A simple rodent subcutaneous assay for identification of new osteoinductive molecules: The key method for screening of novel bone regeneration implants

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Abstract

Treatment of large bone defects and degenerative diseases of the spine is among the most challenging and still unresolved issues in clinical medicine. Therefore, substantial effort has been devoted to the development of novel bone regenerative therapies. Due to their potent osteoinductive properties, Bone Morphogenetic Proteins (BMPs) have been the basis for the development of novel strategies for bone regeneration. The use of animal models is an indispensable part of the preclinical testing of novel therapeutic solutions. The rat subcutaneous assay became the initial screening procedure for the evaluation of promising BMP-based osteoinductive devices for bone regeneration because only osteogenic BMPs can induce new bone at any ectopic rodent site. Moreover, this model is used for research on the mechanisms of ectopic bone formation as well as for the evaluation of the inflammatory response to different materials. In this review, we provided an overview of the assay development and previously conducted studies with different methods (flow cytometry, histological and microCT analyses) for the study outcome evaluation. Moreover, we addressed essential issues in the experimental design such as the follow-up period and the sample size. The rat subcutaneous bone induction assay layed the foundation for isolation and identification of BMPs followed by testing of new osteogenic devices in higher animal species and humans.

Keywords:

Bone morphogenetic proteins, animal models, bone regeneration, subcutaneous bone assay

Sažetak:

Jednostavan potkožni esej u glodavaca za identifikaciju novih osteoinduktivnih molekula: ključna metoda za probir novih implantata za regeneraciju kosti

Liječenje velikih koštanih defekata i degenerativnih bolesti kralježnice predstavlja jedno od najzahtjevnijih i još uvijek neriješenih pitanja kliničke medicine. Znatni napori uloženi su u razvoj novih terapija za regeneraciju kostiju. Zbog svojih osteoinduktivnih karakteristika, koštani morfogenetski proteini (*engl. Bone Morphogenetic Proteins, BMPs*) bili su osnova za razvoj novih strategija za regeneraciju kostiju. Korištenje animalnih modela neizostavan je dio pretkliničkog testiranja novih terapijskih rješenja. Potkožni esej u štakora postao je inicijalni postupak za procjenu potencijalnih terapijskih

rješenja na bazi BMP molekula za regeneraciju kosti jer samo osteogene BMP molekule mogu inducirati novu kost na ektopičnom mjestu u glodavaca. Štoviše, ovaj se model koristi za istraživanje mehanizama ektopičnog stvaranja kosti kao i za procjenu upalnog odgovora na različite materijale. U ovom članku dan je pregled razvoja potkožnog eseja i prethodno provedenih istraživanja koristeći različite metode (protočna citometrija, histološka i mikroCT analiza) za procjenu ishoda. Nadalje, obrađena su bitna pitanja u dizajnu eksperimenta kao što su odabir razdoblja praćenja i veličine uzorka. Potkožni esej nastanka ektopične kosti u štakora postavio je temelje za izolaciju i identifikaciju BMP molekula što je u konačnici rezultiralo razvojem i testiranjem različitih terapijskih rješenja baziranih na osteoindutivnim BMP molekulama kod viših vrsta životinja i ljudi.

Ključne riječi: Koštani morfogenetski proteini, animalni modeli, regeneracija kosti, potkožni koštani esej

1. Introduction

Regenerative medicine is among the most propulsive scientific fields of the 21st century. To achieve the regeneration of different tissues and organs numerous therapeutic approaches have been developed and tested in preclinical and clinical studies. Although bone tissue possesses unique endogenous self-repair properties, large bone defects can not heal spontaneously and may lead to amputation of a limb and subsequently significantly decreased life quality (1). Autologous bone graft (ABG), routinely derived from the iliac crest, possesses osteoinductive, osteoconductive, and osteogenic properties and is currently considered as standard therapy in these conditions (2-4). However, it is also related to several disadvantages including a limited amount of available tissue and morbidities associated with the donor site (deformity and skin scarring, acute and chronic pain) (5, 6). Therefore, there is an imminent need for a safe and effective therapeutic solution that would substitute ABG use.

Bone morphogenetic proteins (BMPs), members of the TGFbeta family of proteins, have been widely used in bone regenerative medicine due to their potent osteoinductive properties (7). To be used at the designated site, BMPs require a carrier that would sustain their concentration and allow the prolonged release of proteins (8, 9). Numerous materials have been evaluated as BMP carriers in preclinical studies, but till now only one BMP-based device containing rhBMP2 on collagen carrier has been approved for anterior lumbar interbody fusion (ALIF), acute tibial fractures, and maxillofacial indications (10-13). However, off-label use of this device in spinal indications resulted in side effects including swelling of the adjacent tissue, ectopic bone formation, osteolysis, and radiculitis (14-18).

