Trauma & Orthopaedics

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Review

Current concerns regarding healing of bone defects

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Abstract

Introduction

Bone tissue usually heals spontaneously, but in complicated conditions such as pathological fractures or those situations leading to large bone defects, the healing process fails. Therefore, it is still a challenge for orthopaedic surgeons to treat and reconstruct large bone defects, delayed unions and non-unions. A variety of therapeutic modalities have been developed to enhance the healing response and fill the bone defects. Different types of glycosaminoglycans, growth factors, stem cells, natural grafts (auto-, allo- or xenografts) and biologic- and synthetic-based tissue-engineered scaffolds are some of the examples. Nevertheless, these organic and synthetic materials and therapeutic agents have some significant limitations, and there are still no well-approved treatment modalities to pass all the expected requirements. Bone tissue engineering is a newer option than traditional grafts, which may overcome many limitations of the bone graft usage. To select an appropriate treatment strategy in achieving a successful and secure healing, more information concerning injuries of bones, their healing process and knowledge of the factors involved are required. Hence, this paper reviews how the bone fractures heal. It is hoped that this review will provide useful information to orthopaedic surgeons and investigators working in the field of bone healing.

Conclusion

There are many natural and synthetic biomaterials, but it is very difficult to treat large bone defects. Finding a composite graft has been very difficult. Development of techniques for bone tissue engineering has shown promise. All strategies show limitations; thus, we call for further studies pertaining to bone fracture healing to help us improve the bone healing methods.

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Introduction

Bone, as a part of the skeletal system, is responsible for mechanical support for the soft tissues and muscles, body shape and movement[1]. In addition, it has essential roles in mineral (calcium) homeostasis and energy metabolism. Under some stressive and continuous compressive conditions, the ability of the bone tissue to tolerate strength decreases. Whenever these forces overcome the toleration of the bone tissue, fracture occurs. Small bone injuries such as stable fractures can heal spontaneously without intervention of orthopaedic surgeons[2]. However, those injuries associated with significant tissue loss, leading to instability and poor alignment of the bony structures, require surgical intervention[3]. Bone fracture healing shows much similarities with soft tissue healing, but its ability to be completed without formation of scar tissue is unique[4]. Bone fracture healing involves a cascade of events including haematoma formation, inflammation, soft cartilaginous callus formation, neovascularization, soft callus mineralization, hard callus formation and osteoclastic remodelling of the hard callus to differentiate the callus to the lamellar bone[5]. Fracture healing is not adequate in large bone defects and may be complicated due to diabetes, aging, neoplastic lesions, infection as well as impaired blood supply[6]. In such circumstances, the traditional bone grafts specially the autografts are the gold standard method of tissue replacement[6–8].

However, the autografts have some disadvantages. Morbidity, pain and cosmetic appearance at the donor site are some of the disadvantages. Time-consuming nature of the procedure is also another disadvantage because an additional surgery is required to harvest the graft, and this increases the cost[7,9]. More importantly, in such injuries leading to production of large bone defects, it is not possible to harvest a proper autograft for filling such large defects. Perhaps there are some limitations in the amount of graft harvesting at the donor site. If done, then the functionality of the donor site is significantly impaired[9,10]. Allografts may be selected to repair large bone defects, but this graft type also exhibits several disadvantages. Perhaps disease transmission such as human immune deficiency virus and hepatitis is one of the most significant limitations of this graft[9,11,12]. The viability of the graft may also be in a doubt, and therefore the graft may not incorporate in the healing response; hence, it may be absorbed by the host immune defence mechanism, a phenomenon known as a rejection. Ethical concerns are other limitations of these types of grafts[13].

Xenografts are another type of bone graft in which the graft is harvested from the animals' body and is used to reconstruct the defect area[7]. Theoretically, the xenografts represent more significant disadvantages than the auto and allografts. Perhaps,

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their resorption rate is higher and they may exaggerate the inflammatory response; hence this may be harmful for bone healing. There are also several zoonotic diseases (bovine spongiform encephalitis, rabies, Epstein–Barr virus, etc.) that could be transferred by xenografts from animals to humans when the reconstruction of the defect area by xenografts is a purpose[14]. Unfortunately, most of these diseases are not well defined in the literature. Despite these limitations, recently, xenografts are more popular than the auto- and allografts, because they are available and have low cost. Therefore, they are used in manufacturing different types of biologic based biomaterials by tissue engineering technologies[12]. Tissue engineering is a new approach. By this technology, several different biomaterials and treatment modalities can be designed in order to abandon the reported significant limitations of the traditional grafts and to induce healing response in a manner to increase the rate and quality of the healing. All these technologies are used to produce a new bone in the defect area so that it would be functionally active and normal with minimum complication during the healing process[15].

