Chapter 11

Bioreactor-Based Bone Tissue Engineering

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Additional information is available at the end of the chapter

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Abstract

The aim of this chapter is to describe the main issues of bone tissue engineering. Bone transplants are widely used in orthopedic, plastic and reconstructive surgery. Current technologies like autologous and allogenic transplantation have several disadvantages making them relatively unsatisfactory, like donor site morbidity, chronic pain, and immunogenicity and risk hazard from infectious disease. Therefore, regenerative orthopedics seeks to establish a successful protocol for the healing of severe bone damage using engineered bone grafts. The optimization of protocols for bone graft production using autologous mesenchymal stem cells loaded on appropriate scaffolds, exposed to osteogenic inducers and mechanical force in bioreactor, should be able to solve the current limitations in managing bone injuries. We discuss mesenchymal stem cells as the most suitable cell type for bone tissue engineering. They can be isolated from a variety of mesenchymal tissues and can differentiate into osteoblasts when given appropriate mechanical support and osteoinductive signal. Mechanical support can be provided by different cell scaffolds based on natural or synthetic biomaterials, as well as combined composite materials. Three-dimensional support is enabled by bioreactor systems providing several advantages as mechanical loading, homogeneous distribution of cells and adequate nutrients/waste exchange. We also discuss the variety of osteoinductive signals that can be applied in bone tissue engineering. The near future of bone healing and regeneration is closely related to advances in tissue engineering. The optimization of protocols of bone graft production using autologous mesenchymal stem cells loaded on appropriate scaffolds, exposed to osteogenic inducers and mechanical force in bioreactor, should be able to solve the current limitations in managing bone injuries.

Keywords: bioreactor, bone, stem cells, differentiation, scaffold

1. Introduction

Tissue engineering is a multidisciplinary field that applies basic concepts and techniques of life science and engineering. It is generally understood as a process of taking human or animal tissues, isolating cells from the tissues, culturing the isolated cells in a supporting material, i.e., scaffold to fabricate cell-scaffold complex, and transplanting the fabricated cell-scaffold complex into human or animal subject. It is applied to fabricate almost every human organ including artificial skin, artificial bone, artificial cartilage, artificial cornea, artificial blood vessels and artificial muscles.

Bone is one of the few organs/tissues capable of spontaneous regeneration rather than simple repair. In other words, after disruption of its structure (fracture), its unique microanatomy and biological properties enable complete structural restoration without the creation of fibrotic scar tissue. However, in certain clinical situations where extensive injury, disease or malformation cause such large defects, it is necessary to resort to bone reconstruction, restoration and/or regeneration by a surgical procedure that replaces missing bone, i.e., by bone grafting. A bone graft is an implanted material that promotes bone healing alone or in combination with other material(s), through osteogenesis, osteoinduction and osteoconduction, in combination or alone [1].

The selection of an ideal bone graft relies on several factors such as tissue viability, defect size, graft size, shape and volume, biomechanical characteristics, graft handling, cost, ethical issues, biological characteristics and associated complications. The materials used in bone grafting can be divided into several major categories, including autografts, allografts, and xenografts. Synthetic and biologically based, tissue-engineered biomaterials and combinations of these substitutes are other options. Altogether, tissue-engineered bone graft requires the optimal selection of cells that are seeded on biomaterial-based scaffolds and exposed to specific biochemical and physical signals known to induce osteogenesis. The development of the successful bone tissue-engineering protocols depends very much on our understanding of bone structure, physiology and development.

Bone is a dynamic biological tissue consisting of metabolically active cells. The cell component of bone consists of the precursor cells (progenitors), osteoblasts, osteoclasts, osteocytes and bone marrow hematopoietic elements. Osteoblasts are metabolically active mature bone-forming cells. They secrete osteoid, non-mineralized organic corpuscle that in turn undergoes mineralization process. Osteocytes are mature osteoblasts trapped within the bone matrix. Every osteocyte extends network of cytoplasmic tubules to the blood vessels and other cells. These cells are involved in the control of extracellular calcium. Osteoclasts are large multi-nucleated cells that degrade bone. Beside cells, bone is also composed of organic and inorganic elements. Approximately 20% of the weight of bone is water until the weight of dry inorganic bone makes calcium phosphate (65–70%) and the organic matrix of fibrous proteins and collagen (30–35%). Bone formation models in vitro are based on the fact that cell differentiation and function can be modelled according to factors that are important for embryonic development. Stem cells represent the building blocks of our bodies, functioning as the natural units of embryonic generation during development and adult regeneration following tissue

damage. Stem cells are undifferentiated cells that can, under certain influence, differentiate into specialized cells and tissues. During development, the potency of stem cells decreases from totipotent stem cells (morula stage), capable of differentiating into all embryonic and extra embryonic tissues, to pluripotent stem cells (PSCs) (blastocyst stage), forming all embryonic tissues, and to multi- or unipotent adult stem cells (ASCs), forming tissues within their germ layer or tissue compartment [2]. Here, we discuss clinically relevant multipotent ASCs found in various adult tissues. Adult stem cells, also called somatic stem cells, in adult organism act as repair system for the body, replenishing adult tissues, prompt tissue homeostasis throughout life and ensure tissue regeneration following damage and they have great potential in regenerative medicine. Mesenchymal stem cells replenish connective tissues including bone. Therefore, they are the first choice among ASCs for regeneration of bone tissue.

Osteogenic differentiation *in vitro* is induced by ascorbic acid, b-glycerophosphate and dexamethasone. Ascorbic acid is essential for the development of osteoblasts, serves as a cofactor in the synthesis of collagen and stimulates the production of extracellular matrix, proliferation and differentiation of cells. b-Glycerophosphate serves as a source of phosphate for the formation of calcium phosphate *in vitro*. It is also responsible for the formation of three-dimensional bone nodules between cells as proof of realized osteoblast phenotype. Dexamethasone (DEX) is composed by a synthetic glucocorticoid, which regulates the expression of osteoblast genes, enhances differentiation *in vitro*, alkaline phosphatase activity and mineralization of bone. Understanding of osteoblast differentiation provided us the information on key pathways' components and enabled us the induction of differentiation using different recombinant proteins like BMP-2, -6 to -9. Also, mechanical stimulation promotes osteoblast differentiation and induces mineralization of extracellular matrix. Mechanical stimulation can be achieved using steady and dynamic fluid flow in bioreactors. For this purpose, different dynamic culture systems have been developed. These systems improve nutrient delivery to the cells and generate shear stress that promotes cell differentiation into osteoblastic phenotype. Bioreactors for bone engineering applications are broadly classified in few main categories, including rotating wall vessels, spinner flasks, perfusion bioreactors and compression systems. In addition to these, combinations of different bioreactors types have been explored in order to better mimic the bone physiological environment *in vitro*, such as for example compression bioreactors with added perfusion [3]. The process of bone tissue engineering in three-dimensional dynamic bioreactor system is a recapitulation of bone healing process *in vivo* in which progenitor cells, due to signals in the microenvironment, are stimulated to differentiate into osteoblasts [4].

2. Cells in bone engineering

2.1. MSCs as the best choice

The important step in bone engineering is the choice of human cell sources that can efficiently produce bone grafts when attached to proper mechanical support with the addition of osteogenic supplements. Cell types that can be potentially used in bone engineering are

primary osteogenic cells isolated from adult bone tissue and periosteum, embryonic stem cells (ESCs), induced PSCs (iPSCs) and ASCs.

