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From morphological basic research to proposals for regenerative medicine through a translational perspective

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Abstract

Basic research, especially morphological research, often fails to get off the ground due to scarcity of opportunities and funding. There is a need to exploit ideas, while starting from a morphological basis, to channel them into pathways with translational value: patents, trademarks, alternative experimental models, etc., aimed at formulating new proposals for applied research.
Among the many sprouts emerging in laboratories where basic research is carried out throughout the country, one reality from Emilia Romagna region is represented by some insights from human anatomy teachers and researchers at the University of Modena and Reggio Emilia. They have developed an original idea whereby they propose to use very small bone segments (the scleral ossicles –SO) taken from the sclero-corneal boundary of lower vertebrates with protruding eyes (particularly from avian species) to supplement 3D scaffolds to be used in regenerative medicine (by triggering/improving angiogenesis and osteogenesis) for the recovery of severe bone injuries, defined as "critical size", i.e., unable to recover autonomously. The idea was followed by the patent application and, subsequently, the filing of a trademark (Pal-OS®) concerning to SO-derivatives (powders, sticks, caps, etc.).
In times when respect for the natural environment and attention to animal health are among the relevant aspects for an ecosystem's welfare, along with these patent and trademark a focus was developed on experimental methods alternative to animal testing, with which to be able to test the efficacy of the proposed products while respecting the 3 R's rules, using a model already known and exploited in the past, the chorio-allantoic membrane – CAM, revisited today from an ethical perspective.
The article traces the observations that led to the idea of patenting scleral ossicles, of filing the Pal-OS® trademark and of using the CAM model to test their validity for regenerative purposes, with the ultimate goal of underlining how morphological observations, interpreted from a translational perspective, can provide interesting insights for clinical applications.

Keywords

Scleral Ossicles (SO); Pal-OS®; chorioallantoic membrane (CAM) model; angiogenesis; osteogenesis; critical-sized bone lesion recovery.

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A local example at UNIMORE: scleral ossicles, Pal-OS®, CAM and their combination

Scleral ossicles – description and development possibilities

In recent years, with the rapid development of sophisticated bio-molecular techniques and the sharpening of the biotechnology mindset, morphology has undergone slowdown that results in term of scarcity of funding and human resources. To emphasize the importance of morphological studies, our research group, starting from mere morphological observations, proposed the use, in a translational key, of a peculiar natural scaffold, the Scleral Ossicles (SO), for bone regeneration.

SO (Fig. 1) are small bony plates, organized to form a ring of 13-14 elements articulated to each other with sutures, located at the sclero-corneal boundary of the eyeball of lower vertebrates with protruding eyes such as teleosts (Franz-Odendaal and Hall, 2006; Franz-Odendaal, 2008a; Lyon et al., 2017), amphibians, reptiles (Franz-Odendaal, 2006; Presch, 1970; Vieira et al., 2007), and birds (Franz-Odendaal, 2008b; Lima et al., 2009; Zhang et al., 2012), whose aim, at the end of their development, is solely to protect the eye from deformation during flight or swimming (therefore submitted lifelong to stereotyped loading), reason why they should not be subject to bone variations dependent on the body's mineral/metabolic requirements (as is the case for all other skeletal segments). Thus, in order not to undergo bone remodeling, once SO reach the final size, all their osteocytes massively go into apoptosis (Palumbo et al., 2012). Therefore, SO are "naturally decellularized" materials, and it has been shown that they do not induce adverse immune reaction (when transplanted into non-immune-depressed animals) and promote both angiogenesis and osteogenesis (Checchi et al., 2018, 2020). On this basis, and after developing and standardizing an appropriate extraction and preparation method, they were thought to be housed in special slots within 3D scaffolds (Fig. 2), to be inserted into critical bone lesions, to