Hence, emergence of modern bone engineering strategies based on osteogenic cells, osteoinductive factors and osteoconductive scaffolds is recognized as potential ways to create biologic tissue substitutes for reconstructing large bone defects[16,17]. A comprehensive knowledge of fracture healing is desirable in improving treatment and management of bone fractures and defects. Although medical technologies and orthopaedic surgical techniques have been much improved, some fractures still heal poorly, others take a long time to heal (delayed unions) and some result in non-unions[18]. Thus, there remains a need to know more about the biology of fracture healing in order to develop strategies for ensuring normal repair of the skeleton[19]. This paper was proposed to review the bone fracture healing process and provide basic information for researchers in the field of bone fracture healing.

Types of bone fractures

To select an appropriate treatment modality for fractured bones particularly those associated with large deficits, it is essential to diagnose what kind of bone fracture has occurred. Classification of the fractures can help orthopaedic surgeons for this purpose. Bone fractures can be classified based on various characteristics[2,20]. Based on shape or pattern of the fractured fragments, fractures are divided into transverse (due to bending or angulation forces and the fracture line is perpendicular the long axis of bone), oblique (due to bending force and the fracture line is oblique), spiral (caused by a torsional force and the fracture line runs in several planes) and comminuted (due to a severe direct force and the fractured bone has more than two fragments[20,21]). Other types include compression or crush fracture (occurs in spongy bones that get compressed), gunshot fracture as well as greenstick fracture (the bone breaks incompletely, so that the outer cortex breaks while the inner bends) and avulsion fracture (occurs when a piece of bone detaches from the main bone). Based on aetiology, there are three types of fractures including traumatic (excessive force), fatigue (due to repetitive stress) and pathological (due to weakening of bone by tumours, bone diseases or disuse). Finally, according to the nature of fracture, there are closed (simple) and open (compound) fractures[2,20,21] (Figure 1). Despite closed fractures, in the open fractures (if the fracture is open from within or outside called internally or externally open fracture, respectively), there is a direct communication between the fracture site and the external environment. Therefore, the risk of infection is higher in external open fractures than that in the closed ones, and open

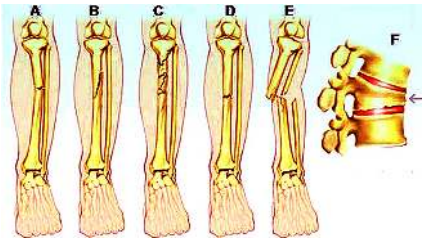

Figure 1: Different types of fracture. (a) Greenstick fracture is an incomplete fracture in which the bone is bent. (b) Oblique fracture in which the fracture has a curved or sloped pattern. (c) Comminuted fracture occurs when the bone is broken into several fragments. (d) Transverse fracture in which the bone breaks at a right angle to its axis. (e) Compound or open fracture is the one in which the fractured bone penetrates the skin. (f) Compression fracture in which cancellous bone collapses and compresses upon itself (arrow).

fractures require special attention in the form of skin wound management by surgery[21]. Massive open fractures are the most serious ones and are difficult to treat because these fractured bones are often associated with delayed healing and non-unions[20]. Also, open fractures require longer time for fracture healing[22].

Bone fracture healing

To design an appropriate treatment modality using different biomaterials and therapeutic agents, it is important to be familiar with the different processes that are involved in healing of the fractured bones. The goal of fracture healing is to regenerate mineralized tissue in the fracture site in order to approximate the intact bone, and to restore mechanical strength and integrity of the injured bone to normalize the functionality of the repaired tissue[3]. The ability of bone regeneration is a key feature for reconstituting original tissue morphology and structure. The type of fracture healing depends on the type of fracture as well as on the method of fracture fixation. Other

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factors that affect the fracture healing pattern are type of biomaterial or graft used to reconstruct the injured area and type of therapeutic agents that have a role in improving the response and pattern of bone healing. However, this is an area of debate, and a number of studies that have defined such patterns in response to various treatment modalities are insufficient[22,23]. In classic histological terms and based on the way of fracture fixation, fracture healing has been divided into primary (direct) and secondary (indirect) fracture healing or union[1,23].

Primary or direct fracture healing

Fixation methods that provide compression across the fracture and stability allow direct healing or union[23]. Primary healing occurs following the open reduction and rigid internal fixation without any gap formation. Primary healing may either occur by contact healing or by gap healing[3]. Unlike contact healing, in the gap healing, bony union does not occur concurrent with Haversian remodelling. Furthermore, in the contact healing, the gap between the bone ends and interfragmentary strain is less than that of gap healing[1,3]. This process involves direct cortical remodelling, which is a process of formation of discrete remodelling units or cutting cones close to the fracture healing site[1,24]. The osteoclasts that exist in the tips of the cutting cones generate longitudinal cavities that are filled with a regenerative bone by osteoblasts. Establishment of Haversian canals is then followed by penetration of blood vessels, leading to direct remodelling into lamellar bone and fracture healing without callus formation[3,24].

