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REVIEW ARTICLE

Biomaterials, substitutes, and tissue engineering in bone repair: current and future concepts

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Abstract Bone is a complex, constantly changing organ comprised of mineralized hard tissue. This important structural component of vertebrate's body serves a variety of functions. Healthy bone system is essential for lifelong execution of these functions. Millions of people worldwide suffer from bone defects due to various reasons, including trauma, tumor, bone diseases, congenital defects, and aging. These defects are increasingly becoming the majority of the clinical cases in orthopedics. For all the aforementioned cases in which the normal process of bone regeneration is either impaired or simply insufficient, there are currently a number of treatment methods available which can be used either alone or in combination for the enhancement of bone healing and regeneration. Accordingly, bone repair has been the focus of many research activities related to clinical therapies. The traditional bone repair procedure widely used in current era involves the use of bone-grafting methods such as autografts, allografts, and xenografts; however, these methods are associated with number of limitations. Therefore, to overcome these problems, tissue engineering as a new and developing option had been introduced recently. In order to provide ideal bone substitutes, a wide range of biomaterials and synthetic bone substitutes are available depending on the goal, each has advantages and disadvantages. The combined use of different bone substitutes together with healing promotive factors, stem cells, gene therapy, and more recently, three-dimensional printing of tissue-engineered constructs may open new insights in bone regeneration in near future. In this review, we describe developments and recognized properties of some of the most utilized materials in bone regenerative medicine heretofore. It may be concluded that presently strong requirements are still to be met in the repair and regeneration of bone defects.

Keywords Biomaterials · Substitutes · Tissue engineering · Bone repair

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Introduction

The skeleton forms the basic frame that supports the locomotory apparatus and forms a mechanical point of view consists largely of a series of lever arms designed to contract the force of gravity and to constrain and direct the forces of muscular contraction (Sarkar and Lee 2015). Bone has several functions which include protection of vital organs, regulation of calcium and phosphorus metabolism, and hematopoiesis (Liao et al. 2011; Clarke 2008; De Baat et al. 2005; McMahon et al. 2013). It also serves as a reservoir of cytokines and growth factors (Liao et al. 2011; Clarke 2008; De Baat et al. 2005; McMahon et al. 2013). Healthy bone system is essential for lifelong execution of these functions (Liao et al. 2011). According to Wolff's law, long bones change shape to accommodate stresses placed on them; this is called "modeling" (Clarke 2008; De Baat et al. 2005). Bone goes through remodeling concurrently, the process by which bone is renewed to

maintain bone strength and mineral homeostasis (Clarke 2008; De Baat et al. 2005). In this process, osteoclasts resorb old bone and osteoblasts replace it with new bone (Liao et al. 2011; Clarke 2008; Buckwalter and Cooper 1987; Zigdon-Giladi et al. 2015). Dynamic balance between these two processes maintains bone structure; therefore, impairment of any of these processes results in bone abnormalities such as fracture, osteoporosis, and osteoarthritis (Liao et al. 2011; Clarke 2008; Buckwalter and Cooper 1987; Zigdon-Giladi et al. 2015). In USA, bone injury occurs to seven million people every year (Wang et al. 2013). Among these, fracture is one of the most important bone injuries that may be defined as a disruption in the continuity and integrity of a bone due to various reasons such as car accidents and falls (Denny and Butterworth 2000). Fracture is classified as complete and incomplete (Denny and Butterworth 2000). A complete fracture is one in which there is total disruption of the continuity of the bone and usually marked displacement of the fragments (Denny and Butterworth 2000). An incomplete fracture is one in which partial continuity of the bone is maintained as in the greenstick fractures of young animals or fissure fractures in mature animals (Denny and Butterworth 2000). Following a fracture, the healing process is initiated (Sarkar and Lee 2015; Kovach et al. 2015; Marsell and Einhorn 2011; Dimitriou et al. 2011). Healing of bone defects in adults closely resembles bone formation during organogenesis (Sarkar and Lee 2015; Marsell and Einhorn 2011; Dimitriou et al. 2011). Fractured bone heals through direct and indirect pathways. Direct pathway is achieved via intramembranous ossification, whereas indirect (secondary) fracture healing consists of both endochondral and intramembranous ossification and is the most common form of fracture healing (Sarkar and Lee 2015; Marsell and Einhorn 2011; Dimitriou et al. 2011). Soon after the bone injury, an acute inflammatory response initiates locally including the production and release of important chemical mediators leading to clot formation (Sarkar and Lee 2015; Kovach et al. 2015; Marsell and Einhorn 2011). The combination of collagen fibers and mineralized osteoids forms the primary soft cartilaginous callus later; gradually, vascularization and calcification lead to ossification of this primary structure and finally remodeling occurs to restore normal bone structure (Sarkar and Lee 2015; Kovach et al. 2015; Marsell and Einhorn 2011). Unlike other tissues, the bone can regenerate and repair itself, this inherent potential causes bone injuries and fractures to heal without scar formation (Sarkar and Lee 2015; Marsell and Einhorn 2011; Dimitriou et al. 2011; Oryan et al. 2014a). Nevertheless, pathological fractures, arthritis, and massive bone defects due to trauma and bone tumor resections may lead to larger bone defects that may have a compromised healing. This biologic process fails or may be insufficient leading to delayed union or non-union of the fracture (Wang et al. 2013; Kovach et al. 2015; Oryan et al. 2014a; Hannouche et al. 2001; Balmayor and van Griensven 2015). It is estimated that, of the 7.9 million fractures sustained in the USA each year, 5 to 20% result in delayed or impaired healing requiring therapeutic intervention (Kovach et al. 2015). However, there are complex clinical conditions in which bone regeneration is required in large quantity, such as for skeletal reconstruction of large bone defects created by trauma, infection, tumor resection, and skeletal abnormalities (Clarke 2008; De Baat et al. 2005; Zigdon-Giladi et al. 2015; Wang et al. 2013; Kovach et al. 2015; Marsell and Einhorn 2011; Dimitriou et al. 2011; Oryan et al. 2014a; Navarro et al. 2008). In these cases, surgery is essential in order to fill the defect with bone grafts or synthetic biomaterials (Clarke 2008; De Baat et al. 2005; Zigdon-Giladi et al. 2015; Wang et al. 2013; Kovach et al. 2015; Marsell and Einhorn 2011; Dimitriou et al. 2011; Oryan et al. 2014a; Navarro et al. 2008). Bone-grafting frequency is indeed the second most frequent tissue transplantation worldwide, coming right after blood transfusion (Oryan et al. 2014a; Campana et al. 2014). Over two million bone-grafting procedures are performed every year, with more than 500,000 implanted in the USA alone (Oryan et al. 2014a; Campana et al. 2014). A bone graft is defined as an implanted material that promotes bone healing alone or in combination with other materials (Oryan et al. 2014a). 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 (Oryan et al. 2014a).