The selection of appropriate cell source for bone tissue engineering depends on several factors such as:

- Possibility of application of patient's own (autologous) cells or another person's (allogeneic) cells;
- Availability and ease of tissue harvesting with minimal donor site morbidity;
- Efficiency of cell isolation and cell yield;
- Potential of cell proliferation;
- Use of cells that have both osteogenic and vasculogenic potential to support the formation of vascularized bone;
- Homogeneity of the obtained cell population;
- Control of induction of osteogenic phenotype;
- Phenotype stability and cell safety;
- Automation and good manufacturing practices production.

Among the mentioned candidates, mesenchymal stem cells (MSCs), as a member of the ASC group, currently possess characteristics that make them the most appropriate cell source for bone tissue engineering. Unlike ESCs which are pluripotent and have unlimited potential for proliferation *in vitr*o [5], MSCs possess multilineage differentiation potential and have limited proliferation capacity [6, 7]. In bone tissue engineering, ESCs gained enormous value as a cell source for the derivation of multiple lineages present in adult bone, such as osteogenic cells, vascular cells, osteoclasts, nerve cells and others. Despite increasing interest in the application of ESCs in bone regeneration strategies, use of this cell source is limited due to political issues and ethical concerns as well as safety reasons. The primary concern is the source from which ESCs are derived. The most commonly referenced pluripotent cells are ESCs derived from the inner cell mass of blastocyst which results in destruction of the embryo [8]. It has also become clear that pluripotency is a double-edged sword; the same plasticity that permits hESCs to generate hundreds of different cell types also makes them difficult to control. Transplantation of hESCs into immune-deficient mice leads to the formation of differentiated tumors comprising all three germ layers, resembling spontaneous human teratomas [9, 10]. Karyotype abnormalities have been observed in ESCs as well as in human iPSCs [11]. Therefore, further studies are needed to ensure the stability and safety of ESC-derived progenitor populations before their potential use in clinical applications. Because these particular cells have created an ethical debate, the researchers have investigated fetal stem cells derived from voluntary interruption of pregnancy as a potential cell source for bone tissue engineering [12]. The cells that have potential medical applications, especially in organ regeneration [13–17], and importantly posess no ethical issues concerning their employment are amniotic stem cells. They are mixture of stem cells that can be obtained from the amniotic fluid [18–20] and the

amniotic membrane [21]. They represent a novel class of PSCs with intermediate characteristics between embryonic and ASCs, as they are able to differentiate into lineages representative of all three germ layers but do not form tumours when injected *in vivo* [22]. They can develop into various tissue types including skin, cartilage, cardiac tissue, nerves, muscle and bone [23–25]. In 2006, Kazutoshi Takahashi and Shinya Yamanaka established for the first time murine ES-like cell lines from mouse embryonic fibroblasts (MEFs) and skin fibroblasts by simply expressing four transcription factor genes encoding Oct4, Sox2, Klf4 and c-Myc [26]. They called these somatic cell-derived cell lines iPSCs. iPSCs exhibit similar morphology and growth properties as ESCs and express ESC-specific genes. The discovery that somatic cells can be reprogrammed into iPS cells has already had major effects on research in stem cell biology and regenerative medicine, but many obstacles remained and need to be resolved to take full advantage of this technology in research and therapy [27]. Therefore, the current clinical protocols are based on the use of autologous MSCs as the cell population that is safe and easy to obtain.

2.2. Sources of human MSCs

Extensive research of adult MSCs started in 1970 when Freidenstein et al. discovered these cells in bone marrow tissue [28]. Later, the presence of MSC-like population was discovered in a wide range of adult tissues, including trabecular bone [29], synovium [30], adipose tissues [31], skeletal muscle [32], periosteum [33], dermis [34], blood [35, 36] deciduous teeth [37], amniotic fluid [38] and umbilical cord blood [39]. Bone marrow-derived mesenchymal stromal cells (BM-MSCs) have become one of the main cell sources for bone tissue engineering [40, 41]. Isolation of MSCs from bone marrow requires invasive procedures that can be quite painful. Bone marrow aspirate could be obtained from the iliac crest, tibia or femur. Typically, the frequency of MSCs in whole bone marrow of adults is between 5×10^{-4} and 10^{-5}, which corresponds to yield of a hundred MSCs per milliliter of marrow. Even though BM-MSCs are rare, they are readily separated from the hematopoietic stem cells in culture by their preferential attachment to the plastic surface [42] and can be easily expanded *ex vivo*. The presence of MSC in adipose tissue has gained considerable attention because of the ease of accessibility of adipose tissue and its abundance in the body. Adipose tissue-derived mesenchymal stem cells (AD-MSCs) were first identified in 2001 by Zuk et al. A major advantage of AD-MSCs is their relative abundance as well as their faster proliferation rate compared to BM- MSCs, which allows more rapid expansion to obtain clinically relevant cell numbers [43, 44]. AD-MSCs have similar osteogenic potential to BM- MSCs with the added advantage of being highly abundant. For example, as many as 1×10^7 AD-MSCs can routinely be isolated from 300 ml of lipoaspirate, with purity greater than 95% [45, 46]. Comparative analysis of human BM-MSCs and AD-MSCs by Li et al. revealed that AD-MSCs have biological advantages in the proliferative capacity, secreted proteins (basic fibroblast growth factor, interferon-γ and insulin-like growth factor-1) and immunomodulatory effects, but BM-MSCs have advantages in osteogenic and chondrogenic differentiation potential and secreted proteins (stem cell-derived factor-1 and hepatocyte growth factor) [47]. These biological advantages should be considered systematically when choosing the MSC source for specific clinical application. Nevertheless, the utilization of human AD-MSCs in scaffolds for bone tissue engineering has been heralded as

the alternative strategy of the twenty-first century to replace or restore the function of traumatized, damaged or lost bone.

MSC-like cells can be derived from the umbilical cord from a newborn baby which contains two arteries and a vein covered with mucus connective tissue rich in hyaluronic acid, referred to as a Wharton's jelly [48]. The blood from the umbilical cord is a rich source for pluripotent cells named as umbilical cord blood derived MSCs (UCB-MSCs). These cells are quite similar to bone marrow-derived MSCs and have osteogenic potential in an optimized culture [49]. Many investigations have thus far been conducted on bone engineering by using these cells and various scaffolds [50].

Several stem cell types in dental tissue have been reported including dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), stem cells of the apical papilla (SCAP), periodontal ligament stem cells (PDLSCs) and dental follicle progenitor cells (DFPCs) [37, 51]. Since DPSCs can be easily isolated by enzymatic digestion of pulp tissue, many studies have been conducted regarding bone engineering with these cells and appropriate 3D scaffolds [52, 53].

2.3. Phenotypic characterization of MSCs

Phenotypic characterization of MSCs is usually carried out using immunocytochemical detection or fluorescence-activated cell sorting (FACS) analysis of cell surface molecule expression [54, 55]. Methods of immunodepletion using such techniques as magnet-activated cell sorting (MACS) have also been used in the negative selection of MSCs [56]. However, the lack of specific markers renders the characterization of MSCs difficult and sometimes ambiguous, especially because many of these epitopes are shared between hematopoietic and mesenchymal stem cells. It is interesting that MSCs from different species do not express the same markers. The use of multiple markers such as cell surface cluster of differentiation (CD) markers, ECM proteins, cell adhesion molecules, integrins, cytokines genetic and proteomic fingerprinting can help identify MSCs. The most commonly used markers to identify MSCs are CD markers. Positive MSC markers include: Stro-1, SH2 (CD105), SH3 (CD73), SH4, CD29, CD44, CD54, CD90, CD133, CD166 and p75LNGFR, whereas negative markers are CD11, Cd14, CD19, Cd31, CD34, Cd45, CD79 and HLA-DR [57]. The International Society for Cellular therapy has provided minimum criteria for defining MSCs. Acceptable MSCs meet the minimum requirements of CD73, CD90 and CD105 positive and CD14, CD34, CD45 and HLA-DR negative expression [58].