Secondary or indirect fracture healing

The most common form of fracture healing is the secondary or indirect healing that involves both intramembranous and endochondral ossification with callus formation. It is enhanced by micro-movement (too much motion leads to delayed healing or even non-healing) and is inhibited by rigid fixation[24,25]. This process occurs in treatment of nonoperative fractures and in particular conditions including external fixation, internal fixation of complicated comminuted fractures or intramedullary nailing in which little motion exists[3]. Contrary to the intramembranous ossification in which the bone is directly formed without cartilage formation, endochondral ossification involves mineralization of cartilage. The secondary fracture healing can be divided into three overlapping phases, including inflammatory, fibroplasia or repair and remodelling (Figures 2 and 3). It needs to be stressed that the events that occur in one phase may be continued in the subsequent phase, and also events that occur in the next phase may start in the previous phase[1,24,25]. These stages include an initial stage in which a haematoma is formed and inflammatory response occurs, a subsequent stage in which angiogenesis develops and granulation tissue and then cartilage begins to form (callus). In subsequent stages, cartilage calcification (endochondral ossification), removal of cartilage and bone formation and ultimately bone remodelling occurs. Finally, the original structure and strength of the bone are restored over months to years[23].

Stages of bone healing

Inflammatory phase

Immediately following trauma, the first stage of fracture healing is blood clotting (haematoma formation) and inflammation, which begin within the first 12 to 14 hours of damage. This process starts with vessels disruption, platelet aggregation, blood coagulation and clot formation in the vessels and the fracture site[26,27]. The blood clot provides a matrix for migration of inflammatory cells, endothelial cells and fibroblasts. The acute inflammatory response peaks through the first 24 hours and is completed by 7 days following injury[27]. The first cells to arrive at the fracture site are neutrophils, then macrophages, lymphocytes and plasma cells replace them. In addition, macrophages not only phagocytose necrotic tissues and other debris, but they also release a range of growth factors and cytokines that initiate the healing process[12,23,26,28–30]. The factors secreted by platelets, macrophages and the bone cells contain transforming growth factor-β (TGF-β), vascular endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), interleukin-1 and -6 (IL-1 and IL-6), tumour necrosis factor-α (TNF-α), bone morphogenetic proteins (BMPs), insulin-like growth factors I and II (IGF-I and IGF-II) and macrophage colony stimulating factor[1,7,31,32]. These factors stimulate the migration of the multipotent mesenchymal stem cells likely originated from the periosteum, bone marrow, circulatory system and the surrounding soft tissues and also induce differentiation of the cells into the mesenchymal cell types including fibroblasts, angioblasts, chondroblasts and osteoblasts that are necessary for tissue repair and regeneration[29,30–33]. Mechanical loading such as weight bearing and physical activities also stimulate the mesenchymal stem cells to differentiate into fibroblasts, chondroblasts and osteoblasts[23]. The growth factors induce the formation of granulation tissue and result in the formation of a soft callus that is followed by hard callus formation in the second phase of bone healing. At this period, the bone has low stiffness and tensile strength, and therefore it fails to tolerate excessive loads[26,31].

Repair or regenerative phase

Fibrocartilage (soft callus) formation

A fibrin-rich granulation tissue is produced after haematoma formation. Next, within this natural scaffold,

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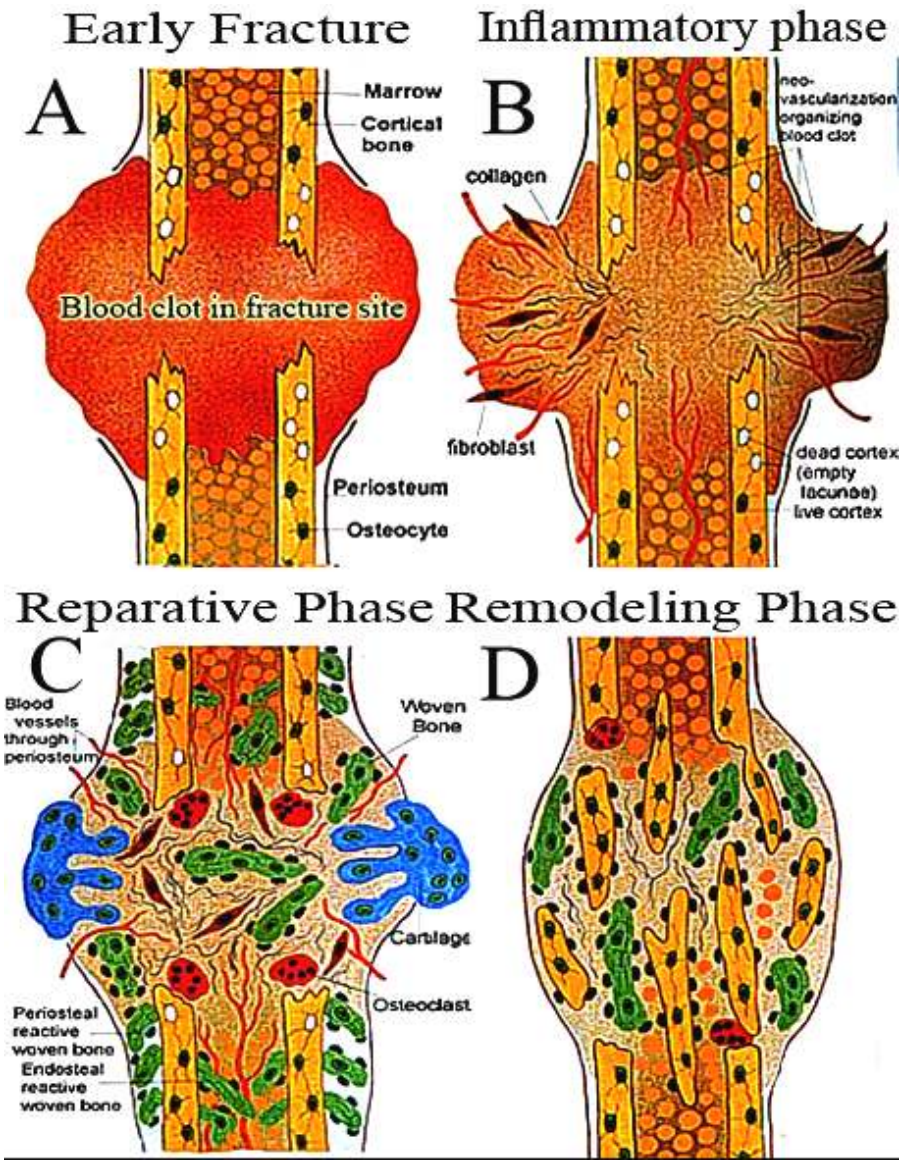