An ideal bone graft material should induce osteogenesis, osteoinduction, osteoconduction, and osseointegration (Oryan et al. 2014a). The materials used in bone grafting can be divided into several major categories, including autografts, allografts, and xenografts (Oryan et al. 2014a). Using natural and synthetic biomaterials is another choice to repair a bone defect (Oryan et al. 2014a; Campana et al. 2014). Repair of bone defects using implanted material commenced millennia ago; ancient Peruvian and Egyptian societies used implants to heal bone defects (Sarkar and Lee 2015). The modern era of bone substitutes commenced with the attempt of the Dutch surgeon Job van Meekeren to repair a soldier's broken skull using a skull fragment from a dog (Sarkar and Lee 2015). Generally, a bone substitute can be defined as "a synthetic, inorganic, or biologically organic combination which can be inserted for the treatment of a bone defect instead of autogenous or allogenous bone" (Campana et al. 2014). A wide variety of bone substitutes have been employed over the past 50 years (Campana et al. 2014). Bone substitutes must meet stringent requirements; they must be non-toxic, mechanically sound, biocompatible, and not evoke any adverse inflammatory response. They should be osteoconductive and osteoinductive, have a three-dimensional (3D) porous structure, and exhibit optimum biodegradation. They should be easily molded into the bone defect within a short-setting time and allow easy fabrication

into the final preforms. The ideal bone substitute should be possibly traceable in vivo; to this aim, radiolucency is ideal to allow optimal radiographic assessment. Also, they should be thermally non-conductive, sterilizable, and readily available at a reasonable cost (Sarkar and Lee 2015; Campana et al. 2014). In this report, we will review some of the available options in bone repair and regeneration including different types of bone grafts and some of the most used biomaterials, their characteristics, advantages, and disadvantages. Moreover, we will highlight the application of tissue engineering techniques to overcome the limitations of the grafts and to enhance bone regeneration.

Grafts

Bone grafting was introduced into general surgical practice early in the twentieth century, and the principles of grafting have been well established for more than 75 years (Piermattei et al. 2006). Bone grafts may be used to bridge major defects or to establish the continuity of a long bone, to aid in fusion of joints, to fill cavities or defects, and to promote bone union in delayed union or non-union fractures (Millis and Martinez 2003). Bone grafting is now a well-established, commonly performed procedure to augment bone regeneration in a variety of veterinary orthopedic surgeries, with autologous bone being considered as the "gold standard" bone-grafting material (Dimitriou et al. 2011; Millis and Martinez 2003). Autograft refers to the transfer of tissue from one site to another in the same animal (Denny and Butterworth 2000). It is the safest and most effective grafting procedure since it contains patient's own bone-growing cells and proteins to enhance osteogenesis and osteoinduction, respectively (Gomez-Barrena et al. 2015). Bone can be harvested from non-essential bones, such as the iliac crest or the fibula, scapula, radius, the chin, the ribs, the mandible, and even parts of the skull. Autogenous bone possesses all the properties essential for bone formation: it is osteoconductive and osteoinductive, and it houses growth factors and osteogenic cells with no associated immune- or infective-related risks; therefore, this method has been suggested as the gold standard (Sarkar and Lee 2015; Zigdon-Giladi et al. 2015; Dimitriou et al. 2011; Oryan et al. 2014a; Campana et al. 2014). Nevertheless, this method also has some limitations such as limited availability, difficulties in harvesting of adequate quantities of bone, the need for general sedation or anesthesia, need for further surgery, longer operative time, postoperative pain and complications, likelihood of blood loss or hematomas, infection, fracture, neurovascular injury, and cosmetic deformity (Sarkar and Lee 2015; Liao et al. 2011; McMahon et al. 2013; Dimitriou et al. 2011; Oryan et al. 2014a; Campana et al. 2014). According to Barnes et al. investigation in 2015, the combination of autogenous cancellous bone graft and extracorporeal shock wave therapy may lead to increased radiographic density of the osteotomy gap in the first 4 weeks after surgery (Barnes et al. 2015). The next solution is allograft. Allograft refers to the transfer of tissue taken from one animal and transplanted to another animal of the same species (Denny and Butterworth 2000). Allografts have lower donor-associated problems, but some of the most important limitations of such transplants are potential antigenic response and disease transmission, lack of osteogenic properties, variable osteoinductivity, limited supply, laborious procedure (tissue processing, harvesting) and loss of biologic and mechanical properties due to its processing (sterilization by gamma irradiation), non-availability worldwide due to religious and financial concerns, and increased cost (Oryan et al. 2014a; Campana et al. 2014). Shafiei et al. compared fresh cortical autograft and fresh cortical allograft, and they reported that autograft was radiographically but not biomechanically and histopathologically superior to allograft (Shafiei et al. 2009). Xenograft refers to the transfer of tissues taken from one animal and transplanted to another of a different species such as bovine, porcine, and ostrich bone which can be freeze-dried, demineralized, and deproteinized or decellularized (Campana et al. 2014). Bovine bone was first introduced by Maatz and Bauermeister in 1957 (Campana et al. 2014). In spite of availability, good physical characteristics, and low cost, xenografts carry the risks of transmission of zoonotic diseases such as BSE (bovine spongiform encephalopathy) or PERV (porcine endogenous retroviruses), and rejection of the graft is more likely and aggressive (Oryan et al. 2014a; Campana et al. 2014). Recently researchers have devised different methods to decrease the disadvantages of xenografts including decellularization. Decellularization of soft and hard connective tissues such as tendons, ligaments, and bones reduces or even eliminates the immunogenicity associated with allografts and xenografts and, therefore, may be effective in enhancing incorporation of these grafts (Oryan et al. 2014a). Multiple physical, chemical, and enzymatic methods have been used to remove cytoplasmic and nuclear antigens with preservation of the extracellular matrix structure and maintenance of mechanical and functional characteristics (Oryan et al. 2014a).