2.4. Nonimmunogenic properties and immunosuppressive nature of MSCs

Previous studies have shown that yield of MSCs is affected by age and health of a donor. The trend is that yield is decreased with donor age. Patients with degenerative diseases, such as osteoporosis and osteoarthritis, tend to have lower MSC yield although they would benefit the most from MSC-based therapies. The alternative is the use of allogeneic MSCs because they have low immunogenic potential and immunosuppressive properties. Immunologic phenotypes of hMSCs are: positive expression for major histocompatibility complex (MHC) class I

molecules, minimal expression for MHC class II and do not express the co-stimulatory molecules CD40, CD40 ligand, CD80 and CD86 [59–62]. MSCs do not fully activate T cells owing to the absence of CD80 and CD86 in their membrane. Apart from not being recognized as alloantigens, MSCs are able to suppress the activation and proliferation of different cells of the host immune system [59, 63–66]. Interleukin-10, transforming growth factor beta (TGF-β), hematopoietic growth factor (HGF), prostaglandin E2 (PGE_2), indoleamine 2,3-dioxygenase (IDO) and nitric oxide (NO) were some of the soluble molecules associated with the immunosuppressive effect of MSCs [67–69]. Another important soluble molecule involved in the immunoregulation of MSCs is HLA-G5, a non-classical human leukocyte antigen (HLA) class I protein that protects the fetus against rejection from the maternal immune system [70]. The HLA-G5 isoform released by MSCs can suppress allogeneic T cell proliferation and can also induce the expansion of $CD4^+CD25^{high}FOXP3^+$ regulatory T cells (Tregs). With regard to innate immunity, HLA-G5 is able to inhibit the lysis of MSCs mediated by NK cells, as well as the secretion of IFN-γ by these cells [71].

2.5. Tumor formation risk in MSCs application

In general, it is believed that MSCs can be safely cultured *in vitro* without risk of spontaneous malignant transformation [72], but there have been no reports of human trials demonstrating the formation of tumors with culture-expanded MSCs [73]. Concerns have been raised about the safety of MSCs for clinical use as there have been some reports of sarcoma formation by cultured murine MSCs *in vitro* and *in vivo* [74, 75]. The mechanism by which MSCs are transformed into malignant cells is known to be related to chromosomal abnormalities, including structural and numeric aberrations, and increases with higher passage numbers. Rubio et al. showed that although MSCs can be managed safely during the standard *ex vivo* expansion period (6–8 weeks), human MSCs can undergo spontaneous transformation following long-term *in vitro* culture (4–5 months), and the transformed cells lead to the formation of tumors in mice [76].

2.6. Osteogenic differentiation of MSCs

Various *in vitro* protocols have been developed to induce hMSCs to differentiate into mesodermal lineages, such as osteoblasts, chondrocytes, adipocytes, as well as transdifferentiate into tissue cells derived from different germ layers, such as neuronal cells or insulin-producing cells [55, 77, 78]. Osteogenic differentiation is a highly programmed process consisting of many stages, including proliferation, differentiation, matrix deposition, mineralization and matrix maturation. The general protocol for *in vitro* bone differentiation of MSCs involves incubation of the cell monolayer in a culture medium containing DEX, β-glycerophosphate and ascorbic acid for a period of 2–3 weeks (**Figure 1**) [79]. DEX is a synthetic glucocorticoid that stimulates MSC proliferation and is essential for osteogenic differentiation [80, 81]. Although the mechanisms underlying DEX's effects are not well known, it has been speculated that DEX upregulates the beta catenin-like molecule TAZ, which results in upregulation of Runx2-related transcription factor and osteogenic differentiation [82]. The optimal concentration of this reagent for MSC bone differentiation is approximately 10 nM, which corresponds to

physiologic concentrations [83]. Organic phosphate released after enzymatic hydrolysis of beta glycerol phosphate plays an important role in matrix mineralization. This free phosphate is usually applied in 5–10 mM concentrations for MSC bone differentiation. Ascorbic acid is a cofactor in the hydroxylation of prolines and lysine moiety of collagen molecules and is an abundant protein in the ECM. This reagent is used in 50–500 μM concentrations [84]. When MSCs are cultured in osteogenic media, they express the same markers as bone-forming osteoblasts that are responsible for laying down the matrix and mineral during new bone formation *in vivo*. The osteogenic differentiation of MSCs *in vitro* has been divided into three stages. The first stage (days 1–4) is the proliferation stage where a peak in the number of cells is seen. This is followed by early cell differentiation (from days 5 to 14), which is characterized by the transcription and protein expression of alkaline phosphatase (ALP). After this initial peak of ALP, its level starts to decline. Also found at an early stage is the expression of a collagen type I matrix onto which the mineral is deposited. The final stage (from days 14 to 28) results in a high expression of osteocalcin and osteopontin, followed by calcium and phosphate deposition [4, 85].

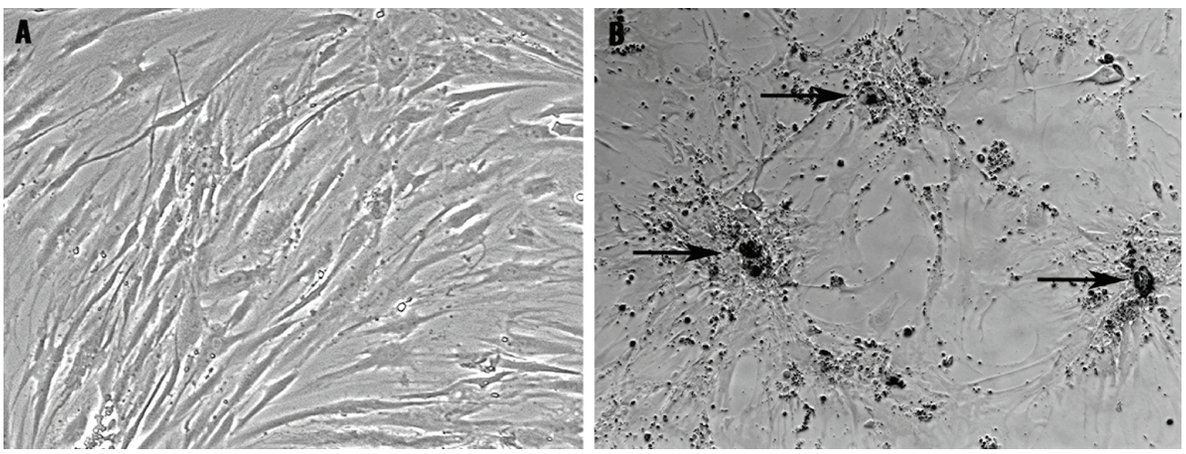

Figure 1. Monolayer of mesenchymal stem cells derived from human bone marrow before (A) and after 3 weeks of (B) differentiation. Arrows mark bone-forming nodules.