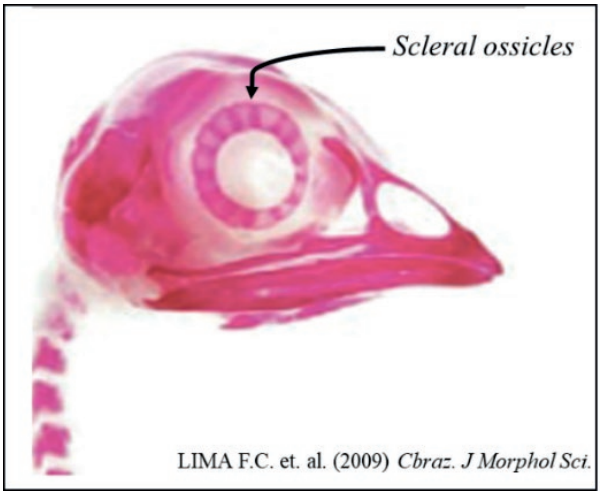

Figure 1. Photograph of the scleral ring in *Gallus gallus domesticus*. The arrow highlights the scleral ossicles.

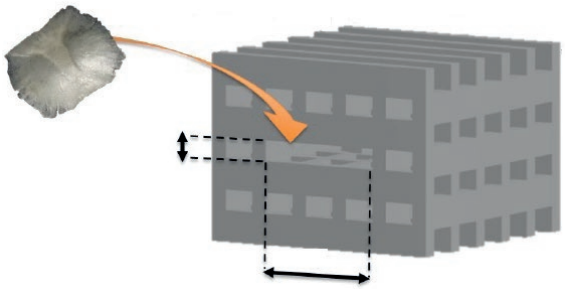

Figure 2. The picture represents a hypothetical 3D scaffold architecture that could host the scleral ossicles in a peculiar slot designed with appropriate dimensions.

trigger an angiogenic and osteogenic response in the host, in order to improve the recovery of critical-sized bone lesions.

In November 2022 (https://www.magazine.unimore.it/site/home/notizie/articolo820058150.html) the University of Modena and Reggio Emilia started the patenting process for the use of Scleral Ossicles (SO).

Pal-OS® – description and development possibilities

Preliminary observations on the realization of prototypes of complex constructs that could accommodate SO suggested, later, that it would be more effective to combine some derivatives of patent-SO to be processed in parallel with the printing of biomaterials that must include them. Thus, a trademark, Pal-OS®, was filed (January 2022)

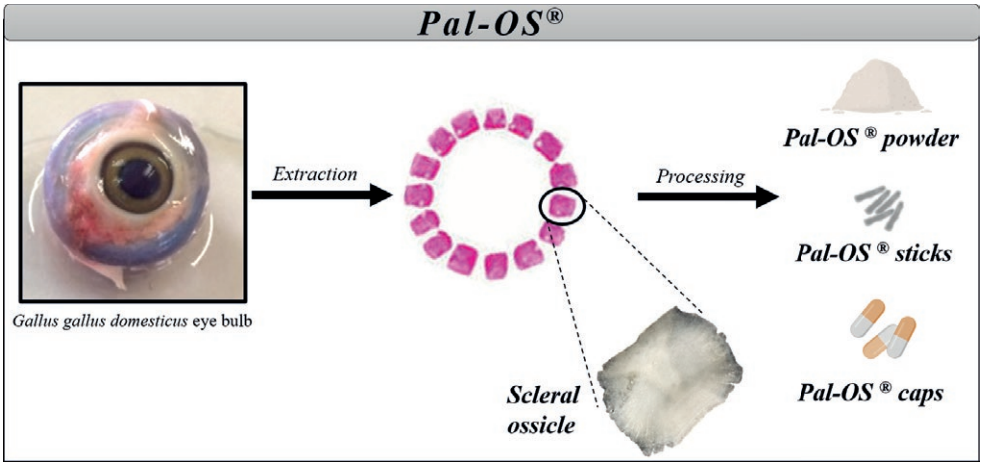

Figure 3. The picture schematically represents the extraction of the scleral ring from the chicken eye bulb. Each scleral ossicle is disjoined and it could be processed in different derivates: Pal-OS® powder, Pal-OS® sticks, or Pal-OS® caps.

denoting any product from comminution of scleral ossicles (powder, filaments, chips, splinters, caps, etc.). The idea is to obtain 3D printed gelatinous biomaterials, such as hydrogels, that include SO derivatives (Pal-OS®), for example Pal-OS® powder sticks (Fig. 3). This will optimize the angio-/osteo-inductive influence of SO on the host bone.