Ceramics

A ceramic is an inorganic solid that is produced by sintering (a heat-treating process) of non-metallic salts to form crystalline structures. In some cases, the surface characteristics become biologically compatible and support bone ingrowth; hence, these ceramics are termed bioceramics (Kraus 2012). Ceramics can be characterized as bioinert, bioactive, and bioresorbable. Alumina and zirconia were the first ceramics to be introduced to orthopedics, mainly as femoral heads of total hip replacements (McMahon et al. 2013; Navarro et al. 2008).

One of the earliest and though oldest bone substitutes firstly implanted in humans by Dreesman in 1892 is gypsum or calcium sulfate familiar as plaster of Paris (Campana et al. 2014; Millis and Martinez 2003; Kraus 2012; Dewi et al. 2015). This ceramic considered to be a fast degradable material that allows complete resorption before the bone defect area is completely filled by new bone and therefore is not suitable for structural support or for long-term presence as an osteoconductive material (Kraus 2012; Dewi et al. 2015). In a study in 2015, the incorporation of $CaCO_3$ hydrogel into plaster of Paris (POP) in different compositions was assessed by Dewi et al., assuming that it may enhance the bone biological activity of POP and decrease its degradability. Histological analysis of the retrieved specimens indicated that the addition of $CaCO_3$ hydrogel into POP increased bone formation, angiogenesis, and collagen density and resulted into faster bone formation and maturation. It was also confirmed that the degradation rate of the POP decreased by the addition of $CaCO_3$ hydrogel (Dewi et al. 2015). In addition, in a retrospectively review by Yongkun et al. on 50 patients in 2014, calcium sulfate grafts (study group) were compared with bone allografts (control group) for the treatment of benign bone tumors. They investigated bone-healing response, complications, and factors affecting bone healing and reported that calcium sulfate bone substitute healing outcome and safety profile is satisfactory in reconstruction of bone defects after benign bone tumor curettage, especially in smaller cavities. Commercial forms of calcium sulfate (Osteoset and MIIG) supplied as cylindrical pellets and injectable formulations are available as bone graft substitutes that could function as bone void fillers (Millis and Martinez 2003; Kraus 2012). Some of the ceramics, such as hydroxyapatite (HA) and tricalcium phosphate (TCP), possess high compressive strength and hardness along with good biocompatibility; therefore, these materials are appropriate as bone substitutes (McMahon et al. 2013). HA is a naturally occurring mineral form of calcium apatite, considered as the material of choice for preparation of bone graft substitutes because the structure of HA resembles the minerals found in natural bone resulting in osteoconductive properties and formation of tight bonds with surrounding native tissues (McMahon et al. 2013; Oryan et al. 2014a). Although HA accounts for nearly 70% of the mineral content of teeth and bone, the occurring HA in the human body exists in a substituted form. Calcium phosphates have been chemically modified in an effort to produce HA that more closely resembles the mineral content of native bone by incorporating Si, Sr, Zn, Mg, alginate, and carbonate as a replacement for hydroxyl or phosphate groups of the apatite structure (Sarkar and Lee 2015; Campana et al. 2014). These chemical modifications enhanced bioactivity and osteoconduction, osteoblastic proliferation and material performance, dissolution rate, densification behavior, mechanical strength, and biocompatibility. For instance, Hawakawa and collaborators experimental study in 2013 revealed that with the increase in the silicon content in the HA lattice, the in vitro degradation rate of the silicon-containing HA increased, while their crystallite size stayed nearly unchanged (Hayakawa et al. 2013). In another study, Zhao et al. demonstrated that electrochemically deposited magnesium-substituted HA promotes osteogenic differentiation of preosteoblasts in vitro and may improve implant osteointegration during the early stages of bone healing compared with electrochemically deposited pure HA-coated surfaces (Zhao et al. 2013). Another widely used strategy is the combined use of HA and TCP as granules that exhibit interconnected pores, each measuring 100–400 μm (Gomez-Barrena et al. 2015). Since it has been proved that high porosity (more than 80%) and a pore diameter distribution in the range of 200–400 μm are optimal for new bone tissue regeneration, porous calcium phosphate ceramic could be ideal as a bone substitute (Liao et al. 2011). For instance, Hu et al. reported in their study in 2015 that mesenchymal stem cells (MSCs) seeded into porous biphasic calcium phosphate ceramics coated with nano-HA may be an effective bone substitute to reconstruct bone defects (Hu et al. 2015). Newer fabrication techniques have been proposed which allow greater control of pore size, porosity, scaffold shape, ease of fabrication, and reliability of physico-mechanical properties. These various techniques include salt leaching, sponge replica and gas foaming, porogen-based method, and 3D printing (Sarkar and Lee 2015).

Healing promotive factors

Growth factors are a large class of cytokines that have been extensively used to treat bony defects. Growth factors are present in the healthy bone matrix and can be provided by clot or the injured bone itself during different phases of tissue healing as well; therefore, they play a significant role in promoting bone regeneration (Oryan et al. 2014a; Hannouche et al. 2001; Roffi et al. 2013). Platelet-derived growth factor (PDGF) and TGF-β are regulatory molecules released by platelets during clot formation; also BMPs, TGF-β, PDGF, IGF-I, IGF-II, and basic and acidic fibroblast growth factors originate from the bone at the site of injury (Oryan et al. 2014a; Hannouche et al. 2001). The mechanisms of growth factor influences are usually cell surface receptor-mediated, which, in turn, results in expression of one or more gene of the target cell (Kraus 2012). Single growth factors may exhibit more than one type of influence (Kraus 2012). Several advantages have been mentioned for healing promotive factors. For instance, these factors induce migration, growth, proliferation and differentiation of an appropriate subset of cells, as well as vascularization regulation in the site of injury; however, growth factors are expensive, instable in vivo, and needed in high supraphysiological doses due to their susceptibility to

burst in the body and their short half-life (Balmayor and van Griensven 2015; Moeinzadeh and Jabbari 2015; Zhao et al. 2015; Civinini et al. 2011). These imperfections lead to a number of undesirable side effects which include bone overgrowth, tumor formation, and immune reactions (Balmayor and van Griensven 2015; Moeinzadeh and Jabbari 2015). Recently, researchers have introduced novel methods to overcome aforementioned disadvantages such as providing controlled-release systems to enhance sustained release of growth factors in vivo and to protect the recombinant growth factors from enzymatic degradation at the injury site (Zhao et al. 2015).