Figure 2: Schematic diagram of fracture healing. There are three processes involved in the healing of fractures: inflammatory, repair and remodelling phases. (a) Early fracture with haemorrhage occurs that is followed by clot formation. (b) Inflammatory phase begins within hours, the inflammatory cells migrate to the facture site as well as neovascularization is followed by organization and resorption of clot. (c) Repair phase. The granulation tissue is replaced by soft (cartilaginous) and then hard callus of woven bone at the fracture site. (d) Remodelling phase. Restoration of bone and modelling of the hard callus to mature lamellar bone occur.

endochondral formation occurs between the fracture ends, and external to periosteal sites that are mechanically more instable and therefore, the cartilaginous tissue forms a soft callus that causes fracture stability[25,31]. Formation of the cartilaginous callus, which is later mineralized, is reabsorbed and replaced with bone, and this is the main feature of this stage[26]. This semirigid soft callus is avascular, but when it is replaced by woven bone, vascular invasion occurs in its architecture.

Chondrocytes originate from the mesenchymal progenitor stem cells, then proliferate and synthesize cartilaginous matrix. This matrix is composed of amorphous collagen materials especially collagen type II and X[31] and glycosaminoglycans. Hyaluronic acid and the polysulphated glycosaminoglycans particularly chondroitin sulphate and dermatan sulphate are predominant and have an important role in cartilage formation. These molecules have excellent water uptake and water-binding capacities and thus absorb water from the surrounding area and increase the size of the extracellular matrix[3]. By proliferation of the chondrocytes within the callus, they undergo hypertrophy and mineralize the cartilaginous matrix[33]. Proliferation of the fibroblasts and chondrocytes is particularly induced by TGF-β, FGF, PDGF, IGF and BMP growth factors[28–30,32]. Sufficient blood supply is a key factor and necessary in fracture healing. In endochondral ossification, chondrocyte apoptosis and cartilaginous degradation as well as removal of cells and extracellular matrix are essential for capillary in-growth in the healing site[3]. Angiogenesis and invasion of vascular endothelial cells into the soft callus is stimulated by proangiogenic factors such as VEGF, BMPs, TGF-β, FGF and angiopoietins (especially angiopoietin I and II)[28,30]. Among these factors, it seems that VEGF plays a critical role in revascularization at the fracture site[26,31,32]. In mice, rats and rabbits, the soft callus formation peaks 7–9 days after the damage[3].

Hard callus formation

When the hard callus is formed and the calcified cartilage is replaced by the woven bone, the callus becomes mechanically rigid and more solid[1,23]. The calcified cartilage acts as a stimulus for angiogenesis to the newly regenerated tissue and brings osteoclasts and osteoblasts into the fracture site. The osteoclasts remove

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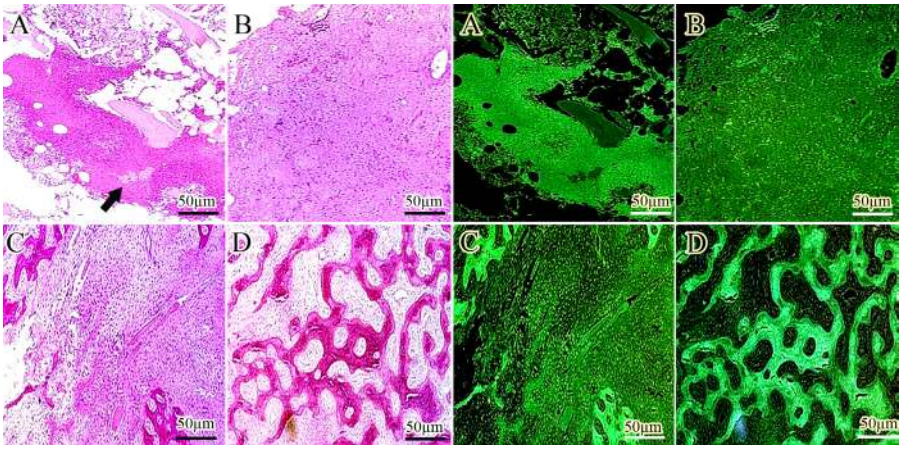