In addition to osteogenic supplements, there are other substances that act as biochemical signals capable of triggering cellular processes like growth, proliferation or differentiation. The most common growth factors in bone tissue engineering are listed below.

Bone morphogenetic proteins (*BMPs*) are a family of cytokines that stimulates the proliferation of chondrocytes and osteoblasts and increases extracellular matrix production. BMPs induce the differentiation of MSCs into osteoblasts. BMPs allow not only skeletal tissue formation during embryogenesis, growth, and adulthood but also bone healing process. In newborns' skeletons, BMPs can be found in the collagen fibers of the bone matrix and also in cells located in the periosteum and the bone marrow. After a fracture, BMPs' growth factors diffuse from bone matrix and activate osteoprogenitor cells which, in turn, produce more BMPs [86].

Fibroblast growth factors (*FGFs*) stimulate the proliferation of mesenchymal cells, osteoblasts and chondrocytes. FGFs enhance the growth of different tissues due to their angiogenic

properties. FGF-2 or bFGF is the most studied cytokine of this family for bone regeneration applications [87].

Insulin-like growth factors (*IGFs*) promote the proliferation of osteoblasts and chondrocytes and induce matrix secretion from both cell types [87]. IGFs stimulate collagen synthesis and mineralization of bone tissue [88].

Platelet-rich plasma (*PRP*) is another known source of various growth factors, namely, platelet-derived growth factor, transforming growth factor-β and vascular endothelial growth factor. The applicability of PRP for the repair of bony defects is well established [89] and several investigators have advocated the use of this product in combination with MSCs [90].

3. Scaffolds in bone engineering

3.1. Scaffold properties

The evolution of bone implant devices has resulted in an increase in knowledge about the microenvironment where the replacement will occur, which results in changes in requirements and properties of the biomaterials used. This evolution can be measured by defining three different generations. However, these generations are not chronological but technological since there is currently active research and development for each. First-generation bone graft substitutes require the biomaterial to match the physical properties of the tissue to be replaced, while maintaining inertness with the tissue microenvironment. These include metals such as stainless steel, titanium and alloys; ceramics such as alumina and zirconia; and polymers such as silicone rubber, polypropylene and polymethylmethacrylate. Second-generation bone graft substitutes are made biodegradable with the aim that the rate of degradation matches the healing rate of the injured bone tissue. These biomaterials are based on the use of synthetic or natural polymers that can provide a controlled chemical breakdown under physiological conditions into inert products that can be resorbed by the body. Examples of the synthetic polymers include polylactide, poly(ε-caprolactone) and polyglycolide; and collagen, chitosan and hyaluronic acid for natural ones. The mechanical and osteoconductive properties of these polymers can be improved by forming composites with bioactive ceramics. Third-generation bone graft substitutes try to get closer to the autograft standard by using biomaterials capable of inducing specific cellular responses at the molecular level, by integrating the bioactivity and biodegradability of second-generation devices. This type of bone graft is based on the concept of bone tissue engineering, which focused on creating a device that enhances bone repair and regeneration by incorporating bone progenitor cells or/and bioactive signals (e.g., growth factors, small molecules) to stimulate cells into a scaffold made of various natural or synthetic biomaterials or their combination and with sufficient vascularization to allow access to nutrients to support this process. Nowadays, many groups worldwide seek to develop scaffolds with osteoinductive properties that would enhance bone healing. These scaffolds have to accomplish certain requirements and have to be:

Biocompatible—cells must populate the scaffold, adhere and proliferate. They should be able to migrate as well as differentiate. Overall, cell function should not be compromised. The

scaffolds should enable unobstructed transport of nutrients, gases, signaling molecules, proteins and waste products in, out and within the scaffold.

Biodegradable—the scaffold should be replaced with host/donor cells (tissues). Therefore, scaffolds must be biodegradable and byproducts must not be toxic. Ideal scaffold degradation should mirror the rate of new tissue formation.

Biofunctional—the scaffold should meet as many as possible functional requirements of the replaced tissue. Good scaffold should have specific mechanical properties and architecture, similar to properties of the replaced bone tissue. Properties like elasticity, permeability, compressibility, viscoelastic behavior, tensile strength and failure strain [91] should be similar and should give shape to the tissue that is regenerated on it [92]. It is very important to have strong, but at the same time, porous bone grafts. The pores should be big enough to allow smooth cell migration and proliferation besides vascularization and small enough to enable cell-to-cell communication and critical cell repopulation of the pores. Pores are crucial in a process of degradation. Their size should allow and promote scaffold degradation.

The main disadvantage of scaffolds is the lack of vascularization. Inspired by the nature of bone, different scaffolds have been studied extensively, and the main challenge is to precisely balance a desired structural strength and porosity. To design bone scaffolds, materials should have the desired biological properties for a specific application and should not be immunogenic causing inflammatory response. The long-term goal is the development of the scaffold that can be applied in a clinical setting. Manufacturing technology should follow good manufacturing practice (GMP) procedures. Ultimately, the main goal is to develop scaffold that fulfills all previously mentioned requirements and has slow-release properties of bioactive molecule. Multiple factors (signaling peptides, adhesion peptides, growth factors, plasmid DNA, antibodies, microRNAs, etc.) can be incorporated into scaffolds to promote osteoblast migration, to manipulate tissue formation and to effectively enhance bone regeneration [93]. For instance, bone morphogenic protein 2 (BMP-2) was photo-crosslinked into biodegradable diblock copolymers PEG-PLA and was slowly released as the polymer degrades [94]. Another approach is to covalently bind the adhesion peptide like well-known arginine-glycine-aspartate ligand or chemotactic factor like platelet-derived growth factors (PDGFs), to attract osteoblast and promote osteogenesis [95, 96] or incorporate angiogenic (FGFs [97]) and anti-angiogenic factors to control scaffold vascularization [98]. MicroRNAs can post-transcriptionally regulate gene expression and alter bone regeneration [99]. There are many problems to that approach, and the major one is controlled release of bioactive substance together with its rapid dilution. To reduce the risk of BMP dilution following release from the scaffold, monoclonal anti-BMP antibodies are encapsulated within the scaffold [100].

3.2. Scaffold types

With respect to source of biomaterials, scaffolds can be divided into two main groups: the ones made from natural and the ones made from synthetic materials. The natural biomaterials are obtained from natural sources and processed to make desired scaffolds. A few decades ago, researchers have discovered that decalcified bone matrix (DBM) possesses inherent osteoinductive properties (http://www.ncbi.nlm.nih.gov/pubmed/4870495), and DBM was used in

