THE EUROPEAN SOCIETY OF MEDICINE
Medical Research Archives, Volume 12 Issue 11

REVIEW ARTICLE

Shaking Up Bone Regeneration: A Review of Nanovibrational Stimulation on Signalling Pathways in the Pursuit of a Cellular Therapy-Based Bone Graft Substitute

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OPEN ACCESS

PUBLISHED
30 November 2024

CITATION
McCormick, M., Dalby, M., et al., 2024. Shaking Up Bone Regeneration: A Review of Nanovibrational Stimulation on Signalling Pathways in the Pursuit of a Cellular Therapy-Based Bone Graft Substitute. Medical Research Archives, [online] 12(11). https://doi.org/10.18103/mra.v12i11.5977

DOI
https://doi.org/10.18103/mra.v12i11.5977

ISSN
2375-1924

ABSTRACT

The demand for a viable alternative to currently available bone grafts continues to grow as the clinical need to fill or augment bone defects increases. Defects in bone due to trauma, infection, malignancy or metabolic bone disease provide unique challenges in treatment. Optimal graft bio characteristics should provide structure, osteogenesis, osteo-induction and osteo-conduction. To achieve this, consideration of cells and materials is required.

Approaches such as nanovibrational stimulation have provided advancements in driving mesenchymal stem cells toward osteoblastogensis, by activating mechanotransductive signalling pathways. Combining the technology of nanovibrational bioreactors with 3D collagen scaffolds has provided further insight into the role of mechanoreceptors in addition to presenting challenges in optimising the stiffness of such scaffolds to transmit the required frequency needed to induce osteoblastogenesis. Advancing this technology could provide opportunity for scalable production of osteoblastogenic cells within natural or synthetic 3D scaffolds.

Successful exogenous osteogenesis of bone forming cells in suitable scaffolding materials could unlock a cascade of treatment avenues previously unattainable, expanding opportunities of reconstructive surgery and improving limb salvage procedures. This article aims to review advances in osteoblast stimulation and scaffolds and discuss the limitations and potential applications of the technologies in a clinical setting.

Introduction

Bone defects occur from a variety of clinical situations such as trauma, impaired healing, infection or malignancy. Bone acts as the largest reservoir of calcium in the body and is actively involved in its homeostasis whilst also being the site of haematopoiesis within the marrow.[1] Further, it possesses the mechanical properties that are essential for providing support and protection to major organs and provide attachment for tendons and ligaments to facilitate locomotion.[1] It is these structural properties of bone that drive the need for bone transplantation. Bone regeneration has been a focus of much research over the last three decades.

The ideal graft material possesses a combination of structural properties, osteoconductivity, osteoinductivity and osteogeneity. Autologous bone graft represents this ideal graft material as it possesses these desirable properties.[2–6] Autologous grafts have limitations on available and accessible donor sites. The Iliac crest is the most common donor site but graft can also be harvested from local anatomy such as the distal radius or proximal tibia. These have limitations on volume of material available for grafting, which may be insufficient to treat the defect. Bone graft harvesting can have structural implications of the donor site leading to donor site morbidity such as fracture, pain or infection. Dimitriou et al published a systematic review demonstrating complication rates of over 19% from iliac crest bone graft harvesting.[7]

Bone allografts are widely used in orthopaedics including femoral head allografts (sourced from patients undergoing total hip replacements for osteoarthritis) and, less commonly, cadaveric grafts (for larger defects requiring complex reconstruction).[8–10] Lomas et al explores two concerns held by surgeons surrounding allografts, namely that they are in scarce supply and that they are unsafe. The authors explain the robust screening and processing systems to reduce risk of disease transmission, and on a basic estimation of demand vs supply, would suggest ample availability of femoral head allograft.[5] This does not reflect the full clinical demand as a femoral head allograft, while suitable for smaller defects are not reliable for critical bone defects. Cadaveric, site-specific grafts in this instance can have a role although both vascular integration and availability of supply become limiting factors.[8,10]

Bone graft substitutes can be used as an alternative. An ideal bone graft substitute should be biomechanically stable, able to degrade within an appropriate time, exhibit the ideal properties of bone graft (osteoconductive, osteogenic and osteoinductive) and provide a favourable environment for invading blood vessels and bone forming cells.[3] Bone graft substitutes can be divided into two main categories, biological and synthetic. Biological materials include demineralised bone matrix (DBM)[11] and natural polymers like collagen type1.[12–15] Materials like porous metals[3,16], synthetic polymers[3,17] and calcium phosphates like hydroxyapatite (HA) and tricalcium-phosphates (TCP)[18,19] are examples of synthetic substitutes. Synthetic materials provide a platform for the attachment and colonization of osteoblasts and subsequent bone formation. Combining these synthetic materials with local growth factors or antibiotics expanded their functionality and efficacy.[1] Studying the effects different materials on cell differentiation improves the understanding of how mechanical signalling plays a significant role in determining the lineage of an MSC.