For instance in Santo and colleagues study in 2012, the tridimensional (3D) structure of chitosan-chondroitin sulfate nanoparticles loaded with platelet lysate included in a poly(D,L-lactic acid) foam was seeded with human adipose-derived stem cells (hASCs) and cultured in vitro under osteogenic stimulus, and the synergistic effect of this combination was suggested (Santo et al. 2012).

Platelet-rich plasma

Orthobiologics such as platelet-rich plasma (PRP) are innovative biological sources which offer exciting new possibilities to promote and accelerate bone and soft tissue healing. Recently PRP has become a field of research interest and has had a wide clinical application (Civinini et al. 2011; Dhillon et al. 2012; Alsousou et al. 2009).

PRP is a volume of fractionated plasma from the patient's own blood (autologous blood) having platelet concentrations above baseline, so that the number of platelets is five times higher than that of the blood (Roffi et al. 2013; Civinini et al. 2011; Dhillon et al. 2012; Alsousou et al. 2009). PRP is an easily obtainable, cost-effective, and valuable source of growth factors with possible beneficial outcomes including reduction of bleeding and pain after surgery, possibility of infection, and rapid tissue healing that might favor the regenerative process (Oryan et al. 2014a; Campana et al. 2014; Roffi et al. 2013; Civinini et al. 2011; Alsousou et al. 2009). In addition, autologous nature of PRP eliminates concerns about immunogenic reactions and disease transmission (Alsousou et al. 2009). When activated by thrombin and calcium, the platelet α granules containing more than 30 bioactive proteins release their contents which play an essential role in bone healing; these include transforming growth factor β, vascular endothelial growth factor (VEGF), insulin-like growth factor, platelet-derived growth factor, epidermal growth factor, hepatocyte growth factor, and basic fibroblast growth factor (Campana et al. 2014; Civinini et al. 2011; Alsousou et al. 2009; Salamanna et al. 2015). Bioactive proteins such as fibrin, fibronectin, vitronectin, and thrombospondin are responsible for osteoblast, fibroblast, and endothelial cell migration (Civinini et al. 2011; Alsousou et al. 2009). For PRP preparation, blood is withdrawn from a patient's peripheral vein and centrifuged to achieve a high concentration of platelets within a small volume of plasma; after that, PRP is reinjected at a site of injury or inserted as a gel or in combination with other biomaterials during surgery (Salamanna et al. 2015). The first evidence of the clinical benefits of PRP in bone reconstruction therapy was reported by Marx et al. in 1998 (Civinini et al. 2011). Heretofore, PRP has been utilized in combination with autograft, allograft, and xenograft to promote bone regeneration; furthermore, many studies have focused on concurrent use of PRP and different natural or synthetic bone substitutes such as HA and β-TCP (Roffi et al. 2013; Civinini et al. 2011; Alsousou et al. 2009; Salamanna et al. 2015; Hakimi et al. 2010; EL Backly et al. 2014; DurmuŞlar et al. 2014). Many studies evaluated the in vitro effects of PRP and confirmed its strong inductive properties; for example, Parson et al. assessed PRP influences on human MSC proliferation, bone morphogenetic protein-2 messenger RNA expression, alkaline phosphatase activity, and bone formation in vitro and reported that PRP can promote bone regeneration (Parsons et al. 2008). In addition, Simson and collaborators detected that the combination of an injectable chondroitin sulfate tissue adhesive and PRP with human MSC could support bone growth. In a newer study in 2015, Fang-Tian et al. showed that different concentrations of PRP have obvious stimulatory effects on proliferation and osteogenic differentiation of human adipose-derived stem cells in vitro (Xu et al. 2015).

Furthermore, numerous studies highlighted PRP effects on bone healing in vivo. For instance, Hakimi et al. in 2010 demonstrated that PRP combined with autologous cancellous graft leads to a significantly better bone regeneration compared to isolated application of autologous cancellous graft in an in vivo critical size defect on load-bearing long bones of mini-pigs (Hakimi et al. 2010). Shafiei-Sarvestani et al. in 2012 reported promising results from combined use of HA and Persian Gulf coral by macroscopic, histologic, radiologic, and biochemical evaluations in an experimental study on rabbit bone (Oryan et al. 2014a). In a similar study in 2014, EL Backly et al. proved that PRP can enhance osteoconductivity of HA and β-TCP composite (EL Backly et al. 2014). Also, Neves et al. subjected rabbits to a total osteotomy of the proximal portion of the right fibula with or without hyperbaric oxygen therapy and autologous platelet concentrations in 2013 and revealed that hyperbaric oxygen therapy and autologous platelet concentrates combined increased the rate of bone healing in this experimental model (Neves et al. 2013).

Although PRP has been used pervasively, clinical effectiveness and its mechanism of action have not been fully recognized, and enhancement of tissue healing by

PRP remains largely anecdotal and controversial (Civinini et al. 2011; Alsousou et al. 2009; Salamanna et al. 2015; Hakimi et al. 2010).

Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are a wide and heterogeneous family of secreted proteins within the transforming growth factor beta superfamily introduced by Urist in the 1960s, which can induce ectopic bone formation (Zigdon-Giladi et al. 2015; Dimitriou et al. 2011; Campana et al. 2014; Kraus 2012; Miyazaki et al. 2009; Harwood and Giannoudis 2005). They comprise only 0.1% by weight of all bone proteins and are not accessible until the bone matrix has been demineralized; therefore, they remain rare and very expensive (Miyazaki et al. 2009). Intracellular cascades which resemble endochondral ossification are activated subsequent to adhesion of BMPs to specific receptors on the surface of the osteogenic progenitors, which leads to mitogenesis of MSCs and other osteoprogenitors and their differentiation toward osteoblasts (Dimitriou et al. 2011; Miyazaki et al. 2009; Poth et al. 2015). Most highly studied members of this family are BMP-2, BMP-4, and BMP-7 (Oryan et al. 2014a; Kraus 2012; Miyazaki et al. 2009). Bone morphogenetic proteins are soluble and are rapidly cleared from a local environment easily and become inactivated in vivo if not in a carrier matrix that attenuates clearance and releases them consistently over time (Kraus 2012; Miyazaki et al. 2009). Additionally, carrier matrices may also possess osteoconductive capacities or structural strength (Miyazaki et al. 2009). Several carriers have been utilized; some of them had distinct problems such as technical difficulties, inflammatory reactions, and formation of bony voids within the healed bone (Kraus 2012; Harwood and Giannoudis 2005).