Figure 3: Histopathological changes during fracture healing. (a) There is haemorrhage forming haematoma (arrow) in the bone tissue. (b) In the inflammatory phase, numerous inflammatory cells infiltrate in the damaged site. Then collagen is produced after fibroblasts migration and proliferation. (c) Repair phase is characterized by the newly regenerated fibrocartilage zone with the blood vessels and then formed the bone through the endochondral ossification with chondrocytes. (d) Remodelling phase in which normal microstructure of the bone containing different types of its cells has been restored. A1 to D1 are the inverted images of A to D. The pattern of bone healing can be better seen in the invert figures.

the cartilage matrix to be replaced by a woven bone structure[3]. A key feature in fracture healing is the formation of the cartilaginous callus which is later mineralized, reabsorbed and replaced by bone[1,3].

Bone remodelling phase

Differentiation of the woven bone

The third stage of fracture healing involves the remodelling of the woven bone into lamellar (cortical or trabecular) bone structure[3,24]. In addition, neovascularization is still maintained in this phase. This phase may last months to years, and its goal is to restore the normal and original architecture and integrity of the bone[24]. A lamellar bone gradually replaces the hard callus so that the cortex and medulla of the bone are gradually developed[31]. The osteoclasts are the major cells involved in resorption of the calcified bone. Several growth factors and cytokines such as ILs, TNF-α, BMPs and TGF-β promote osteoclastogenesis[3]. The osteoclasts remove the cartilaginous tissue and allow the osteoblasts to secret osteoid, and this results in mineralization of the fractured area[3,31].

Alignment and maturation of the newly formed tissue

The osteoclasts reabsorb the newly differentiated bony tissue in the injured area to shape its architecture to be comparable to the intact bone. At this stage, the osteoblasts deposit more osteoid and calcium phosphate in the newly regenerated bone and increase the density of the mineralized matrix[31]. Therefore, due to the osteoclast activity, the transverse diameter of the bone decreases but the density of the internal architecture of this tissue increases, and these alterations approximate the architecture of the healing tissue to the intact bone[3]. As this stage is continued, the cellularity gradually decreases and the bone density is gradually enhanced due to improved weight bearing and increased physical activities[23].

Selection of animal models

It is well established that *in vitro* results are of low value in a clinical setting. This is because of the significant differences between the *ex vivo* and *in vivo* situations; translation of the *in vitro* results to clinics is generally hard[32]. Animal experimentation is a better approximation than *in vitro* tests, and usage of animal models is often essential in extrapolating the experimental results and translating the information in a human clinical setting[34,35]. In addition, usage of animal models to study fracture healing is useful to answer questions related to the most effective method to treat humans[36]. There are several factors that should be considered when selecting an animal model. These include availability of the animal; cost; ease of handling and care; size of the animal; acceptability to society; resistance to surgery, infection and disease; biological properties analogous to humans; bone structure and composition; as well as bone modelling and remodelling characteristics[34,37]. Animal experiments on bone healing have been conducted on small and large animals, including mice, rats, rabbits, dogs, pigs, goats and sheep. Each of the species has unique advantages and disadvantages in terms of their appropriateness as a model for healing of bone tissue (Table 1)[34,38,39]. Nevertheless, rabbits are commonly used animals for medical researches (approximately 35% of musculoskeletal research works)[34]. Radial, fibular and clavarial bones of rabbits have been reported to be suitable because there is no need for external or internal fixation of the defect site after experimental induction of bone defect model[36,40,41].

Assessment of fracture healing in animal studies

Morphological analyses are performed by clinical, radiographic, biomechanical, light microscopic, scanning electron microscopic and transmission electron microscopic

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Table 1 Comparison of bone properties in several laboratory animals with humans

Animal model	Advantages	Disadvantages
Dog	Similar bone mineral density and organic composition, tractable nature and Haversian systems to humans	Ethical issues, higher rate of bone turnover, more resistance to ultimate strain than human, quadrupedal
Sheep	Similar body weight to humans, suitable dimension of long bones for implants, similar mineral component and rate of bone remodelling to humans	Housing, handling and availability issues, age-dependent bone remodelling, quadrupedal
Goat	Tolerant to ambient conditions, similar bone composition and remodelling to humans, similar haversian systems to humans	Inquisitive and interactive nature, others similar to sheep
Pig	Similar bone mineral density, anatomy, morphology, healing and remodelling to humans	Difficult to handle, housing and availability, aggressive, higher growth rate and excessive body weight, quadrupedal
Rabbit	Housing and availability, short skeletal and sexual maturity, cost-effective, similar haversian systems to humans	Size limitation, faster bone turnover than humans, quadrupedal
Rat	Ease of handling, housing and availability, cost-effective	Size limitation (too small), lacks haversian systems, quadrupedal