the treatment of clinical orthopedic situations which has shown favorable results [101, 102]. Decellularized ECM (mammalian extracellular matrix) scaffolds recovered from allografts (tissue from individuals of same species) and xenografts (tissue from individuals of different species) have a desired three-dimensional porous structure and can be repopulated by host bone-forming cells. ECM is a complex of different glycosaminoglycans, glycoproteins and huge number of different small proteins. The cells can easily attach, grow and differentiate with excellent viability. Decellularization and treatments such as freeze-drying, irradiation and washing with acid minimize their immunogenicity, but some epitopes can still be recognized by the host. These treatments prevent any infection to be transferred from the tissue, but can affect their mechanical and biological properties [97]. Most commonly used biological materials for bone tissue engineering are chitosan, collagen, hyaluronic acid, alginate, elastin, cellulose, fibrin, gelatin, etc. Chitosan is a hydrophilic, linear polysaccharide (suh, matthew, application of chitosan-based) obtained by alkaline deacetylation of chitin from shrimp and other crustacean shells. It has many beneficial properties, such as biocompatibility (no inflammatory or allergic reaction, (chatelet, damour, influence of the degree), biodegradability (it is naturally degraded by hydrolytic enzymes such as lysozyme) and no toxicity [103]. Since collagen is the most abundant protein in various tissues including bone, scaffolds made of collagen are very attractive for biomedical applications. Collagen is composed from two α1 chains and one α2 chain wrapped together by hydrogen and covalent bonds to form right-handed triple helix. These fibers spontaneously pack together to form long thin fibrils of similar structure. Collagen is an attractive material for a scaffold synthesis because its mechanical properties can be altered by crosslinking, either with different chemicals (glutaraldehyde, formaldehyde, etc.) or with physical treatments (UV irradiation, heating, etc.) [104–106]. Hyaluronic acid is a simple linear polysaccharide composed of a repeating disaccharide, and it is hydrophilic, nonimmunogenic, and easy to modify and produce. It is easily replaced by extracellular matrix produced by host cells due to hyalurodinase degradation. These materials have a huge biological activity; they promote cell adhesion as well as cell growth. They are biodegradable, allowing host cells to replace the scaffold with their own extracellular matrix. The main drawbacks are their poor mechanical properties limiting their use as bone grafts and the reproducibility of their synthesis. Immunogenicity, limited physical and mechanical stability as well as limited resource of biomaterials have encouraged researches to develop composites using synthetic materials.

Typically, two individual groups of synthetic biomaterials are used in the fabrication of bone grafts: ceramics and synthetic polymers. Ceramics polymers (inorganic oxides and salts), such are hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and biphasic calcium phosphate (BCP) are mechanically stiff and have very low elasticity, making them suitable only for bone tissue grafts. Ceramics perfectly imitate natural bone structure, and cell interaction with ceramics promotes proliferation as well as differentiation of osteoblasts [107].

Synthetic polymers, such as polystyrene, polyglycolic acid (PGA) and poly-l-lactic acid (PLLA) acid, have the rewarding and satisfying properties because their architecture can be adjusted and changed by the composition of the polymer as well as by altering the synthesis method. However, cell might have difficulties to attach and proliferate on their surface, so there is

always the risk of rejection due to reduced bioactivity. Degradation of synthetic polymers becomes the major issue because most of them are degraded by hydrolysis, causing lower local pH and cell necrosis [108].

Since ceramics have excellent osteoinductive properties but low mechanical strength, and synthetic polymers exhibit poor osteoinductivity but better mechanical strength and degradability, in the past decade researches have been trying to develop the scaffolds made of ceramic and polymer composites. Most commonly used 3D composites are made of synthetic polymers such as poly(lactic) acid (PLA), PGA, poly(ε-caprolactone) (PCL), poly(lactic-co-glycolide) (PLGA), poly(propylene fumarate) (PPF) and natural polymers such as collagen type I and chitosan. These composites have rigid sponge-like structures often containing HA (133-138 from three-dimensional alexander). Hydroxyapatite increases attachment of mesenchymal stem cells, differentiation to osteoprogenitors and promotes cell survival [109, 110].

3.3. The ideal scaffold for bone tissue engineering

The ideal scaffold is difficult to obtain and should be biocompatible, bioresorbable, osteoconductive (must allow bone cells to adhere, proliferate and secrete extracellular matrix), osteinductive (with the ability to induce new bone formation), osteogenic (should act as MSCs and osteoblasts reservoir), structurally similar to bone enabling formation of strong bonds with surrounding bone tissue, as well as it should be easy to use and cost-effective. New approach includes development of methods to isolate and transplant bone tissue-forming cells, bioactive matrix materials that act as tissue scaffolds mimicking what happens in nature, and delivery of bioactive molecules within scaffolds. In the past two decades, many 3D systems have been

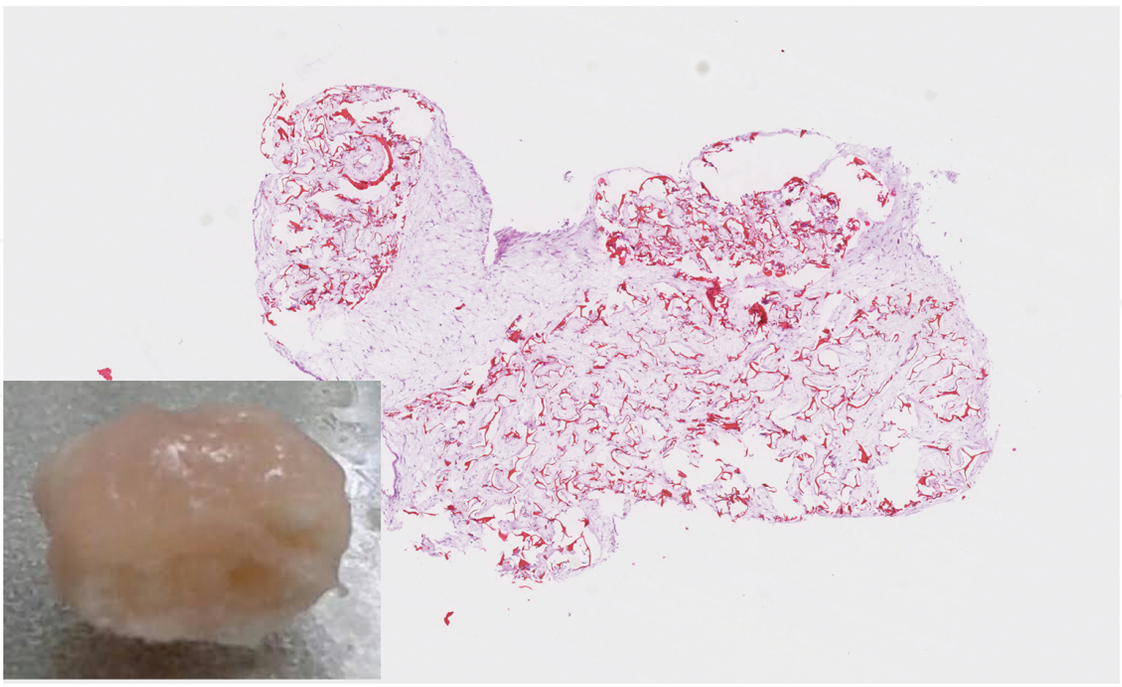

Figure 2. Bone graft grown on scaffold made of chitosan and hyaluronic acid. Section of graft stained with hemalaun/eosin staining shows equal cell distribution, formation of extracellular matrix and scaffold residues.

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studied and some have been commercialized for clinical application. The main advantage of this system is that cells grown in 3D environment that is more representative to natural bone tissue. Final goal is to produce a "living" scaffold providing mechanical support, bioactive signal as well as cells with osteogenic potential. Researchers have developed bioreactors to mimic physiological conditions. The main advantage is that this system enables controlled manipulation of all variables. In contrast to classic static *in vitro* cultures, bioreactors allow to apply mechanical stimuli that are very important in osteogenic differentiation [111]. Many different bioreactors to promote good osteogenesis (Koller reactors [112]), spinner flask bioreactors, [113] have been investigated. Recently developed perfusion bioreactors have shown high efficiency in uniformed cell seeding on a scaffold, enhanced proliferation, great supply of oxygen and nutrients throughout the scaffold as well as enhanced osteogenic differentiation because the pump forces the medium to flow through the scaffold (**Figure 2**) [114–117].