Cellular therapies

Cellular therapies were established as a safe treatment for haematological disorders such as lymphoma and leukaemia in the 1990s, early cellular therapies utilised advances with haematopoietic stem cells (HSCs). [20] Research into mesenchymal stem cells' (MSCs) ability to differentiate into multiple lineages including osteoblasts, myocytes, chondrocytes and adipocytes was also advancing.[21] In 1991, Caplan proposed the use of MSCs in self-cell therapy, where autologous MSCs can be expanded in vitro and reintroduced to repair skeletal tissues such as bone and cartilage.[21] This therapeutic approach utilises the body's natural repair mechanisms.

Understanding the intracellular and extracellular influences of the MSC or skeletal stem cell (SSC), proposed as a more appropriate term to describe such cells,[22] has been a focus of research aiming to optimise the cellular microenvironment for osteogenesis. The understanding of mechanoreceptors and their influences of cellular differentiation has been one such focus which has allowed the development of a nanovibrational stimulating bioreactor to stimulate SSCs toward osteogenesis.[23]

Osteoblast Development

During embryological development, osteoblast formation progresses via two distinct pathways: endochondral ossification and intramembranous ossification.[24] In fractured bones both types of ossification can be utilised dependant on the environment of the injury. Intramembranous ossification is akin to primary bone healing which relies on direct reduction and absolute stability of the fracture,[25,26] two basic science concepts familiar to orthopaedic surgeons. This allows direct new bone formation underneath the periosteum. Conversely endochondral ossification is present during secondary bone healing where the fracture is immobilised via relative stability. This process utilises the formation of callus prior to new bone formation.[25,26]

There are several key signalling pathways which contribute to and regulate osteoblast differentiation from SSCs. These include Wnt, bone morphogenetic protein (BMP), transforming growth factor-β (TGF-β), hedgehog, fibroblastic growth factors (FGFs), and the more recently discovered piezo1/2 pathways.[24] These pathways co-ordinate the differentiation process by regulating various transcription factors, such as Runt-related transcription factor 2 (Runx2), osterix (Osx), activating transcription factor 4 (ATF4), and special AT-rich sequence- binding protein-2 (SATB2), all critical for bone formation.[24] The most significant are Runx2 and core binding factor β(Cbfβ, a RUNX co-activator), which orchestrate osteoblast-specific gene expression, and factors such as β-catenin that promote osteoblastogenesis while inhibiting chondrocyte and adipocyte differentiation.[24,27] It has been shown that nanovibrational stimulation can influence

some of these signalling pathways to upregulate these transcription factors.[28]

Cellular environment

With an understanding of the signalling pathways and transcription factors that have influence over the fate of an SSC, a focus of research has been on optimising the conditions for promoting osteoblast differentiation. Understanding the influences of the cellular environment can help improve the in vitro culture and development of osteoblasts.[29] Donnelly et al have employed bioengineering techniques to create an in vitro niche that mimics the extracellular matrix (ECM) structure and mechanical properties of the bone marrow environment. The niche was based on bone forming polymer-fibronectin (FN)-BMP2 endosteal surfaces[30] that support MSC osteogenesis overlaid with soft collagen I hydrogels driving expression of niche factors.[29]

Structural scaffolds have been studied to investigate the influences on osteoblast development.[3,17,31,32] Surface characteristics, including chemistry, topography and roughness, significantly influence scaffold integration and cell behaviour, influencing cell adhesion, proliferation and protein binding. [3,17,33] Hydroxyapitate (HA) [$Ca_{10}(PO_4)_6(OH)_2$] and collagen scaffolds are an established way of mimicking the cellular environment.[34] With collagen being the predominant organic component to bone and HA being the main inorganic component, the combination of these lead to improved mechanical properties of collagen + HA composites. Collagen compensated for poor fracture toughness of HA and HA provided collagen a higher stability while improving mechanical properties.[34,35] HA is the main inorganic component of bones contributing around 70% of bone tissue.[34] HA can combine with many different materials including natural or synthetic polymers, growth factors or cells to imitate the natural structure of bone. [33,34] The mechanical properties of HA and biocompatibility make it a valuable recourse with wide reaching applications for bone regenerative therapies.