examinations[42]. In some instances, several other tests such as molecular and biochemical analyses are also applied. Each of these tests provides particular information on certain characteristics of bone morphology and its functionality. Clinical evaluations of the fracture healing potential consist of weight bearing on both forelimbs without pain and tenderness. At radiologic level, the defect area is observed at different consequential times, and the quality and morphology of the healing tissue regenerated in the defect area is scored. These scores include presence of bone indicating a complete union (+3 score), presence of cartilage (+2 score), existence of soft tissue within the defect indicating a possible unstable union (+1 score) or complete instability at the defect area indicating non-union (0 score)[7]. To investigate different stages of healing and to measure the rate of bone formation, union and remodelling, radiographs of the fracture site are taken. Radiographic examination is also considered as a diagnostic tool for evaluating the type of fracture and for the surgical scheming[43].

The biomechanical characteristics are extracted from both generated force-displacement and stress-strain curves including stiffness (the coefficient of inclination for the linear region (tan-α) of the load-deformation curve, N/mm), stress or tensile strength (the ratio ultimate strength/cross-sectional area, N/mm^2), strain (the fractional increase in length of the material due to an applied load, %) as well as ultimate load (the highest registered load, N)[44]. Light microscopy is the most commonly used morphological technique and allows histomorphometric measurement of different parameters in the healing bones (e.g. callus size, cartilaginous and mineralized volume of callus, transverse cross-sectional area of bone cortex, transverse cross-sectional area of bone marrow cavity, the ratio of the latter/former, cell constituents and counts in the healing area and many other parameters such as collagen typing and glycosaminoglycan composition)[45,46]. The histopathological examination of healing bone by light microscopy is routinely carried out, using haematoxylin and eosin staining, and is usually scored for example according to the Emery's scoring system as follows: when the gap is empty (score = 0), filled with fibrous connective tissue only (score = 1), more fibrous tissue than cartilage (score = 2), more cartilage than fibrous tissue (score = 3), cartilage only (score = 4), more cartilage than bone (score = 5), more bone than cartilage (score = 6) and ultimately if it is filled only with bone (score = 7)[41,44,47].

Scanning electron microscopy gives a three-dimensional view of the structures with greater resolution and a better view at both micro- and nanoscale level. This morphometric technology is popular in the field of biomaterials, but nowadays, its popularity has been increased in the field of experimental medicine because it provides a better view of the healing tissue, and different types of characteristics including bone density, cellularity, differentiation of the cortex and medulla and several other parameters can properly be viewed by this microscope; hence better judgment can be made compared to the traditional light microscopy[42,45].

Transmission electron microscopy, on the other hand, reveals cell

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morphology and activity, the macromolecular arrangement of the matrix components, collagen arrangement, maturation and density and many other criteria at nanoscale level[42].

Immunohisto/cytochemical, molecular and biochemical analyses are other methods whose popularity is recently increasing in the field of experimental medicine. These methods give many mechanistic data and help the extracted results from the traditional methods to be logically judged and discussed. For example, collagen types I (as an indicator of osteogenic activity), II and X (as indices of hypertrophic chondrocytes); TNF-α (stimulator of chondrocyte apoptosis); mRNA levels, expression of several extracellular matrix proteins including matrix metaloproteinase-13 (which is much important in cartilage remodelling); aggrecan; proteoglycan; VEGF (has an important role in angiogenesis, consequently resorption of cartilage by chondroclasts); alkaline phosphatase and some non-collagenous proteins such as osteonectin and osteopontin are assessed by polymerase chain reaction[48,49,50]. Other techniques that could be used include magnetic resonance imaging, microcomputed tomography scan (to measure bone volume of the callus and bone densitometry) and microcomputed tomography angioscan are used to assess quality of bone healing both in experimental *in vivo* and in clinical practice[22,43,51,52].

Enhancement of fracture healing

Several studies have tested the effectiveness of various natural and artificial substances and implants, grafts, scaffolds with or without cell seeding in experimentally induced bone fractures or defects. A number of these studies are presented in Table 2. Each substance or method has its own significant limitations and therefore has not been suggested as a perfect method to enhance the healing of bone defects and non-unions. Therefore, many investigators have focused on application of various bone graft substitutes such as autografts, allografts and xenografts to promote bone repair[7,44]. Of these, autografts provide three essential and interdependent ingredients necessary to enhance bone formation and repair. These properties include progenitor cells for osteogenesis, growth factors that regulate activities of bone cells for osteoinduction (non-collagenous bone matrix proteins) and scaffolding for osteoconduction (collagen and bone minerals)[7,53,54]. Autografts are still considered as the gold standard of bone graft materials in orthopaedic surgery[8]. Application of autografts decreases the risk of transmissible infectious disease. They also have optimal osteoconductive, osteoinductive and osteogenic properties and they do not induce immune response after implantation, increasing their ability to incorporate into their new site[41,44,54]. Nonetheless, autografts are usually associated with morbidity, pain and infection at the donor site, increased donor site recovery time and other significant limitations as discussed before. Such limitations of autografts have encouraged the researchers to search for a more suitable bone graft substitute. Hence, allografts and xenografts that decline the donor site morbidity, are simply available, have easy usage and reduce the operating time are the major candidates in this regard[54]. However, several disadvantages have limited their usage including transmission of infection or disease, induction of immune responses with inflammatory reaction, delayed union and non-union as well as slower incorporation and possible graft rejection or sequestration[53,54]. It is possible to preserve them to minimize the recipient's immune response for example by freezing or freeze-drying; however, this processing steps may alter the basic characteristics and architecture of the grafts[8,9,55].