4. Systems for 3D cultivation of bone tissue

Ex vivo tissue-engineering (TE) strategies for *de novo* generation of bone tissue enclose the combined use of autologous bone-forming cells and three-dimensional scaffold materials serving as structural support for the cells [118]. Bioreactors are used as a tool for studying and mimicking *in vivo* conditions in an *in vitro* environment for the growth of tissue substitutes and represent the device used to develop biological processes by closely monitoring and controlling the environment [119]. Parameters that must be controlled and appropriately adjusted in order to perform controlled and successful experiments are:

- Temperature
- pH
- Oxygen diffusion
- Nutrient transport
- Waste removal

Tissue-engineering bioreactors can be used to aid the *in vitro* development of new tissue by providing biochemical and physical regulatory signals to cells, encouraging them to undergo differentiation and produce extracellular matrix prior to *in vivo* implantation [120].

This 3D cell expansion on a scaffold poses several challenges. The first challenge is the **transport of nutrients** to cells and **removal of waste** metabolites from the interior of the scaffold. In 2D cell culture diffusion provides nutrients and oxygen to all cells as well as waste removal, but in 3D constructs diffusion is insufficient [121]. That represents an important issue in tissue engineering, limiting the tissue growth due to insufficient nutrient transport [122]. To overcome this problem, scientists developed more complex bioreactor systems 3D tissue culture to improve the media flow and transport of nutrients to cells which contribute to balanced development of tissue [118]. Dynamic bioreactor culture systems are essential for *in*

vitro cultivation and maturation of tissue-engineering bone grafts, in particular for larger bone grafts where the core of the scaffold is more than 200 μm from the surface. Bioreactors improve the mass transport of nutrients and overcome the diffusion limitation of traditional static culture [123]. Bioreactors bring several advantages into the culture of functional tissues. They do not only increase mass transport inside three-dimensional structures but also reduce the handling steps, hence reducing contamination potential.

Fluid shear stress caused by mixing or perfusion the medium is also very important for bone tissue engineering because it exposes the cells to mechanical stimulation. *In vivo*, mechanical loading increase production of prostaglandins, alkaline phosphatase, collagen type I, along with osteoblast proliferation and mineralization [124]. Mechanical loading of the skeleton causes interstitial fluid flow through lacunar and canalicular space of bones. The cells lining these spaces are then influenced by the mechanical stimulation provided by the fluid flow, differentiating or proliferating accordingly [125, 126]. Based on this knowledge, it is clear that the recapitulation of these mechanisms *in vitro* is essential for the growth and the regenerative properties of human osteoprogenitor cells seeded onto scaffolds [127]. *In vitro*, mechanical stimulation can encourage cells to produce extracellular matrix (ECM) in a shorter time period and in a more homogeneous manner than in static culture [128]. A benefit of ECM production is the increase in mechanical steadiness of the scaffold and tissue graft. Another important advantage of bioreactors is **induced cellular differentiation**. Mechanical stimuli can be used to encourage stem cells down a particular path and hence provide the cell phenotype required [129].

As well as providing mechanical stimulation, bioreactors can also be used to improve **cellular spatial distribution**. A heterogeneous cell distribution is a major problem in developing three-dimensional tissue or organ *in vitro* [130]. Scaffolds in larger size range are easily fabricated, but problems arise with culturing cells on these scaffolds. As the size of the scaffold increases, diffusion of cells to the center becomes more difficult. Static culture conditions result in scaffolds with few cells in the center [131]. Thus, bioreactors can be used in tissue-engineering applications to overcome problems associated with traditional static culture conditions, improve cellular distribution and accelerate construct maturation [132] while applying biophysical signals to constructs to improve tissue formation *in vitro* prior to *in vivo* implantation [120].

The ultimate design of a tissue engineering bioreactor system must: (i) ensure a controlled and rapid cell growth; (ii) facilitate uniform cell distribution; (iii) provide and maintain the physiological requirements of the cell (nutrients, oxygen, growth factors); (iv) increase mass transport both by diffusion and convection using mixing medium systems (v) expose cells to physical stimuli; and (vi) enable reproducibility, control, monitoring and automation. For this purpose, different dynamic culture systems have been developed. These systems improve nutrient delivery to the cells and generate shear stress that promotes cell differentiation into osteoblastic phenotype. Bioreactors for bone engineering applications are broadly classified into few main categories, including rotating wall vessel, spinner flask, perfusion bioreactor and compression systems. In addition to these, combinations of different types of bioreactors have been explored in order to better mimic the bone physiological environment *in vitro* and

all these systems for tissue culture are used to achieve a homogeneous cell growth within the scaffold [120].

4.1. Rotating wall vessel bioreactor

Cells that grow *in vitro*—outside the body in 2D layers do not behave in the same way as cells grown *in vivo*—inside the body. *In vivo* cells grow three-dimensionally and form tissues that have modified their structure to perform a specific function and secrete extracellular matrix. Two-dimensional growth represented a limit to the scientists who wanted to understand mechanisms that govern cell behavior and tissue formation. In the 1970s, a small NASA group of scientists began to think about space as a possible answer. The group believed if cells could be grown without the Earth's gravity influence, they would not settle to the bottom of the culturing container, instead they would be suspended in the medium and therefore might compound and form tissue that more closely resembles the tissue in the body [133]. The rotating-wall vessel (RWV), developed by NASA, was originally designed to protect cell cultures from high shear forces generated during the launch and landing of the space shuttle. When the device was tested on the Earth for cells in suspension, cells aggregated and formed structures similar to tissues. These observations led to the possibility that the bioreactors might be used to study co-cultures of multiple cell types and their association, proliferation and differentiation during the early steps of tissue formation [134].

The RWV bioreactor provides a low turbulence culture environment which promotes the formation of large, three-dimensional cell clusters. Due to their high level of cellular organization and specialization, samples constructed in this bioreactor more closely resembled the original tumor or tissue found in the body. Cartilage, bone marrow, heart muscle, skeletal muscle, pancreatic islet cells, liver and kidney are just a few of the normal tissues cultured in rotating bioreactors [133].

The RWV bioreactor (**Figure 3A**) consists of a cylindrical growth chamber with a gas exchange membrane. The solid-body rotation is accomplished by a vessel rotating horizontally around its axis, randomizing the gravitational forces acting on the cell surface. The culture chamber is completely filled with medium and is oxygenated through a silicone rubber membrane by an air pump that draws incubator air through the filter. As the vessel rotates, the liquid inside accelerates until the entire fluid mass is rotating at the same angular rate as the wall. Thus, this environment eliminates most of the disruptive shear forces associated with a conventional bioreactor, scaffolds and cells obey simple kinematics and are uniformly suspended in the culture medium with minimum shear forces. In this environment, cells aggregate and undergo three-dimensional growth to form tissue-like structures. As aggregates grow during culture, the speed of vessel rotation is increased to contrary gravitational sedimentation [134].