The use of animal models is an indispensable part of the development of novel therapeutic solutions in regenerative medicine (19, 20). Animal models used in the development of novel devices for bone regeneration might be categorized as ectopic models that are primarily used for the evaluation of osteoinductive properties and models that mimic target clinical indication. In ectopic models tested implants are placed subcutaneously or intramuscularly in rats and mice and only exceptionally in larger animals like rabbits, minipigs, sheep and NHPs (21-26). Osteoinductive devices are intended for the rebridgment of large segmental defects and for achieving spinal fusion in patients with degenerative diseases of the spine by inducing bone between adjacent vertebrae. These models might be also classified based on the stage of preclinical development into models used for initial evaluation (rodent ectopic and critical-size defect models), intermediate testing (rabbit segmental defect and PLF models), and advanced (dog, sheep, and non-human primate segmental defect and PLF models) evaluation of novel osteoinductive devices (20). Due to ethical reasons and the high cost of the experiments on larger animals (dog, sheep, and non-human primates), the majority of studies have been conducted on rodents while only a few devices have been evaluated in advanced stages of preclinical testing and eventually in clinical trials. Apart from the evaluation of a novel osteoinductive devices, ectopic models in rodents might be used for basic research on the mechanisms of the bone induction and properties of different molecules and biomaterials (27-30).

We have recently reviewed a broad range of aforementioned animal models in the development of novel bone regenerative therapies (19, 20). In this review, we focused specifically on the rat subcutaneous implant assay, a well-established model for the evaluation of osteoinductive properties of tested implants as well as evaluation of the inflammatory response of the living organism.

2. Rat subcutaneous bone induction assay

Rodent ectopic models might be conducted in rats or mice, however, due to the higher similarity of bone biology with humans, rat is the preferred species for conducting such experiments (19). Moreover, ectopic models are further subdivided into subcutaneous and intramuscular based on the site of device implantation. Among these sites, the subcutaneous site is preferred because it contains fewer cytokines and growth factors than muscles providing a more independent assessment of implant properties (31). Therefore, the rat subcutaneous assay is a subtype of rodent ectopic models optimal for the initial testing of novel osteoin-

ductive devices. The assay has been introduced and well-defined along the discovery path of BMPs via purification of the bone induction activity from bovine pulverized and decalcified bone matrix (32-36). Namely, the purified fractions from demineralized bone showed bone activity when implanted subcutaneously in rats only when bound to inactive, pulverized, demineralized and extracted bone particles, but not when implanted as a solution without a carrier (Figure 1).

Rat subcutaneous assay is in brief conducted as follows: After experimental animals are anesthetized, a vertical midline skin incision is done over the thoracic region and subcutaneous pockets are created in the axillary region by blunt dissection (37-39). Previously prepared potentially osteoinductive implants are placed into the pockets and the incision is sutured. To minimize the number of used experimental animals, implants should be implanted bilaterally. Apart from subcutaneous implantation in the axillary region, in the other variant of this model implants might be subcutaneously implanted in the rat dorsal region (40). Experiments should be approved by the Ethics committee and conducted according to the national legislature on the use of experimental animals and FELASA recommendations.

Methods of evaluation of the newly induced bone in rat subcutaneous implant assay include histological and microCT analyses as well as cellular analyses using flow cytometry (Figure 2). The choice of the evaluation depends on the aim of the study as well as the time point of evaluation and these methods are discussed in the following sections.