Tests on the application potential of Pal-OS® derivatives are currently in progress and propose the embedding of Pal-OS® within matrices of different nature, that can be customized by the manufacturer in order to adapt perfectly to the size of the bone defect to be to deal. "Critical size" bone defects are fractures which, due to their excessive extension, do not undergo self-regeneration and therefore require a method or therapy that allow complete healing. Essential requirements for the healing of a critical fracture are the restoration of an efficient vascular system and the reduction of the gap between the two bone stumps. Vascularization is an essential preliminary element without which ossification cannot proceed. As mentioned above, SO have been tested for biocompatibility and angiogenic potential *in vitro* and *in vivo* resulting biocompatible and able to induce angiogenesis; therefore, the use of these ossicles would ensure the angiogenic potential necessary for the recruitment of all the elements (cells and cytokines) to initiate the process of bone regeneration (Ferretti and Palumbo, 2021).

Our final goal is to use customized constructs containing Pal-OS® derivatives as triggers for bone regeneration. To do this, 3D printed constructs containing Pal-OS® derivates were tested onto the Chorioallantoic Membrane (CAM) in order to select the best combination between bio-ink and Pal-OS® derivatives in terms of angiogenetic response.

Description and ethical value of CAM

An important aspect of our research activity which deserves attention is performing (after the preliminary *in vitro* tests) *in ovo* experiments by means of Chorioallantoic Membrane (CAM) assay (Fig. 4A). The CAM is a highly vascularized membrane that performs the functions of the primitive respiratory organ of the chick embryo and contains high amount of both oxygen and growth factors (Ribatti, 2010; Schneider-Stock and Ribatti, 2020).

CAM forms on days 3–4 of embryo development by the fusion of the chorion and the allantois. It acts as natural bioreactor and largely vicariates the use of animal experimentation (in fact, if used within day 17 of development, it does not require ethics committee approval). In the past, it has been used mostly in cancer research to study the molecular mechanisms of anticancer drugs and the correlated angiogenic response (Ribatti, 2014; Ribatti et al., 2001, 2003; Ribatti and Tamma, 2019), as well as for various types of tests (Kundeková et al., 2021): angiogenic potential assay (in both physiological and pathological purview), experimental embryology, tumor growth, experimental metastasis (Ribatti, 2021), teratogenesis and toxicology, drug delivery assay (Fonseca et al., 2021). Recently, instead, CAM has been re-discovered as an alternative to the *in vivo* experimentation, which underlines its ethical value. In fact, according to the 3R's principles of the animal experimentation (Reduction, Refined, Replaced), CAM assay has been exploding in recent years, where animal *welfare* is absolutely to be pursued and developed to make research sustainable, in relation to the "health" of both the ecosystem in general and the animal world in particular (Fig. 4B). The authors were recently funded by the Emilia Romagna Region, which has