Hybrid scaffolds techniques based on Poly(lactic-co-glycolic acid) (PLGA) and silk to recreate structural aspects of the cellular environment have also been tested demonstrating that the PLGA-silk-HAp scaffolds demonstrated superior bone formation compared to control, silk-only, and PLGA-only scaffolds.[31]

Nanotopography has been studied for its influence over the stimulation and differentiation of SSCs. It has been shown that nanotopography can effectively induce osteogenesis in MSCs through a combination of adhesion signalling, BMP co-signalling, and the regulation of microRNAs (miRNAs).[36] Geometric environmental patterning and cell shape can independently influence the differentiation of SSCs. Different shapes, aspect ratios, and subcellular curvature modulate whether SSCs differentiate into adipocytes or osteoblasts. Specifically, high contractility promoted by certain geometric shapes (e.g. star-like structures) favours osteogenesis, while shapes with low contractility promote adipogenesis.[37] A recent review found that nanotopographic surfaces (e.g., nanopits, nanowires) enhance bone cell activity by promoting osteoblast differentiation and cell adhesion.[38] Understanding nanotopography and the influence that geometry, roughness and porosity can have on cell behaviour has expanded implant design in orthopaedics, trabecular metals have demonstrated improved osseointegration of implants.[39]

Porosity, pore size and interconnectivity are additional considerations shown to have influence on bone regeneration in scaffold design.[33,34] Larger pores (>300 µm) have been shown to be favourable for cell culture and bone ingrowth as pore occlusion occurs later than with smaller pores allowing space for nutrient and oxygen supply including revascularisation.[33,40] Jaio et al demonstrated that higher porosity metals better support osteogenic proliferation, differentiation and bone ingrowth than lower porosities.[39]

Modern approaches to cellular scaffolds have combined this knowledge, attempting to create an environment that reacts even more biomimetically with the extracellular matrix and growth factors for additional biological support for cell culture.[30] The polymer poly(ethyl acrylate) (PEA) in combination with fibronectin (FN) nanonetworks allow for simultaneous availability of both integrin and growth factor binding regions promoting enhanced BMP-2 signalling. [30] Cheng et al demonstrate this technique in a successful veterinary murine case study using ultra-low dose BMP-2 with a PEA polymer to treat a critical bone defect in a Münsterländer dog with a humeral non-union.[32]

Mechanical signalling

Mechanical stimulation within the microenvironment has been shown to have influence on osteoblast differentiation. [13,23,28,41–43] Appropriate mechanical stimulation activates calcium channels on the cell membrane to promote the transport of calcium into the cell, promoting osteogenesis. Piezo1 and Piezo2 have been identified as important mechanosensitive ion channels, originally in excitable cells, but latterly, more generally. [44,45] Piezo channels are now implicated in MSC/SSC and osteoblast response to changes in mechanical load.[13,46] Piezo1, for example, has been shown to regulate bone remodelling. [47]

Use of nanovibrational bioreactors[23] to expose MSCs/SSCs to vibrations, has been seen to stimulate osteogenesis [28,41] Such mechanical stimuli can be delivered through piezoelectric actuators using the reverse piezo effect to deliver known expansions (vibrations) based on electrical signal input.[41] It has been demonstrated that exposure to a frequency of 1 kHz stimulation leads to the up-regulation of osteogenic markers, including RUNX2 and BMP2. Lower frequencies, such as 500 Hz, do not show as strong an effect,[41] while higher frequencies have been implicated in stimulating off-target as well as osteogenic effects.[48] This suggests that frequency specificity is crucial for triggering osteogenesis.[41] Nikukar et al. also identified that the Ras homolog gene family, member A (RhoA) and its downstream effector Rho-associated protein kinase (ROCK) (RhoA/ROCK signalling pathway) are central to the osteogenic differentiation of MSCs under nanoscale mechanical stimulation. Inhibition of ROCK prevented osteoblast differentiation, confirming its role in mechanotransduction. The authors highlight the potential for scaling up this method to develop large-scale

osteoblast bioreactors, which could be used for producing bone tissue in regenerative therapies without relying on complex biochemical factors.[41]

Further studies have implicated ion channels, such as piezo channels, but with more focus on transient receptor potential cation channel subfamily V member 1 (TRPV1) as a driver of osteogenesis.[13]