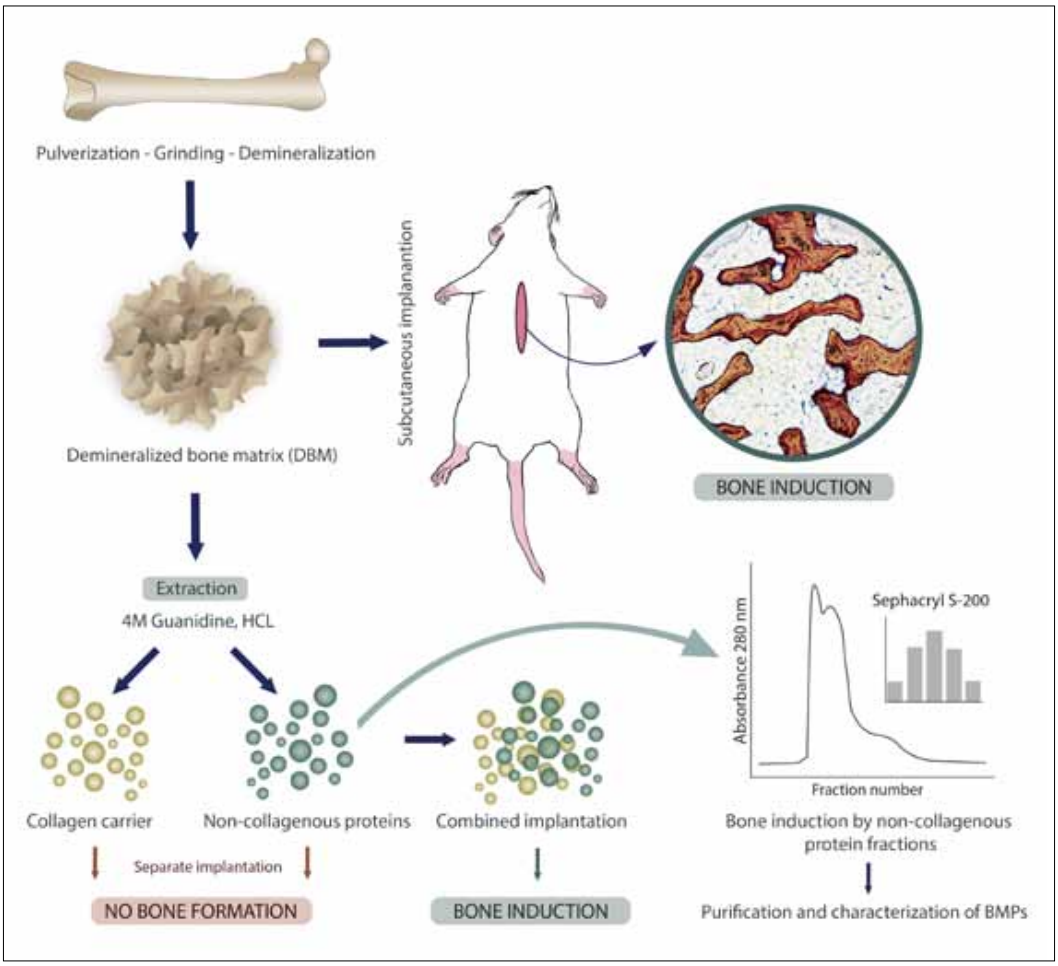

Figure 1. In vivo bone assay for characterization of BMPs. Rat bone is subjected to pulverization, grinding and demineralization. Demineralized bone matrix (DBM) implanted subcutaneously in the axillary region induces ectopic bone formation. Extraction of DBM with 4M guanidine and hydrochloric acid (HCL) leads to separation of non-collagenous proteins and collagen carrier and when implantated separately in the rat no bone is formed. However, when the collagen carrier and non-collagenous proteins are premixed and implanted, new ectopic bone is formed. The subcutaneous rat bone assay was a critical tool for further characterization of osteogenic protein fractions and BMP genes cloning.