Based on the literature, it seems that bone tissue engineering is the newest option in promoting and accelerating the healing potential of the bone defects[56]. In this regard, gene therapy (transfer of genes that code growth factors such as BMPs to target cells with the help of a plasmid or viral vector) may provide promising results[46].

Discussion

The authors have referenced some of their own studies in this review. These referenced studies have been conducted in accordance with the Declaration of Helsinki (1964) and the protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed. All human subjects, in these referenced studies, gave informed consent to participate in these studies.

This study presents crucial information about bone injury, healing and regeneration. These basic information are necessary for those researchers who are going to design better treatment modalities to enhance bone healing and regeneration. The present study showed that bone healing is a complicated process that can be affected by many variables. To design a suitable treatment strategy, it is necessary to have knowledge about several bone healing characteristics such as osteoinductivity, osteogenesis and osteoconductivity. Tissue engineering is a new approach and has been introduced in recent years[15]. Generally, it is divided into three categories including tissue scaffolds, healing promotive factors and stem cells[15]. All these consequents are present in autografts, and for this reason autografts has been suggested as a gold standard method of bone reconstruction for many years[10,44]. However, its major

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Table 2 Effects of various agents and biomaterials on bone fracture healing

Reference	Therapeutic agent	Animal model	Mode of study	Effects
53	Bone marrow graft	Rabbit, radial defect	Radiological and biochemical	Positive effects on bone healing and bending stiffness
52	Indomethacin, rofecoxib, celecoxib	Rat, closed femur fracture	Radiographic, histological and mechanical tests	Delayed fracture healing
4	Bone morphogenetic protein-2 (BMP-2) gene therapy	Sheep, iliac crest defects	CT scan, histological analysis	Retarded bone formation due to antibodies produced against the adenovirus (viral vector)
54	Magnesium and calcium cements	Horse, metatarsal osteotomy	Clinical and radiographic investigations	Both helped fracture healing by osteogenesis and fragment stabilization
55	Xenogenic demineralized bone matrix (DBM) and fresh autogenous cortical bone	Rabbit, radial defect	Radiological, histopathological and biomechanical tests	Enhanced bone healing
56	Bovine foetal growth plate	Rabbit, radial defect	Radiological, histopathological and biomechanical tests	Satisfactory healing with this xenograft
40	Bovine DBM and bovine foetal growth plate	Rabbit, radial defect	Radiological, histopathological, biomechanical tests	Improved healing occurred in the defect
8	Fresh cortical autograft and allograft	Rabbit, radial defect	Radiological, histopathological and biomechanical tests	No significant differences between two groups
57	Zolendronate (a bisphosphonate)	Rabbit, fibular osteotomy	Histomorphometrical evaluation	Stimulated primary bone formation, but inhibited bone remodelling
58	Low- and high-dose methotrexate (MTX)	Rat, femoral gap	Immunostaining and histomorphometrical evaluations	High-dose MTX delayed new bone formation but low-dose MTX no effect
44	Hydroxyapatite and human platelet-rich plasma	Rabbit, radial defect	Radiological, macroscopic, histopathological and biomechanical tests	Hydroxyapatite-hPRP Promoted bone regeneration
7	hPRP plus Persian Gulf coral	Rabbit, radial defect	Radiological, histological, macroscopical and biochemical tests	Promoted bone regeneration
41	Hydroxyapatite-hPRP and coral-hPRP, hPRP alone	Rabbit, radial defect	Radiological, biomechanical, macroscopic, histopathologic tests	hPRP enhanced bone formation in comparison with hydroxyapatite-hPRP and coral-hPRP
51	Adipose tissue stem cell and greater omentum	Dog, radial defect	Radiologic and histopathological examinations	Both promoted osteogenic potential in bone healing
47	Periosteal free graft and pedicle omentum	Dog, radial defect	Gross, radiological and histopathological evaluation	Pedicle omentum accelerated bone formation
59	Mesenchymal stem cells	Mice, femoral defect	Micro-CT and histological analysis	Promoted bone regeneration and healing
45	Thalidomide	Rat, bone damage (osteoporosis) by prednisolone	Histomorphometric measurement	Prevented inhibitory effects of prednisolone on the osseous tissue