Cell culture scaffolds and mechano-response

Using scaffolds, we can understand MSC/SSC mechanical response in 3D and on osteogenic implant surfaces also. In 3D collagen gels, for example, peizo1 and transient receptor potential vanilloid 1 (TRPV1) were identified as having key roles in converting mechanical stimuli into biochemical signals that promote osteogenesis. Osteoblast differentiation was inhibited on blocking these channels within the 3D scaffold. [13] Further, in 3D co-cultures with macrophages and MSCs, protein kinase C (Akt) has been implicated in promotion osteogenesis while reducing osteoclast formation.[49]

Also in collagen gels, increased vibration amplitude has been seen to increased reactive oxygen species (ROS) production and inflammation.[43] ROS and inflammation are marks of wound healing, as increased cell activity drives mitochondrial oxidative phosphorylation. Low levels of ROS and inflammation can, therefore, be a sign of positive osteogenic response, but care needs to be taken that it does not tip to pathological levels.[43,50]

Nanotopography has been implicated in osteogenesis, specifically 120 nm diameter pits with 100 nm depth and average 300 nm centre-centre spacing in a 'near square' patterns where the pit centre placement has a random offset of up ±50 nm in X and in Y.[51] Using the osteogenic nanopattern in parallel with nanovibration was seen to have an even more stimulatory effect with respect to osteogenesis of MSCs/SSCs.[48]

Applications of nanovibrational stimulation

The theoretical applications of nanovibrational technologies are wide ranging and still in development. While Caplan's proposed autologous in vitro expansion of SSCs and re-introduction to patients holds value in elective, planned procedures it would have limited value in emergency setting such as trauma, due to the time needed to culture sufficient cells. Having a 'graft bank' of implantable bone, grown in vitro from healthy donors of allogenic SSCs, could provide a solution to this. Advancements of in vitro scaffolds to mimic the extracellular environment could provide options for customisable bone graft substitutes allowing precision in the graft size and shape. Clinical applications of customisable bone graft substitutes are wide ranging, from grafting defects due to trauma, infection or malignancy, to improved surgical options for arthrodesis procedures.

With any new or advancing cellular therapy, safety is key. Proof of concept studies are required to translate in vitro research into clinically valid treatment options. While nanovibrational stimulation has shown to stimulate osteogenesis, early-stage trails could combine nano vibrational allogenic cells with existing scaffolds already on the market. This would provide understanding of how the SSCs would react in vivo and potentially expand the translation of cellular engineering of bone graft substitutes.

Williams et al have developed a wearable device to deliver continuous nanovibration to the hindlimb bones of rats with complete spinal cord injury (SCI). This intervention aimed to reverse SCI-induced osteoporosis. However, the results showed that the intervention did not reverse established osteoporosis in this model.[42]Despite not reversing osteoporosis, the study found an elevated concentration of the bone formation marker procollagen type 1 N-terminal propeptide (P1NP) in rats receiving 40 nm amplitude nanovibration. This suggests increased synthesis of type 1 collagen, indicating a potential positive effect on bone formation.[42] Demonstrating early potential for a wearable device to alter bone production. Similarly, the use of Low intensity pulsating ultrasound (LIPUS) in combination with whole body vibration has also shown to improve bone to implant integration in rat studies.[52] The heterogeneity of available evidence for LIPUS makes it difficult to justify its use in fracture managment.[53]

Conclusion

Innovation in bone regenerative therapies require a broad understanding of the molecular and environmental factors which can influence osteogenic cells. It is with advancements in the understanding of the extracellular microenvironment that has borne experimental innovation of Campsie et al's nanovibrational bioreactor; putting into practice the influences of mechanical signalling pathways to stimulate in vitro osteogenesis.

Utilising the role of mechanical signalling pathways with a combination of nanovibrational stimulation and nanotopography it is possible to stimulate an SSC toward an osteogenic lineage. Application of this knowledge and technology could range from developing cellular therapies for bone graft substitutes, implantable or wearable devices delivering nanovibrational stimulation to a patient. Additionally advances in nanotopography could advance implant design making them potentially better suited for osteo-integration, improving implant longevity and thus reducing the need for further surgeries.

Future challenges lie in the translation of in vitro studies into clinical trials. Evidence of successful implantation of osteoblast cells, grown in vitro, could open the possibility of custom-made bone graft as an alternative to currently available bone or bone graft substitutes. Nanovibrational stimulation acts as a realistic, scalable method to stimulate osteogenesis. The advancements in understanding and directing mechanical stimulation has allowed cellular engineering of osteoblasts in absence of chemical or genomic alteration.

Author contributions

MM primary author, MD, DWS, RDM critical revision and final approval of the manuscript.

Conflict of interest

The authors declare no competing interests.

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