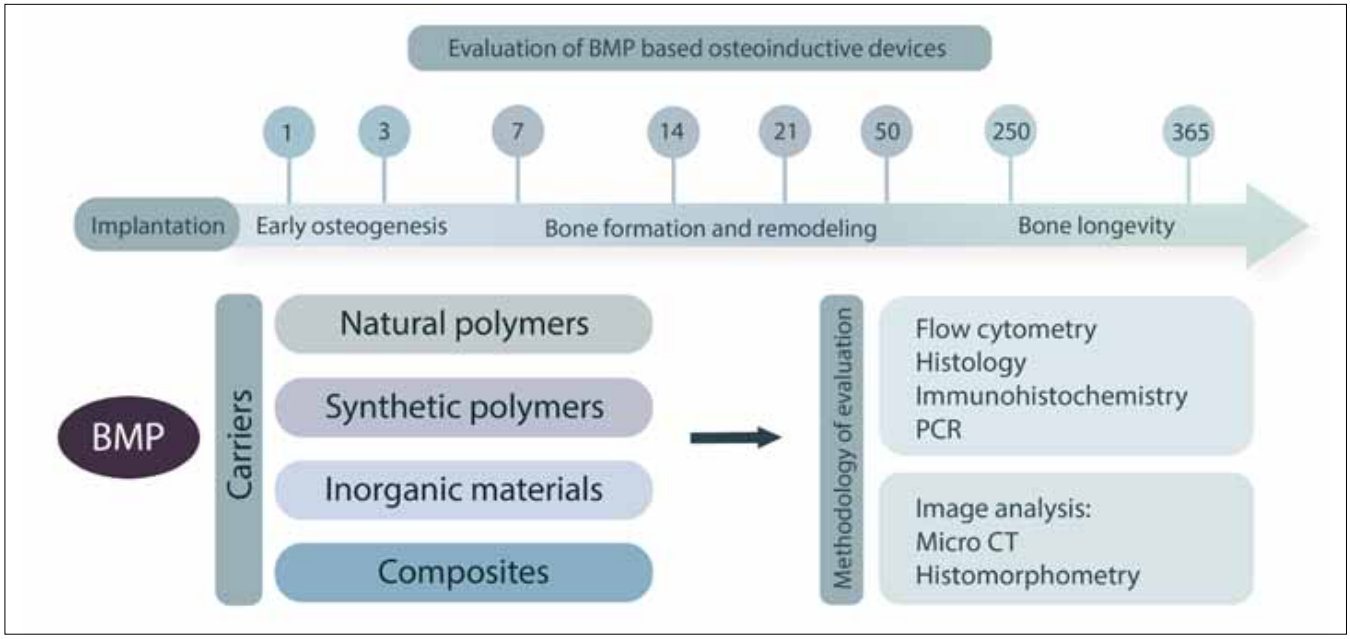

Figure 2. Rat subcutaneous bone assay serves for the initial evaluation of BMP based osteoinductive devices. Early osteogenesis, bone remodeling and bone longevity using various materials (natural and synthetic polymers, inorganic materials and their composites) have been evaluated by flow citometry, histology, immunohistochemistry, PCR or by image analyses including micro CT and histomorphometry.

3. Preclinical studies employing rat subcutaneous bone induction assay

Rat subcutaneous assay has been widely used for the evaluation of BMP-based devices comprised of osteoinductive BMPs (BMP2, BMP6 and BMP7 were most commonly used) and their carriers (39-46). BMP carriers might be divided into natural and synthetic polymers, inorganic materials, and composites of these materials (8, 9). Due to their advantages including biodegradability and resorbability, natural polymers including collagen, gelatin, fibrin, hyaluronic acid, chitosan, and alginate have been extensively studied in preclinical studies (41, 47-53). However, the aforementioned materials also possess important disadvantages including rapid BMP release from the carrier due to low affinity for these proteins, immunogenicity, and small but still existing risk of viral transmission (8, 9, 54). To overcome some of these disadvantages, synthetic polymers including polylactic acid (PLA), polyglycolic acid (PGA), poly(D, L-lactide-co-glycolide)(PLGA), polyethylene glycol (PEG), polypropylene fumarate (PPF) and poly-E-caprolactone (PCL) have been introduced and tested in preclinical studies (55-66). However, testing of these materials has revealed that they might cause bulk degradation, chronic inflammation, and decreased pH due to acidic breakdown byproducts (9). Importantly, both natural and synthetic polymers are compressible in the surroundings in which compressive forces are present due to weak biomechanical properties (8, 9, 54). Inorganic materials such as calcium phosphate ceramics, calcium phosphate/calcium sulfate cement, and bioglass are resistant to compression and have been considered as a BMP carrier (8, 9, 40, 42, 67-70). Moreover, these materials might be combined with natural or synthetic polymers taking advantage of different materials (5, 41, 71, 72).

We have developed Osteogrow, a novel osteoinductive device comprised of recombinant human Bone Morphogenetic Protein 6 (rhBMP6) delivered within autologous blood coagulum as a BMP carrier (37-39, 44-46, 73-78). Similar to natural and synthetic polymers, ABC is susceptible to compression. Therefore, compression resistant matrix (CRM) such as synthetic calcium phosphate ceramics should be added to ABC to enhance its biomechanical properties (38). We have recently conducted extensive studies using rat subcutaneous assay in which we evaluated these CRMs and elucidated how particle size and chemical composition of ceramics affected the outcome of BMP-induced bone formation (39, 44, 46). Moreover, we have shown how the structure of newly formed bone might be tailored with the addition of bisphosphonates, potent anti-resorptive agents, providing novel strategies in the engineering of new bone (45).

Apart from the studies focused on the BMP-mediated bone formation and evaluation of novel osteoinductive devices, rat subcutaneous assay might be used for studying the inflammatory response of the organism to the aforementioned materials used as a BMP carrier (79).

4. Time course of ectopic bone formation and follow-up period in rat subcutaneous bone induction assay

The time course of bone induction by BMPs depends on the properties of the BMP carrier and the applied BMP dose, however, a general sequence of BMP-mediated bone formation has been established (37, 38, 44, 45). In the first few days following implantation BMP attracts mesenchymal stem cells (MSCs) while the carrier attracts inflammatory cells which participate in the foreign body response (37, 38). Till the end of the first week, MSCs differentiate into chondrocytes and osteoblasts allowing endochondral ossification to occur at the peripheral parts of the implants (37, 44). At the end of the second week after implantation newly formed bone is typically present throughout

the implant (38). Along with newly formed bone, bone marrow containing blood vessels is formed between bone trabeculae (39). In the following weeks, newly formed bone is being remodeled, adipogenic differentiation occurs in the bone marrow and carrier is being resorbed (38, 44, 45). Importantly, the resorbability of the BMP carrier depends on its properties and might extremely differ among them (77). For example, blood coagulum is resorbed within a few days while calcium phosphate ceramic particles might still be unresorbed even one year following implantation (37, 45).