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Table 2 (*continued*)				
22	Botulinum toxin A	Rat, closed femoral fracture	Biomechanical and histopathological analysis	Increased healing power, fixation rigidity and decreased callus diameter
60	Deep-freezing block allograft with low-level laser therapy	Rabbit, calvarial defect	LM, SEM evaluations	Increased collagen deposition, moderated bone remodelling, incorporated the graft–host interface
46	Nonviral copolymer-protected gene vector (COPROG) of BMP-2 plasmid	Rat, tibial fracture	Histomorphometric analysis, polymerase chain reaction	Showed positive effect on fracture healing
61	Human mineralized bone and bone marrow mesenchymal stem cells (MSCs)	Rabbit, tibial fracture	Histopathological examination	Increased new bone formation and healing

limitations have been discussed as well as the worse limitations of allo- and xenografts. A proper tissue-engineered graft should have similar characteristics as autografts[12]. It should have a similar architecture, composition and biomechanics as autografts. Therefore, the best tissue replacement designed for bone reconstruction should be three dimensional in nature[12]. For example, it should accelerate osteoinductivity and increase cell migration, proliferation and release growth factors.

Some immune-potent activity by such scaffolds is acceptable because it has been shown that inflammation, if not exaggerated, has a key role in tissue regeneration[12]. Such a scaffold should have strong water uptake and water-binding capacity as well as water delivery characteristics in order to absorb the inflammatory mediators and cellular elements in its architecture, preserve them and slowly deliver the beneficial growth factors and proinflammatory mediators in order to provide an optimum environment during different stages of bone healing[12]. Such a scaffold also should not be rejected acutely, but also it is impressive to be gradually absorbed and thus could potentially incorporate at different stages of bone healing[12]. Alignment of the scaffold although is not an important factor when reconstruction of an injured bone is a purpose; however, the scaffold should guide the healing tissue between the bony ends, in order to reduce the chance of malunion or non-union. Therefore, such scaffold/s should have this capability to align the callus and establish the continuity in the defect area (osteoconductivity). The porosity of the scaffold is also important. Unlike tendon or ligament tissue-engineered scaffolds, the bone scaffolds should be highly porous, and the pores should be present at all sides of the scaffold in order to provide an optimum environment for tissue regeneration and matrix deposition[12].

As a second category of tissue engineering, healing promotive agents such as glycosaminoglycans, especially hyaluronic acid, chondroitin sulphate and dermatan sulphate, play a significant role during bone healing[57,58]. Most of the newly regenerated cartilaginous tissues in the defect area at mid-level of bone healing are composed of chondroitin sulphate and dermatan sulphate; hence by assembling these glycosaminoglycans, it may be possible to accelerate bone healing. Glycosaminoglycans also incorporate in tissue remodelling and maturation and have strong roles in collagen maturation and development of three-dimensional osteogenesis[57,58]. Growth factors are other tissue promotive agents[32]. As discussed earlier, they have major roles in regulating different stages of bone healing[28–30] as they are necessary for cellular migration, differentiation, maturation and matrix deposition. Assembling these growth factors to tissue-engineered scaffolds results in enhanced healing capability of the injured area; therefore, a better healing control can be achieved and a better outcome can be expected. Application of pure growth factors is an expensive way. The platelet-rich plasma contains several growth factors and can be a proper substitute in this regard. A recent review has shown the advantages and disadvantages of this reagent and comprehensively discussed its effectiveness from basic to clinic[32].

Stem cells are another category of tissue engineering. Such scaffolds

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could be assembled with stem cells and growth factors in order to design the most similar graft as autografts. In such a model of treatment, we can expect appropriate tissue regeneration with an optimum healing pattern as those of the autografts. However, studies in this area are expensive, but the results could be encouraging and performing such researches is reasonable[15]. As a final suggestion, it should be highlighted that *in vitro* tests are an initial level of evidence. *In vivo* studies are the best approximation in translational medicine, but several clinical studies are needed after *in vitro* and *in vivo* approval of the tissue engineering approaches in order to confirm the effectiveness of each treatment strategy, and as a final confirmation, comprehensive review or meta-analysis studies are needed to discuss the advantages and disadvantages of each novel treatment strategy in order to introduce a well-accepted method for bone tissue healing and regeneration.

Conclusion

Despite existence of large variety of natural and synthetic biomaterials, it is still a great challenge in orthopaedic surgery to treat large bone defects or non-unions. The gold standard of therapeutic strategies is still autografts. Given the limitations of the types of bone grafts including autografts, allografts and xenografts, development of an ideal composite graft has been one of the most considerable challenges in finding the optimal scaffold vehicle for delivering osteogenic cells, osteoconductive materials and osteoinductive growth factors. Recently, development of the techniques of bone tissue engineering has indicated great promise for this purpose. In addition, gene therapy is considered as a probable option to promote the healing. Nevertheless, it seems that all these strategies have limitations; hence, further studies must be conducted to improve bone fracture healing. An understanding of the fracture healing process is critical for future advancement of fracture healing. It is hoped that large bone defects and non-unions will be successfully treated in future with novel therapeutic strategies.

Abbreviations list

BMP, bone morphogenetic protein; FGF, fibroblast growth factor; IGF, insulin-like growth factor; IL, interleukin; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor

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