For instance, Cha and colleagues evaluated the efficacy of a bovine HA/collagen carrier loaded with *Escherichia coli*-derived bone morphogenetic protein 2 (BMP-2) at 0.1, 0.5, and 1.5 mg/ml to augment bone formation in a mongrel dogs nasal sinus in 2014, and they reported that bone morphogenetic protein in this carrier even at low concentrations induces osteogenic activity, enhancing local bone formation in a canine sinus model (Cha et al. 2014). In a similar study in 2015, Taniyama et al. utilized a porous HA-collagen composite as a drug delivery carrier of recombinant human BMP-2 by impregnating of the composite with different amounts of BMP-2, and they concluded that this implant is effective for the repair of osteochondral defects generated in the patellar groove of Japanese white rabbits (Taniyama et al. 2015). Furthermore, Poth et al. in 2015 reported the potential of biodegradable chitosan-tripolyphosphate nanoparticles for a fast release of unmodified BMP-2 at the titanium implant surface in order to enhance the osseointegration of endoprosthesis after revision operations (Poth et al. 2015).

Although many reports confirmed the beneficial effects of BMP on bone regeneration and quality, some others showed their ineffectiveness on regeneration of non-weight-bearing bone healing (Oryan et al. 2014b). For instance, the efficacy of commercially available rhBMP-2-based system to regenerate canine calvarial defect was assessed, and in their result defects treated with rhBMP-2 were significantly less protective against trauma than native bone at 6 months (Cray et al. 2014). It has also been shown in an animal model that artificially administered BMP can cross the placenta and subsequently be detected in the growing embryo. As this area has been little investigated, use in pregnancy is currently contraindicated (Harwood and Giannoudis 2005). In addition, BMPs showed adverse effect in cervical spine and are hence contraindicated in this application (Campana et al. 2014). Finally, the comparison of bone morphogenetic protein-7 and platelet-rich plasma was done by Calori et al. for treating 29 cases of long-bone non-unions. The results indicated that BMP-7 is more efficacious that PRP as there was a significant failure rate of 6.2 versus 38.5% between BMP-7 and PRP, respectively (Civinini et al. 2011; Calori et al. 2006).

Gene therapy

Another promising method in bone repair is the application of gene therapy. The first idea related with a gene therapy approach evolved as early as 1966 and was mentioned by Edward Tatum when he speculated that viruses could be used effectively to introduce new genes into defective cells of particular organs (Balmayor and van Griensven 2015). In 1969, the first isolation of a gene was succeeded by Beckwith, promising a brilliant future to the so-called human genetic engineering (Balmayor and van Griensven 2015). Gene therapy consists of transfer of genetic information into the genome of the target cell, allowing expression of bioactive factors from the cells themselves for a prolonged time where the bone is intended to be regenerated, though it is a safe and effective strategy to induce bone healing (Sarkar and Lee 2015; Dimitriou et al. 2011; Oryan et al. 2014a).

Genetic material can be introduced directly into a distinct area and then can be translocated into the nucleus of the cells by physical mechanisms such as electric-pulsed or ultrasonic waves (Kraus 2012). Alternatively, gene delivery can be performed by viral (transfection) vectors or non-viral (transduction) vectors such as liposomes, cationic polymers, lipids, peptides, and even calcium phosphate (Dimitriou et al. 2011; Oryan et al. 2014a; Balmayor and van Griensven 2015; Kraus 2012; Heyde et al. 2007). Also recently, sonoporation seems to be a promising means (Balmayor and van Griensven

2015). With the in vivo method, viral vectors (adenovirus, retrovirus, and adeno-associated virus) containing the desired genetic material transfer genes directly into the host, and the virus is used to transport the gene into the cell and facilitate its expression (Dimitriou et al. 2011; Oryan et al. 2014a; Kraus 2012). This technique is relatively easier; however, a number of safety issues currently limit using this procedure (Dimitriou et al. 2011). Another method is the indirect ex vivo technique which is performed by harvesting cells from tissues, their genetic modification in vitro, and then re-implantation (Dimitriou et al. 2011). Although this method is technically more demanding, it is a safer method, allowing testing of the cells for any abnormal behavior before re-implantation and selection of those with the highest gene expression (Dimitriou et al. 2011). Adenovirus vectors encoding for bone morphogenetic protein genes have been used (Balmayor and van Griensven 2015; Kraus 2012). In a study by Schwabe et al. in 2012, a COPROG-coated implant and hBMP-2 plasmid, a newly developed non-viral vector, was used to stabilize rat tibial fracture, and a positive effect on fracture healing was shown (Balmayor and van Griensven 2015). Gene therapy has many advantages such as flexibility to express the protein locally and focally, or in a disseminated fashion, as needed and reducing the amounts of therapeutic molecules (Balmayor and van Griensven 2015). Indeed, there are several advantages of gene therapy; this approach has a series of limitations including transinfection of the target cells with the foreign genes and hardship of targeting the right gene at the right location in the right cells. Additionally, another unresolved issue of gene therapy is to express it for sufficiently long time, while minimizing adverse reactions (Oryan et al. 2014a).

Tissue engineering

Tissue engineering, an important therapeutic strategy to be used in regenerative medicine in the present and in the future, can be defined as "a complex and dynamic process that affects the structure and architecture of any viable and non-viable tissue with the aim to increase the effectiveness of the construct to restore, regenerate, maintain, or improve function in defective tissue or lost tissue caused by different pathologic situations" (Oryan et al. 2014a; Gong et al. 2015; Rodriguez-Vazquez et al. 2015). The fundamental concept of tissue engineering, first defined in 1988, is to combine different biocomponents, such as living cells, biomaterials, and biologically active factors, to form tissue engineered constructs to promote the repair and regeneration of tissues; therefore, the triad formed by stem-cells, signaling molecules, and scaffolds or extracellular matrix is considered as the principle of tissue engineering (Rai et al. 2015; Jeong and Atala 2015). Tissue engineering is possible by using different strategies such as introducing isolated MSCs from different origins into defect site, application of growth factors as tissue inducers, and arranging the cells on biologic or synthetic constructs to develop a structural scaffold or matrix to promote bone regeneration (Oryan et al. 2014a; Kraus 2012; Rodriguez-Vazquez et al. 2015).