The follow-up period of the study is chosen according to the aim of the study. The majority of the studies aimed to evaluate properties of osteoinductive devices and had a follow-up period between 7 and 28 days (37-44, 46, 50, 67, 80-82). However, a shorter period than 7 days might be chosen to investigate the cellular response to implants while a longer follow-up period (up to one year) might be chosen to evaluate the longevity of the new ectopic bone (20).

Choice of evaluation methods depends on the aim of the specific study and the time point at which evaluation occurs. The cellular response of the organism to implants is evaluated in the first few days following implantation using flow cytometry or histological sections supplemented with immunohistochemistry. The structure of newly formed bone formed at later time points is analyzed by microCT and histological sections (39). Moreover, both microCT and histomorphometry might be used for the quantification of new bone but microCT analyses are considered more accurate since they analyze the entire implant.

5. Evaluation methods

5.1. Histology

Histological analysis is among the most important methods for evaluating ectopic bone formation since it provides insight into the structural properties of newly formed bone (Figure 3). Histomorphometric analyses is used to obtain the quantity of bone, bone marrow, and unresorbed carrier (39). Moreover, key molecules and cells involved in ectopic osteogenesis and cellular response to implants might be identified and localized using immunohistochemistry or immunofluorescence (44, 46).

At the end of the follow-up period, implants are harvested and fixed using formaldehyde solution. Bone tissue specimens might be further processed undecalfied or using decalcification in chelating agents such as EDTA or formic acid and embedded in resin (undecalcified specimens) or paraffin (decalcified specimens) and cut to 5-6 μm thick sections (39, 45, 75-77). Obtained sections are mounted on glass slides and stained with one or more appropriate methods including Goldner, hematoxylin-eosin, Von Kossa, or toluidin blue stain.

Histological sections obtained using both undecalcified and decalcified processing methods are equally valuable for structural analyses of the newly formed bone. However, undecalcified tissue processing of specimens containing calcium phosphate ceramics has certain advantages over decalcified processing since after decalcification ceramics appear as void spaces (39, 45, 75-77). Moreover, Von Kossa stain is used to detect mineralization and therefore it might be applied only on undecalfied specimens.

5.2. MicroCT analyses

MicroCT analysis is along with histological analyses the most important morphological method of evaluation of ectopic bone formation in a broad range of animal models including rat subcutaneous assay (Figure 4). Obtained microCT sections allow a descriptive structural analysis of newly formed bone. Moreover, microCT analysis determines the volume of newly formed bone as well as the volume of residual ceramics (77). It is important to emphasize that the bone volume determined by microCT analysis is in general more accurate than the amount of bone determined histomorphometrically since it takes into account the whole implant while histomorphometry is conducted using a limited number of sections through the implant. In addition, using microCT analysis it is possible to calculate trabecular parameters of newly formed bone including trabecular number, trabecular thickness, and trabecular separation (39).

One of the most important microCT scanning parameters is resolution. Contemporary microCT scanners provide superior resolution up to several microns (29). However, an increase in scanning resolution is followed by significantly increased scanning time, increased size of obtained data, and subsequently prolonged time of microCT analyses. Therefore, it is necessary to choose a scanning resolution that provides sufficient resolution with reasonable scanning time and data size. Based on our previous studies we suggest that a scanning resolution of 18 μm is optimal for microCT analyses, while a higher resolution (4-9 μm) might be used to show the structure of newly formed bone on a limited number of samples (39, 45, 76, 77).

MicroCT scanning might be conducted *in vivo* or using specimens *ex vivo* following euthanasia. *In vivo* microCT scanning is conducted during a short anesthesia and allows the follow-up of the same implant through different time points. However, obtained sections often contain artifacts due to respiratory movements. Therefore, scanning of implants *ex vivo* provides optimal quality and should be used for microCT analysis, while *in vivo* scanning might be used to obtain preliminary results.

5.3. Flow cytometry

Flow cytometry is a sophisticated laser-based technique that measures scatter and fluorescence to determine a set of unique cellular properties. It allows fast, relatively quantitative and multiparameteric analysis of a single cell in a heterogeneous cell population as well as isolation of those cells with a process called cell sorting (83, 84). Such sorted specific cell types can be sequenced or used for gene expression analysis on a population or a single-cell level, and as well combined with ELISA, proteomics, or Western blots analysis directly (85).