Cultures using an RWV bioreactor proved useful for growing tissues, such as bone. Many studies showed enhanced proliferation, distribution and differentiation of osteoprogenitor cells on scaffolds when cultured in a free fall manner in RWV-based bioreactor systems [135]. Until today, many designs of rotating bioreactor systems have been developed for dynamic 3D bone tissue engineering. One of them is RWV bioreactor with the scaffolds attached to the external wall by use of stainless steel clamps. External and internal cylinders were driven by

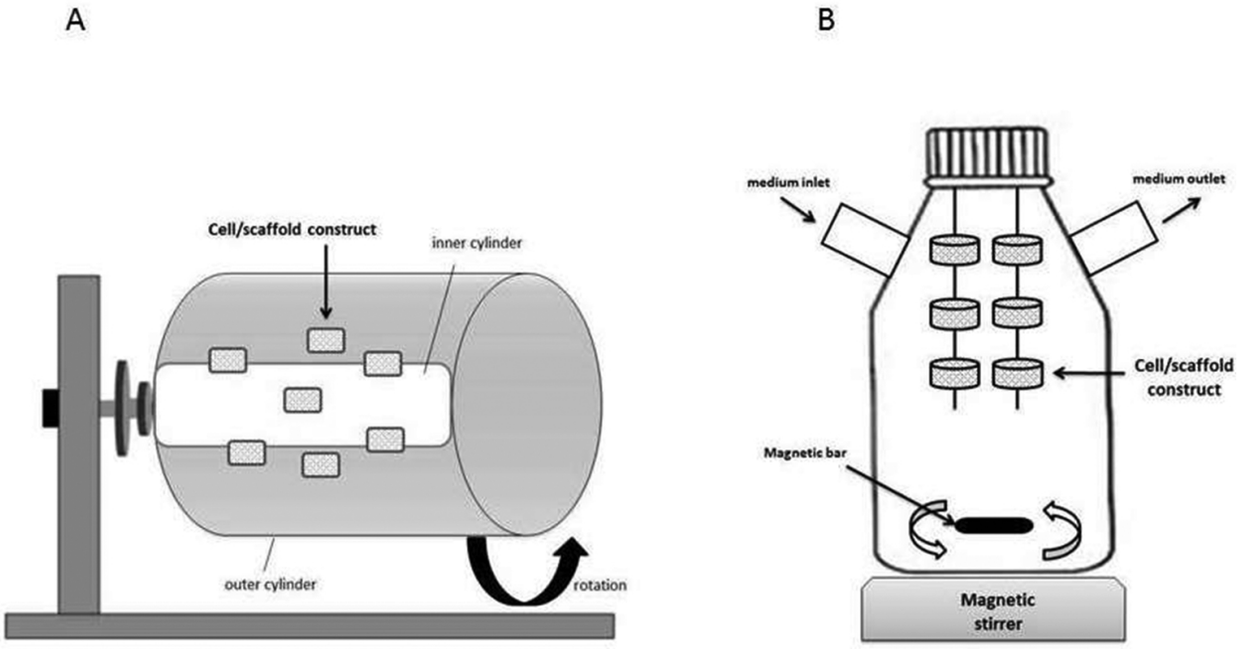

Figure 3. Design of rotating vessel (A) and spinner flask (B) bioreactor systems.

step motors and compared to the cultivation in static culture. The use of RWV resulted in better cell proliferation and differentiation. The second one is a rotating bed bioreactor (RBB). In an RBB, constructs are attached directly on the axis and moved between gas and liquid phases in an alternating manner. One major benefit of the system besides the positive effect in terms of proliferation and differentiation is the compatibility with good manufacturing practices (GMP) standards. Disadvantage of RWV system is the collision of scaffolds with the bioreactor wall, which may damage scaffolds and disrupt seeded cells. This can be omitted by the use of the RBB concept. Another major disadvantage of the rotating system is that mineralization is limited to the outer part of the scaffold. Internal nutrient transport limitations could not be eliminated by rotation-based bioreactor systems [118]. Rotating wall vessels are limited to the small-sized constructs due to insufficient transport inside the scaffold. Additionally, because of the low range of values of shear stress, these systems may not be efficient in promoting robust osteogenic differentiation. On the other hand, rotating wall vessels allow the accompanying culture of several cell/scaffold constructs. These systems could be adopted to engineer thin bone substitutes for the reconstruction of flat bones or as bone patches for restorative applications of the skeletal system [3].

4.2. Spinner flask bioreactor

A simple bioreactor system based on media mixing is the spinner flask (**Figure 3B**). Spinner flasks are composed of a glass media reservoir with side arms that can be opened to remove scaffolds and media and also to allow gas exchange. The flask has a stir bar mechanism that stirs the media in the flask. They are often used in bone tissue engineering because of the ability to increase expression of early osteoblastic marker alkaline phosphatase, late osteoblastic marker osteocalcin and calcium deposition as compared to static culture and rotating wall

bioreactors. This effect is the result of convective transport of nutrients to the surface of the scaffold in contrast to the purely diffusional transport in static culture. It also increases the concentration of oxygen throughout the scaffold [136].

Scaffolds are hanging attached to vertical needles from the top of the vessel and are submerged in the medium. The top of the vessel is usually used for gas exchange and medium oxygenation. Mixing of the medium is maintained by stir bar mechanism at the bottom of the vessel. The convective forces generated during stirring moderate the nutrient concentration gradients at the surface of the scaffold and produce turbulences that enhance mass transport according to the center of the samples [3].

Commonly, spinner flasks are around 120 ml in volume (up to 8 liters), are run at 50–80 rpm and 50% of the medium used in them is replaced every 2 days [137]. An important advantage of the spinner flask design is its maintenance of well-mixed environment within the flask. However, spinner flasks are not always an ideal solution, since the constant mixing motion causes turbulent flow within the capsule and the associated high shear stress. Spinner flasks have been modified form their original design to reduce the turbulent flow. Current designs induce small waves during mixing instead of the rough, turbulent flow induced from traditional spinner flasks. Spinner flasks are intended for small-scale production and do not appear to be used as much as other types. They are primarily used for the seeding of cells in 3D scaffolds until they are ready for more large-scale cell culture procedures [119].

4.3. Perfusion bioreactor

Spinner flasks and rotating wall bioreactors do not effectively perfuse media through the center of the scaffold. Bioreactors that use a pump system to perfuse media directly through a scaffold are known as perfusion bioreactors [136]. In perfusion bioreactors, scaffolds are placed in the perfusion chamber (**Figure 4A**) in a press-fit manner so that the medium is forced to pass through the center of the samples [3]. Flow perfusion bioreactors have been shown to provide more homogeneous cell distribution throughout scaffolds. This has resulted in greater cellularity throughout the scaffold in comparison to static controls, suggesting the better nutrient exchange [120].