Isolated stem cells

A stem cell is defined as an unspecialized cell that can renew and maintain itself for a longer period of time with the potential to commit to a cell or tissue lineage with specialized functions (Rai et al. 2015). Multipotent MSCs are non-hematopoietic clonogenic cells of mesodermal derivation residing in several postnatal organs and connective tissues (Barba et al. 2013; Gomez-Barrena et al. 2011). They were first described in the early 1960s, as an adherent, fibroblastoid cell population with inherent osteogenic properties and isolated for the first time from bone marrow by Friedenstein et al. in 1976 (Barba et al. 2013; Gomez-Barrena et al. 2011). These are the most popular stem cells used in bone tissue engineering because of their relatively decreased morbidity during isolation and potential for expansion, proliferation, production of necessary cytokines, differentiation into a variety of cell types such as adipocytes, chondrocytes, and osteocytes, invoking a vascular response, and produce matrix and new bone (Kovach et al. 2015; Campana et al. 2014; Kraus 2012; Rai et al. 2015; Barba et al. 2013). MSCs that contribute to healing are primarily derived from the periosteum, endosteum, and marrow cavity (Marcucio et al. 2015; Knight and Hankenson 2013). MSC harvest, however, requires aspiration from the iliac crest which only yields 10–40 ml of marrow or from bone marrow biopsies, both of which can be painful and yield low numbers; so as an alternative, these cells can be isolated from many other types of tissues and organs such as the adipose tissue, brain, thymus, lung, liver, spleen, kidney, dental pulp, and also embryonic tissues, such as Wharton's jelly and umbilical cord blood (Kovach et al. 2015; Barba et al. 2013; Marcucio et al. 2015; Aurrekoetxea et al. 2015; Levi and Longaker 2011). An example of stem cell application is Schimming and collaborators study in 2004 which revealed that periosteum-derived osteoblasts can form lamellar bone within 3 months after transplantation (Liao et al. 2011). Another study by Kim et al. in 2009 demonstrated promising results from injection of autologous cultured bone marrow osteoblasts for treating large bone defects (Kim et al. 2009). In a more recent study by Kisiel et al. in 2012, proliferative capacities of bone marrow, adipose tissue, muscle, and periosteum-derived MSCs were compared, and periosteum was reported as a superior tissue source for MSCs (Kisiel et al. 2012). Restrictive factors in clinical use of stem cells are immune rejection of cells that are not of autologous origin, donor-related differences (e.g., age and systemic conditions) that affect cell function, absence

of potency test that can predict the in vivo function of cells before transplantation, and the necessity to perform safety and regulation of these procedures before clinical trials (Zigdon-Giladi et al. 2015; Gomez-Barrena et al. 2011).

Scaffolds

Since cell therapy alone is not sufficient to regenerate large tissue defects and replace whole organs, the approach of combining stem cells and biocompatible scaffolds is a more promising strategy. To achieve this, after harvesting stem cells from selected tissues and organs they will be introduced into a natural or synthetic scaffold to assemble a structure similar to desired injured tissue. Scaffolds as the most important issue in this field can be defined as a permanently or temporarily placed three-dimensional porous and permeable natural or synthetic biomaterial that is biocompatible (Oryan et al. 2014a; Rai et al. 2015). Scaffolds act as a matrix for cell adhesion, migration, and proliferation and differentiation, and though properties of scaffolds such as biodegradability, resorbability, hydrophilicity, and hydrophobicity, porosity, suitable pore size and shape, internal and external architecture, stiffness, and strength are responsible for osteoinduction (Oryan et al. 2014a; Rai et al. 2015). In addition, scaffolds for osteogenesis should mimic bone morphology and structure in order to optimize integration into the surrounding tissue and to provide a suitable microenvironment for MSC adhesion, proliferation, and differentiation (Barba et al. 2013). Several major technical advances have been achieved in this field during the past decade. For instance, in the study of Parrilla et al. in 2011, suitability of a synthetic scaffold seeded with adipose tissue-derived stem cells for mandibular defect healing was reported in comparison with the same scaffold seeded with differentiated human dermal fibroblasts and naked scaffold by histologic and computerized tomographic analysis (Parrilla et al. 2011). In a similar study performed by Liao and colleagues in 2013, the osteogenic potential of porcine adipose-derived stem cells was compared among three-dimensional fabricated polycaprolactone, polycaprolactone, and β-TCP and collagen-coated polycaprolactone and β-TCP scaffolds (Liao et al. 2013). The results showed no significant difference in porosity of aforementioned scaffolds; but collagen-coated scaffold was superior in hydrophilicity and swelling ratios and also osteogenic differentiation in vitro (Liao et al. 2013). In addition, better woven bone and vascular tissue formation was yielded on collagen-coated scaffold in vivo (Liao et al. 2013). Moreover, Liu et al. showed in 2013 that hybrid scaffold consists of collagen, and demineralized bone powder seeded with human periosteal-derived cells has good osteoinductive potential (Oryan et al. 2014a). In another study, Xuan et al. studied polycaprolactone/HA tissue scaffolds with individualized grooves manufactured by fused deposition modeling technique alone and seeded with bone marrow-derived MSCs to reconstruct partial sternal defect and they resulted that the scaffold seeded with MSCs could induce new bone union and enhance the amount of bone ingrowth (Xuan et al. 2014).