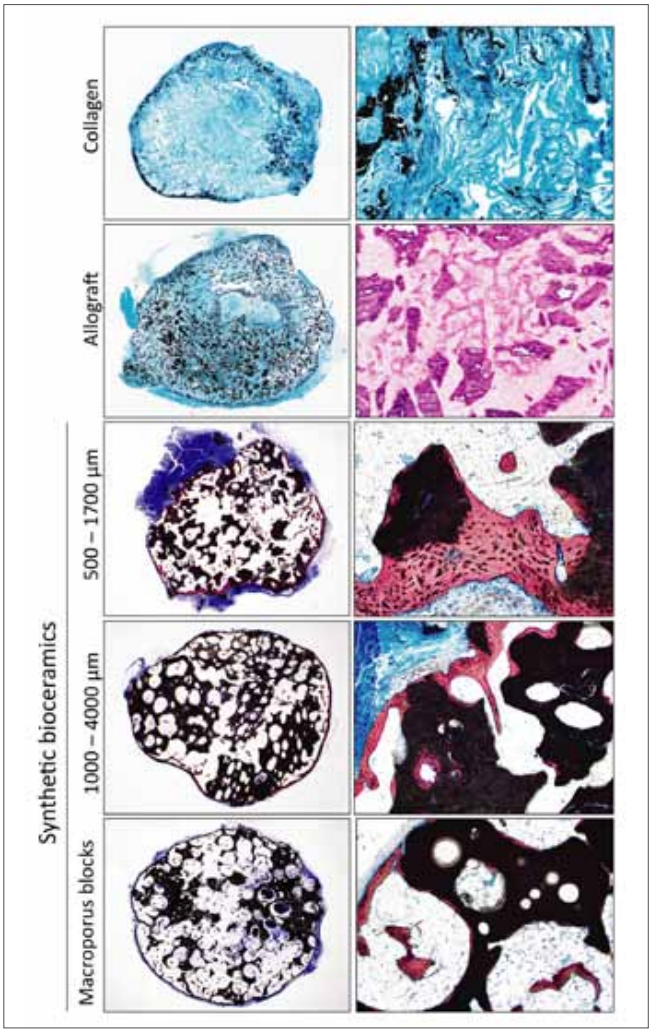

Figure 3. Histology sections of bone induced by rhBMP6 implanted with different carriers like collagen, autologous blood coagulum with or without addition of compressive resistant matrices in form of allograft or synthetic ceramics of different particle size. Histological sections were stained by Von Kossa, Hematoxylin-Eosin (HE), or Sanderson's Rapid Bone Stain with Van Gieson picrofuchsin.