These bioreactors have an advantage over the others because they provide a uniform mixing of the media, enabling better control of the environment and the physical stimulation of the cells in the bone tissue [121]. They are very effective for the culture of mesenchymal stem cells and have been shown to induce osteogenesis. This is attributed to the ability of the perfusion system to increase the transport of oxygen and nutrient through the scaffold and expose the cells to the mechanical stimulation [137]. The optimization of the perfusion bioreactor protocols for tissue engineering must ensure balance between the transport of substances and waste metabolites and hold newly synthesized tissue within the scaffold, taking care of the fluid flow rate which goes through the pores [120]. Many different perfusion bioreactor systems have been developed, but most systems are based on the similar basic design—media reservoir, pump, tubing circuit and scaffold chamber. The scaffold is sealed within the chamber so media cannot flow around it. Thus, media flows directly through the pores of the scaffold [136]. Scaffolds should have interconnected pores and should have between 70 and 99% porosity in

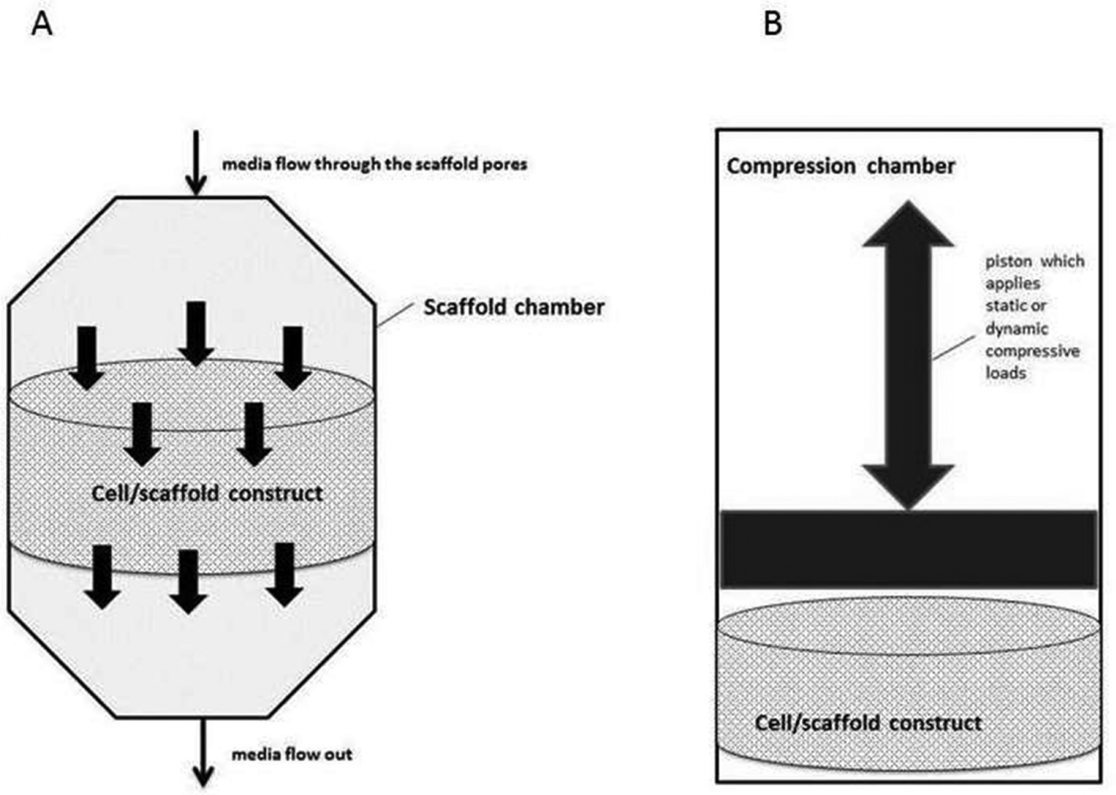

Figure 4. Design of the scaffold chambers in perfusion (A) and compression (B) bioreactor systems.

order to facilitate direct perfusion. In most cases, the major difference between the systems is the design of the perfusion chamber because it is the key element to ensure deep perfusion of the scaffold center [121]. Despite these difficulties, many perfusion bioreactor systems have been developed and tested for bone tissue-engineering purposes [136]. The pump produces a force that travels through the tubing circuit and perfuse the media through the scaffold pores in a continuous or noncontiguous way [120]. This force represents the perfusion flow rate applying mechanical stimulation in the form of shear stress to cells in the scaffold. This mechanical stimulation proved to be a powerful tool to stimulate osteogenic differentiation, and data show that cell-matrix and cell-cell junction molecules are capable of converting mechanical stimuli into biochemical signals.

One of the most important parameters when optimizing a bioreactor is the flow rate. It depends on the composition, porosity and geometry of the scaffold. The pump is capable of precisely and consistently pumping flow rates from 0.01 to 6.0 ml/min through each chamber [138]. Still, there is a big variation of values and there are not many studies that compare a significant range of flow rates. It appears that the increase in flow rate leads to an increase in the deposition of mineralized matrix. Very low flow rates such as 0.01 ml/min have been reported to lead to higher cell viability, but this does not seem an optimal flow rate for bone tissue engineering as it might be too low to actually accomplish an adequate distribution of nutrients, oxygen and removal of waste products. It is also necessary to bear in mind that lower values of flow rate will provide lower values of shear stress, which might facilitate cell attachment and spreading,

hence leading to higher values of cell viability. Despite the wide variation of the flow rates tested, it seems that the optimal values would range from 0.2 to 1 ml/min, depending obviously on the system being used. This is the range of values that seems to have a more positive effect on osteoblastic differentiation, ECM deposition and distribution [121]. Perfusion bioreactor is so far the only system that produces such a force, making it ideal for growing large bone grafts *ex vivo* [139].

4.4. Compression bioreactors and combined systems

Compression bioreactors (**Figure 4B**) were intended to mimic the bone physiological *in vitro* environment, characterized by repeated mechanical stimulation required for functional bone regeneration. Many studies provide evidence that mechanical loading, when combined with flow perfusion, can play a main role in promoting survival and functional osteogenic differentiation of the cells within the scaffold. Short-term mechanical stimulation enhanced the expression of several genes encoding for factors involved in osteogenesis, including *RUNX2*, *osteopontin*, *integrin-β1*, *TGFβR1*, *SMAD5*, *annexin-V* and *PDGFα* [3]. These experiments demonstrate that even short mechanical stimuli can be sufficient to activate the osteogenic differentiation pathways in human mesenchymal stem cells. Compression bioreactors systems consist of a motor, a system providing linear motion and a compression chamber in which one or more clips apply static or dynamic compressive loads directly to the scaffold [3]. The bioreactor chamber holds the scaffold in place and ensures hermetic sealing as well as force transmission onto the cell-seeded scaffold. It consists of medium flow distributors, a flexible force transmitting disk and the intended space for scaffold placement. The power transmission rack includes a plunger, a pre-load screw and a cam-shaft. The chamber is placed on the clip and fixed via tightening of the pre-load screw. The camshaft moves the clip in order to apply a sinusoidal compression pattern onto the bioreactor chamber [140]. The system can be controlled by a signal generator, and load response can be measured by using linear variable differential transformers and load cell, respectively. In contrast to static culture, mass transfer is considerably improved in compression bioreactor culture since compression leads to the fluid flow through the scaffold [141]. The compression bioreactors provide a promising tool for bone fracture research and for *in vitro* estimate of alternative fracture treatments based on engineered tissue grafts, allowing the reduction of animal experiments.

5. Conclusion

Bone defects that are due to trauma or pathological and physiological bone resorption represent a global health problem. The need for bone regeneration is one of the central issues in regenerative medicine. Tissue engineering is becoming a useful addition to medical therapies for repairing and restoring the function of bone tissue. Bone constructs elaborated with tissue-engineering principles are a promising substitute for autologous bone graft and have long been considered the golden standard for repair of large bone defects. Mesenchymal stem cells from adult tissues are the most suitable cell source for bone tissue engineering. Although the application of MSCs as cellular material facilitates the construct fabrication, more

work needs to be done to fully determine the clinical potential, efficacy and safety of stem cell-based treatments. There is a constant need in the development of new scaffolds that have optimal characteristics, and are affordable as well as easy for manipulation. Bioreactor dynamic setting enables better culture conditions and mechanical stimuli for improved bone tissue growth. In spite of the existing problems, advances in the field are enormous and therapy using scaffolds, healing signals and stem cells together should be able to solve the current limitations in managing bone injuries.

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