Another approach in bone tissue engineering is using a natural or synthetic scaffold without seeded stem cells to allow host cell ingrowth and neovascularization and stimulate bone repair and regeneration (McMahon et al. 2013; Oryan et al. 2014a). Natural-based scaffolds have several remarkable advantages in comparison to synthetic scaffolds as a suitable bone substitute which include superior biocompatibility, biodegradability, osteoconduction, osteoinduction, osteogenesis, and osteointegration, but their immunological behavior is variable in different species and is also related to the type of application (Oryan et al. 2014a). Acellularization as a novel manufactural technique for constructing scaffolds reduces or even eliminate immunological associated issues; though recently, it has opened a new insight in tissue engineering. Heretofore, researchers have used various decellularization methods to construct antigen-free collagen-based scaffolds from animal sources (Oryan et al. 2014a; Quan et al. 2014; Funamoto et al. 2010; Dong et al. 2012; Chen et al. 2015; Chen et al. 2016; Farnebo et al. 2014). It should be noted that present article authors have used decellularization techniques on different tissues of ostrich in order to provide suitable xenograft scaffolds promoting healing and regeneration (Saadinam et al. 2014; Farahani et al. 2015; Fatourehchi et al. 2015). In these studies, acellular cornea, skin, tendon, meniscus, and biphasic osteochondral composite (unpublished data) derived from ostrich was grafted to rabbit and guinea pig, and promising results were obtained. Since bone is mainly composed of collagen and HA, scaffolds containing these organic materials function better than other bone substitutes. Nakamura et al. investigated high hydrostatic pressure as a modern method of decellularization in 2014 and tested the obtained scaffold in vivo and in vitro (Quan et al. 2014). They assured that this method would completely remove cells, antigen molecules, and viruses while preserving structure and the composition of the native tissue (Quan et al. 2014). In a study performed by Chen and collaborators in 2015, cell-free bone coated with collagen and HA was used as a three-dimensional scaffold for evaluating repair efficacy in vivo and osteogenic differentiation in vitro (Chen et al. 2016). Recently, cultural and religious limitations and the high risk of zoonotic disease transmission have attracted researchers toward utilizing other sources of collagen such as marine sources (Silva et al. 2014; Pati et al. 2012; Chou et al. 2014; Yamamoto et al. 2014). Accessibility, availability, cost-effectiveness, and possessing similar composition to bone are the most important advantages of marine sources (Oryan et al. 2014a; Silva et al. 2014). Hoyer et al. in 2012 used mineralized salmon skin collagen and HA to assemble an appropriate

scaffold for bone regeneration, possessing good elasticity, absorbability, and porosity (Hoyer et al. 2012). In another study in 2014, Cheng-Hung and colleagues used acellular fish scale to prepare a pin for internal fixation of femoral fracture in rabbit and achieved promising results (Chou et al. 2014). Presently, the role of fish scale and its decellularized scaffold with PRP on healing of tibial bone defect in rabbit is under investigation by present article authors.

To overcome limitations of bone repair methods and to find an ideal bone substitute, calcium-phosphate-based biomaterials are widely used in the past decades (Dimitriou et al. 2011; Oryan et al. 2014a; Navarro et al. 2008). Of these, HA, the most important structural component of natural hard tissues, is attractive as a source for biomedical applications nowadays due to its non-toxicity, excellent biocompatibility, osteoconductivity, and osteogenicity (Wang et al. 2013; Kim et al. 2014; Ivankovic et al. 2010; Venkatesan et al. 2014; Michel et al. 2015). The scaffold constructed from HA acts as a temporary substrate or template, providing the necessary support for the cell and vascular ingrowth such as oxygen and nutrients and maintains their differentiated functions. Furthermore, its architecture defines the final shape of the new bone (Ivankovic et al. 2010; Michel et al. 2015). Heretofore, several natural sources of calcium and phosphate have been used as raw material to provide HA scaffolds including egg shell, animal skeleton, and marine sources like coral and nacre (Kim et al. 2013; Cadman et al. 2012; Ni and Ratner 2003; Gao et al. 2007). Coralline-based xenografts introduced as bone graft substitutes in the mid-1970s could be used either simply in their natural calcium carbonate form for better resorption of the graft by the natural bone or transformed industrially into HA through a hydrothermal process for promoting biological activity (Hannouche et al. 2001; Campana et al. 2014; Kim et al. 2013). For instance, Gao et al. in 2007 obtained HA from coral exoskeleton under different thermal conditions and intervals (Gao et al. 2007). In addition to coral, marine environment is rich in porous organisms containing minerals that make them appropriate for bone replacement (Kim et al. 2013; Clarke et al. 2011). Cuttlebone, the internal hard organ of cuttlefish, is an ultra-lightweight highly porous structure consists of calcium carbonate which plays an important role in organism protection and functions as a floating tank. Its chemistry and crystallography are similar to coral, and it is also low cost and readily available (Kim et al. 2014; Kim et al. 2013; Cadman et al. 2012). It also has osteoconductivity, biocompatibility, and plasticity due to its morphological and chemical characteristics (Dogan and Okumus 2014). It should be noted that its pore diameter varies between 200 and 600 μm in different species and therefore, it is suitable for new bone formation and neovascularization (Hoyer et al. 2012; Dogan and Okumus 2014). Several researches have investigated the effect of raw or processed cuttlebone in bone repair and regeneration during the last decades (Hoyer et al. 2012; Kim et al. 2014; Ivankovic et al. 2010; Kim et al. 2013; Cadman et al. 2012; Dogan and Okumus 2014; Hongmin et al. 2015; Tkalčec et al. 2014; Kim et al. 2012; Yi et al. 2011; Liu et al. 2013; Ivankovic et al. 2009). For instance, a porous polycaprolactone (PCL) scaffold incorporating cuttlebone-derived HA powder was fabricated using the solvent casting and particulate leaching method by Kim and collaborators in 2013 for application in tissue engineering (Kim et al. 2014). In a similar study, Hongmin et al. demonstrated that cuttlebone-derived HA has a high surface protein adsorption, it supports MSC differentiation as a scaffold and it also induces new bone formation without using exogenous growth factors and cells after subcutaneous implantation (Hongmin et al. 2015). Presently, the role of calcium carbonate and cuttlefish-derived HA with PRP on healing of tibial bone defect in rabbit is under investigation by present article authors.