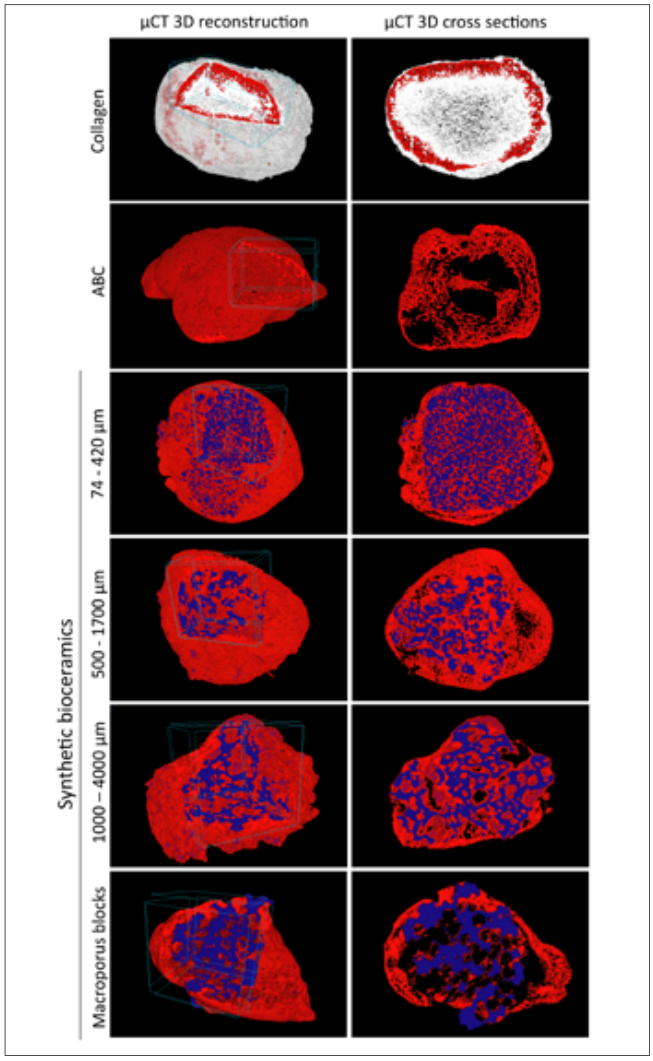

Figure 4. MicroCT 3D reconstruction of bone induced by rhBMP6 with different carriers including collagen, autologous blood coagulum alone with or without the addition of compressive resistant matrices in form of allograft or synthetic ceramics of different particle size.

Any foreign material implanted in the body will stimulate an immune response, and flow cytometry has been emerged as a critical tool in the study of the immune system. With the growing appreciation of the immune system in tissue engineering and regenerative medicine, flow cytometry is increasingly being implement in the analysis of biomaterials response (85, 86).
The host's innate and adaptive immune systems are the major mediators in the response to foreign implants, and a surgical implantation of an osteoinductive device that contains carriers such as natural and synthetic polymers and inorganic materials into the human body have the ability to trigger host responses (20, 87). In general, biomaterial properties, such as macroporosity and surface microstructure, manage the host response as well as ultimate bone healing cascade, and the understanding of the intercellular communications during the inflammatory reaction, its resolution and the bone regeneration phase, is crucial for improving current therapeutic strategies or developing new approaches (88).
In addition, flow cytometry is also used for characterization of mesenchymal stem cells cultured *in vitro* which are detected by positive and negative specific surface markers to evaluate suitability of the cells for further usage in development of implants for ectopic implantation *in vivo* and applications in bone tissue repair (89-92). Moreover, flow cytometry can be used for characterization of ectopic implants to assess bone marrow formation and tissue vascularization in the implants themselves (89).

6. Determination of sample size

Defining an adequate sample size to obtain reliable results is among the most important aspects while planning each experiment (19). According to the 3R principle and recommendations on the rational use of laboratory animals, the number of used animals should be reduced to minimal. However, if the sample size is too small, reliable conclusions can not be made and obtained results might not be useful. Therefore, the sample size should be carefully determined based on the study aim, the variability of the outcome determined in the preliminary experiments, and the recommendations for each specific animal model (20). In the previously published studies employing rat subcutaneous bone induction assay the number of specimens was between 3 and 10

