondrocytes [31, 32]. In the absence of chondrocyte stress EMFs may be ineffective which may in part explain the lack of EMF effect in some studies. It also needs to be evaluated whether degeneration of tissue or the age of the donor are responsible for the EMF effects. For general statements about degeneration and EMF effects, further studies are necessary to clearly define this mechanism.

Conclusions

The application of 5 mT EMFs has a positive effect on degenerated OA chondrocytes by increasing expression of *COL2A1* and *ACAN*. The effect of 5 mT EMFs on non-OA chondrocytes is markedly less pronounced. 8 mT EMF did not exert an effect on OA or on non-OA chondrocytes. Further studies are necessary to evaluate EMF effects in different settings, which may be a valuable tool in treating OA patients.

Abbreviations

ABC: Avidin-biotin-peroxidase-complex; ACAN: Cartilage-specific proteoglycan core protein; ARs: Adenosine receptors; COL1A1: Collagen type 1 alpha$_1$ chain; COL2A1: Collagen type II alpha$_1$ chain; DAB: 3.3 Diamino benzidine tetrahydrochlorhide; DMEM: Dulbecco's modified eagle's medium; EMFs: Electromagnetic fields; GAPDH: Glycerinaldehyd-3-phosphat-dehydrogenase; hMSCs: Human mesenchymal stem cells; IHC: Immunohistochemistry; IL–1β: Interleukin-1 beta; IL-6: Interleukin-6; mABs: Monoclonal mouse antibodies; NF- κB: Nuclear factor κB; OA: Osteoarthritis; PBS: Phosphate buffered saline; qRT-PCR: Quantitative real-time polymerase chain reaction; RT: Room temperature; SOX9: SRY (sex determining region Y)-box 9; TGF-β1: Transforming growth factor beta 1; TNF-α: Tumornecrosis factor alpha

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

JR, BS, AB, CB, VJ and SM-W designed the experiments; JR, BS, CB and AB performed the experiments; JR, BS and SM-W analysed data; JR, BS, AB, CB, VJ and SM-W wrote the paper. All authors have read and approved the final submitted manuscript.

Ethics approval and consent to participate

The study was approved by the Ludwig-Maximilians-University medical center ethics committee.
Informed consent was obtained if necessary.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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