Growth factors as tissue inducer

Promising results from several experimental and clinical trials have suggested administration of growth factors and other bioactive molecules to promote bone formation and repair in preclinical and clinical conditions (Campana et al. 2014). Heretofore, many researchers have studied a combination of different growth factors and supportive scaffolds (Oryan et al. 2014a; Oryan et al. 2014b). For instance, Ozturk et al. evaluated the efficacy of VEGF combined with HA-containing gelatin scaffold in treatment of critical-sized tibial bone defect in rabbit model and showed that it is more effective in early phase of fracture healing than the scaffold without VEGF (Oryan et al. 2014b). Another prospect in using growth factor is as a tissue inducer. A variety of administration methods have been investigated including bolus injection, surface-adsorbed protein release, osmotic pumps, and controlled release from biodegradable scaffolds (Campana et al. 2014). More recently, drug delivery techniques such as entrapment within a matrix allowing growth factors to be released at a desirable rate and concentration from the scaffold to aid the regenerating tissue have been applied (Campana et al. 2014). For instance, Subbiah et al. designed a novel dual growth factor delivery system by combining polylactic-co-glycolic acid (PLGA) nanoparticle-encapsulated bone morphogenetic protein and VEGF which included alginate microcapsules via an electrodropping method. They assessed the scaffold for umbilical cord blood-derived MSC osteogenesis in vitro. Furthermore, they applied the scaffold in an 8-mm diameter rat calvarial defect model with collagen and obtained positive results including vascularized bone regeneration, 82.3% bone healing, and 12.6% vessel-occupied area confirmed by computed tomography and histology analyses (Subbiah et al. 2015). Some other newer approaches for controlled growth factor delivery

are under investigation recently; for example, a unique scaffolding system is synthesized by Ma and collaborators via encapsulating BMP-2-binding nanospheres into nanofibrous microspheres. They used heparin-conjugated gelatin as a domain for BMP-2 to stabilize this growth factor, protect it from denaturation and proteolytic degradation, and subsequently prolong its sustained release. Finally, they reported that this microsphere is an excellent osteoinductive scaffold for enhanced bone regeneration by evaluating the system in calvarial defect model (Ma et al. 2015).

Three-dimensional printing

Among all the biofabrication approaches, three-dimensional bioprinting technology based on inkjet printing of a liquid binder onto powder biomaterials is becoming a dominant technological platform and is suggested as a new paradigm for twenty-first century tissue engineering (Oryan et al. 2014a; Gao and Cui 2016). This approach has several advantageous properties such as delivering and creating biomimicked tissue with high throughput, digital control, and the capacity of single cell manipulation. Therefore, this enabling technology may be our next step in surpassing the hurdles and limitations of conventional scaffold-based tissue engineering and has great potential in regenerative medicine and translational applications (Jeong and Atala 2015; Gao and Cui 2016). In this regard, Zeng et al. evaluated the efficacy and feasibility of three-dimensional printing (3D printing)-assisted internal fixation of unstable pelvic fracture from minimal invasive pararectus abdominis approach in 38 patients, retrospectively (Zeng et al. 2015). They reported that all patients have achieved clinical healing, with mean healing time of 8 weeks, and suggested that this method has the advantages of trauma minimally, bleeding less, healing rapidly and satisfactory reduction, and worthwhile for spreading in clinical practice. In another study with similar purpose, Ishack and colleagues created custom 3D scaffolds of 15% HA and 85% β-TCP coated with dipyridamole, bone morphogenetic protein 2, or saline and assessed it by implanting in a 3-mm cranial critical bone defect. Histological analysis showed increased bone formation and a trend toward increased remodeling in both coated scaffold and suggested that these coated scaffolds may be very useful for treating critical bone defects due to trauma, infection, or other causes (Gomez-Barrena et al. 2011). 3D bioprinting is yet to successfully overcome the many challenges related to building 3D structures that closely resemble native organs and tissues, which are complex structures with defined microarchitecture and a variety of cell types in a confined area. An integrated approach with a combination of technologies from the fields of engineering, biomaterial science, cell biology, physics, and medicine is required to address these complexities (Zhang and Zhang 2015).

Conclusion and perspectives

Heretofore, researchers have been using various methods such as grafts, natural and synthetic bone substitutes, healing promotive factors, stem cells and gene therapy, and 3D-printing in order to enhance bone regeneration; each of these options possesses specific advantages. For instance, bone grafts are ideal for restoring the continuity of injured long bones, may be helpful in fusion of joints, filling major bone defects, and achieving some degrees of union in delayed and non-union fractures (Millis and Martinez 2003). Ceramics can be characterized as bioinert, bioactive, and bioresorbable. Some of the ceramics, such as HA and TCP, possess high compressive strength and hardness along with good biocompatibility; therefore, these materials are appropriate as bone substitutes (McMahon et al. 2013). Healing promotive factors are appropriate when migration, proliferation and differentiation of cells, and vascularization is required in the site of injury (Balmayor and van Griensven 2015; Moeinzadeh and Jabbari 2015; Zhao et al. 2015; Civinini et al. 2011). However, growth factors are expensive, instable in vivo, and needed in high supraphysiological doses due to their susceptibility to burst in the body and their short half-life (Balmayor and van Griensven 2015; Moeinzadeh and Jabbari 2015; Zhao et al. 2015; Civinini et al. 2011). The effectiveness of BMP, one of the popular healing promoters, is still controversial (Cray et al. 2014). The use of gene therapy depends on weighing the advantages like flexibility to express the protein locally against the disadvantages including transinfection of the target cells with the foreign genes and hardship of targeting the right gene at the right location in the right cells (Oryan et al. 2014a). Although stem cell therapy showed promising results in several studies, some restrictive factors such as immune rejection of cells that are not of autologous origin and variations in individual characteristics affecting on cell function have been mentioned (Zigdon-Giladi et al. 2015; Gomez-Barrena et al. 2011). In addition, cell therapy alone is not sufficient to regenerate large tissue defects and replace whole organs; therefore, the approach of combining stem cells and biocompatible scaffolds is a more promising strategy (Oryan et al. 2014a; Rai et al. 2015). 3D printing is a novel approach that has great potential in regenerative medicine and translational applications and could resolve conventional scaffold-based tissue engineering-related problems (Jeong and Atala 2015; Gao and Cui 2016).

It is somehow difficult to note a single method as an ideal and best option to promote bone regeneration in different clinical conditions. We tried to discuss advantages and disadvantages of each method but there are still some blind aspects in bone regenerative medicine in which bone regeneration is insufficient or compromised such as large bone defects, delayed and non-unions, and skeletal abnormalities. Therefore, these conditions demand further investigations, and bone

regeneration is an active research area. Development of innovative approaches is expected in the near future due to progressive expansion of bone biological and molecular knowledge. Natural materials often entail a certain level of immunoinertness and biodegradability and can be included in scaffolds for differentiative purposes. Conversely, synthetic materials are modifiable and often mass producible, a desirable trait when considering scale-up for various patients and/or large defect areas. By combining synthetic and natural materials, the benefits of each can be combined into a single scaffold (Michel et al. 2015).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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