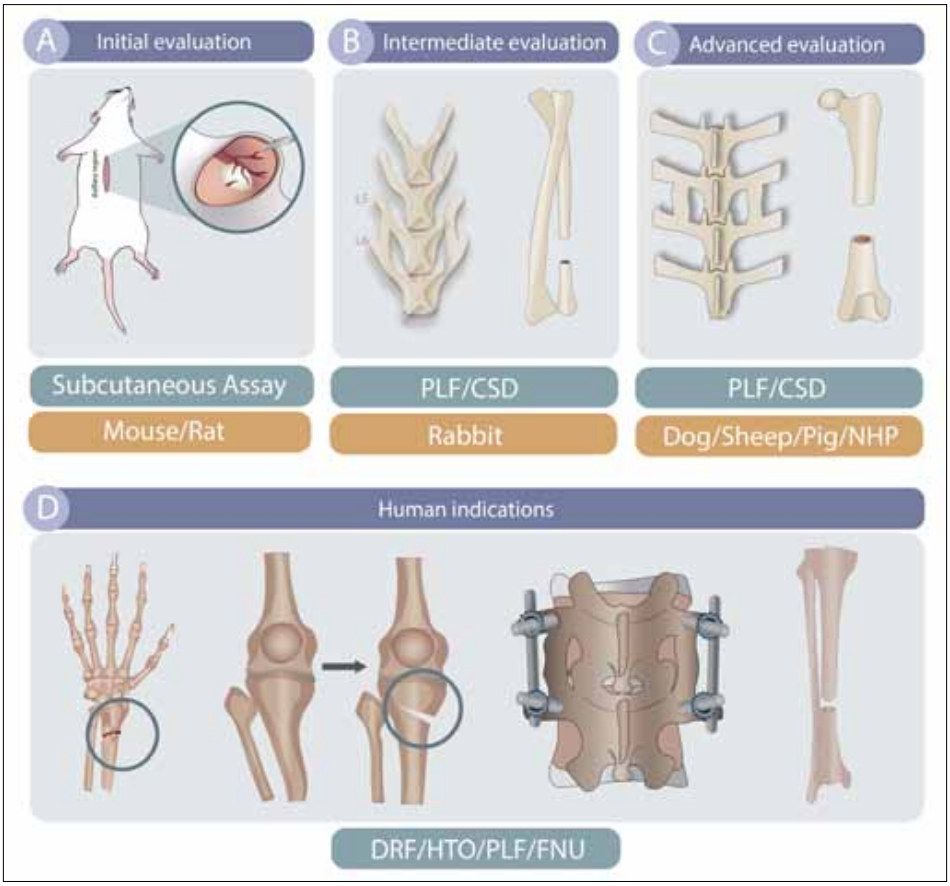

Figure 5. Animal models for evaluation of osteoinductive devices based on the stage of preclinical development ranging from mouse (A), rabbit (B) to non-human primate (NHP) (C), namely classified as initial, intermediate and advanced leading to clinical trials in humans (D). PLF – posterolateral spinal fusion; CSD – critical size defect; DRF – distal radius fracture, HTO – high tibial osteotomy; FNU – fracture nonunion.

per group (40-43, 50, 67, 68, 80-82, 93-98). However, in the majority of studies the sample size was higher than 4 per group. Therefore, based on the previous studies and our own extensive experience with this animal model we recommend the number of samples of at least 5-6 per group.

7. Translatability of the rat subcutaneous bone induction assay model

Rat subcutaneous assay is along with other experimental models conducted on rodents among methods for initial evaluation of novel osteoinductive devices. Moreover, it is an ectopic bone model primarily used for the evaluation of the osteoinductive potential of tested implants and does not simulate any target clinical condition. Therefore, following the successful outcome in this model, promising osteoinductive devices should be tested in models on larger animals that mimic the intended clinical indication (20). Segmental defects and posterolateral spinal fusion (PLF) are among the most challenging orthopedic conditions which might be treated with an osteoinductive device. Subsequently, relevant animal models that mimic segmental defects and PLF have been developed in rabbits, dogs, sheep, and non-human primates. The aforementioned models in rabbits are widely used because they allow for testing the safety and efficacy of the osteoinductive device in the target clinical indication (37, 50-53, 61-64, 66, 69, 71, 76, 77, 99-115). Animal models in sheep and NHP are the final step of preclinical testing and they provide the most similar conditions to humans (20). However, only few osteoinductive devices which were evaluated in rat subcutaneous assay and rabbit segmental defects/PLF models have been subsequently tested in sheep/NHP models (5, 70-72, 75, 116).

We have recently conducted an extensive preclinical evaluation of Osteogrow, a novel osteoinductive device comprised of rhBMP6 within autologous blood coagulum (ABC) and its formulations with allograft (Osteogrow-A) and synthetic calcium phosphate ceramics (Osteogrow-C) (37-39, 44, 46, 73, 75-77). At the beginning, we used the rat subcutaneous bone induction assay to test a large number of potential formulations (38, 39, 44-46) and to select optimal formulation for further evaluation in rabbit and sheep PLF models (38, 75-77) (Figure 5). In this series of experiments, we have demonstrated that findings observed in the rat subcutaneous bone induction assay were translatable to higher-order animals. Moreover, following preclinical testing on animals, Osteogrow was successfully tested in phase I/II clinical trials in patients with distal radial fracture (DRF) and high tibial osteotomy (HTO) (117, 118) and Osteogrow-A in patients undergoing PLIF procedure for lumbar back pain (https://www.genera-research.com/) (Figure 5).

8. Conclusions

Animal models are an indispensable part of the preclinical evaluation of the safety and efficacy of novel therapeutic solutions. Rat subcutaneous bone induction assay, originally developed for testing individual protein samples from demineralized bone purified extracts during isolation of BMPs, is a very useful animal model for the initial evaluation of osteoinductive implants and findings observed in this model are translatable to higher-order animals and humans. All experiments employing rat subcutaneous assay should be carefully planned and prepared to obtain reliable results and achieve a rational use of laboratory animals. Moreover, proper sample size and follow-up period as well as adequate evaluation methods should be chosen according to the aims of the study.
A large number of BMP-based osteoinductive devices have been tested employing rat subcutaneous bone induction assay but only a few were further evaluated in higher-order animals and none of them eventually resulted in the development of a therapeutic solution for segmental defects of the bones and posterolateral spinal fusion which are among the most complex and frequent indications in clinical medicine. However, the development of Osteogrow, a novel osteoinductive device, provides hope that effective therapeutic solutions might be soon available for the aforementioned indications.

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Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Not applicable.

Conflict of Interest

S.V. is a founder of Genera Research and a coordinator of the EU HORIZON 2020 grant OSTEOproSPINE, funding clinical studies for new bone repair drugs (patent WO2019076484A1).

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