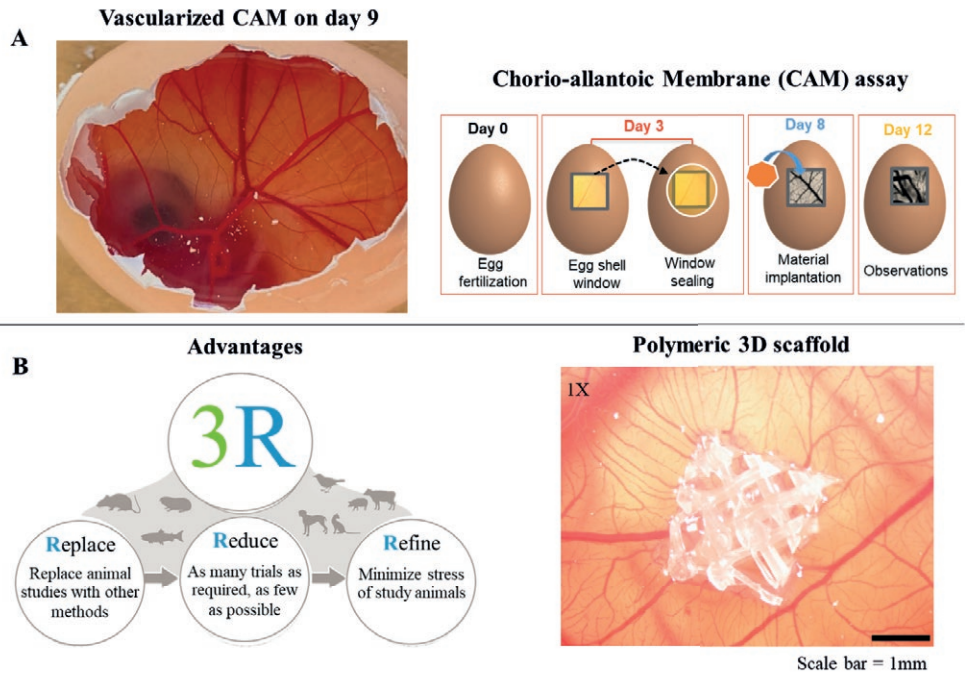

Figure 4. A. On the left a photograph of Chorio-allantoic Membrane (CAM) on day 9 of incubation. On the right, a diagram representing the CAM assay during 12 days of incubation: on day 3 eggs are opened in order to detect that fertilization has occurred. On day 8 the CAM is ready to host the material and the implantation is performed. Finally, on day 12 all the observations take place. B. All the advantages deriving from CAM assay are represented on the left of the picture: the 3R's principles are respected. On the right, a picture of a polymeric 3D scaffold on chorio-allantoic membrane that shows a large number of vessels around it.

been funding precisely alternative research models to animal testing for several years. The use of CAM assay cuts across several topics: from tests of biocompatibility, cytotoxicity and mechanisms of action of bioactive molecules, to validation of materials for tissue engineering; just to this last topic, our interest is particularly focused and declined in various projects.

Conclusions – Proposed development of eco-sustainable strategies for skeletal tissue regeneration

Studies on scleral ossicles and their derivatives (Pal-OS®) in combination with the use of ethical CAM assay are a clear demonstration of how morphology can overcome the risk of being confined and considered obsolete following the exuberant development of other disciplines. Morphology, instead, represents the indispensable basis of innovative proposals with translational significance and applicative possibility.

Moreover, our group's recent studies are absolutely based on environmentally sustainable research as well as respect of animal welfare: scleral ossicles are obtained

from poultry waste (whose extraction and processing procedure was patented by UNIMORE-Palumbo), thus no animal sacrifice is required for their procurement. CAM is a simple, quick, low-cost and ethically sustainable model, that replaces the use of animals in the primary stages of experimentation and does not require administrative ethics committee approval (unlike animal experimentation), because the chick embryo is not considered as living animal until 17th day of embryo development in most countries (moreover, the chick embryo younger than 10th day are assumed to be unable to experience pain (Institutional Animal Care and Use Committee (IACUC) - Brown University, 2019)).

In conclusion, the purpose of this prospective article is to emphasize how the "serendipity" (Dong et al., 2021; Hartl et al., 2021; Thomson, 2021) of morphological evaluations like the observation at the onset of this "story" concerning the generalized osteocyte apoptosis in some types of ossicles (Palumbo et al., 2012), can provide new application insights in the regenerative field, when combined with the scientific creativity, declined from a translational perspective, allowing the complementation or improvement of approaches that are already exploited by regenerative medicine.

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