

Enhancing bone healing and regeneration: present and future perspectives in veterinary orthopaedics

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Summary

Methods currently used to restore bone defects in human and veterinary orthopaedics are often not satisfactory. This is especially the case in the healing of large, irregular defects which result in the formation of tissues with inferior qualities compared to the original structures. For these reasons, several new approaches are currently being explored to improve bone healing capacities in different situations. This review will examine the different techniques used to enhance bone regeneration, highlighting both experimental and clinically applicable methods with regard to veterinary orthopaedics.

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Introduction

Fracture healing typically results in restoration of the original structure and function of the bone tissue; a process which is unlike the healing of muscle or skin tissue, both of which are not able to regenerate without scar tissue formation. In fracture repair, proper reduction and immobilisation are essential to achieve optimal bone healing. This can be accomplished through the use of specific reduction techniques, surgical instruments, and orthopaedic implants (1).

Intimate contact of the fracture fragments is required for secondary osteons to progressively advance from one fragment to another, although smaller defects will also heal spontaneously without the need for additional 'bone healing enhancers'. Larger bone-defects, specifically those defined as 'critical sized defects', represent a huge challenge in both human and veterinary orthopaedics as these defects do not show any spontaneous closure and require additional means to enhance bony union (2). Traditional techniques are mainly based on the transplantation of autologous bone tissue, which is known to be incorporated more rapidly than any other type of graft. Despite the development of improved surgical techniques, human literature still reports substantial morbidity associated with bone graft donor sites, especially those for posterior iliac crest graft harvest (3, 4). Comparable morbidity has not been reported in veterinary literature. The quantity of available bone graft tissue however is often limited in small-sized patients, especially when dealing with large bone-defects, which encourages the use of allografts, xenografts and different alloplasts as substitutes. The use of 'foreign' substances to

replace bone defects carries specific risks depending on the characteristics of the applied bone substitute. Consequently, the search for the 'ideal bone graft' – one which would deliver osteogenic cells directly (osteogenesis) or stimulate differentiation of bone cells from undifferentiated mesenchymal cells (osteoinduction), as well as provide a matrix as a scaffold for new bone ingrowth (osteoconductive), and support the bony column during the healing process – is still ongoing. With large bone-graft constructs, the generation of an adequate blood supply (angiogenesis) is required in order to provide the graft with the necessary nutrients for enabling long-term incorporation and remodelling of the graft tissue. An adequate blood supply will also allow the bone to counteract possible infection and to receive the needed circulating factors and nutrients (5). Most commercially available bone grafts only carry one or more of these properties when incorporated into the host tissue. The final selection of which bone graft material to use is subsequently based on the specific requirements for the actual clinical situation (6). During the last decade, the search for the 'ideal bone graft' has led to the development of several alternatives.

Indications for use of enhanced bone regeneration techniques

The use of bone enhancing grafts can be indicated in various human surgical disciplines including surgery of the head, dentistry, long-bone and joint surgery. Its use in veterinary surgery is currently limited,

but promising results in human studies might result in similar new surgical techniques and opportunities for veterinary surgery in the near future.

Surgery of the head and dentistry

Cleft lip and cleft palate are relatively common congenital abnormalities of the head which are found in both humans and animals. Bone enhancement techniques play an important role in cleft repair (7). Head trauma represents a common pathology encountered in both small and large animal practices (8). It most often results in fractures amenable to classic osteosynthesis techniques for repair, but can sometimes lead to substantial bone loss. The reconstruction of large bone-defects in the cranio-maxillo-facial area still represents a major surgical challenge despite the considerable progress which has been made in the field of enhanced bone regeneration.

Dentistry related bone-graft application in humans focuses on repair of alveolar bone defects caused by periodontal and peri-implant related bone destruction and alveolar ridge height preservation for aesthetical purposes, and to provide a basis for future implant placement (9). An edentulous upper jaw is a frequent handicap found mainly in humans and domestic small animals. Loss of teeth and aging induce bone resorption resulting into progressive atrophy of the maxillary bone. Rehabilitation of this atrophic maxilla with dental implants is impossible without bone grafting. In humans, this is routinely achieved in the posterior maxilla by using a sinus floor elevation procedure whereby the thickness of the maxillary sinus floor is increased with a suitable bone substitute (10).

Application of bone substitutes in veterinary dentistry has been advocated for both dogs and cats to preserve the alveolar bone height or provide jaw stability following specific tooth extractions (11–13).

Long-bone and joint surgery

Enhancement of the bone healing process can be an essential part of the surgical treatment for many orthopaedic conditions.

Bone grafts can be used to bridge major defects or to establish the continuity of a long-bone (e.g. after trauma or tumour resection). These grafts are indicated in procedures involving fusion of joints, filling of cavities or defects, and to promote bony union in delayed union or nonunion fractures (6). The aetiology of a non-union may involve multiple factors. A poor blood supply to the affected area together with a poor general nutritional status can predispose to a non-union fracture. Poor apposition of the fractured bone-ends, pathological fractures, presence of foreign bodies, large quantities of necrotic bone, infections or non-justified corticosteroid therapy have also been reported as possible aetiological factors (14). Enhanced bone regeneration is justified in cases of non-unions not only to provide support and fill existing lacunae, but also to enhance biological repair when the skeletal defect reaches the so-called critical size (15). Bone enhancement techniques are a real challenge in the treatment of critical sized defects, which have been defined as the defect size whereby normal complete calcification of the defect will not occur during the remaining lifetime of the animal or man (16).

Different substitutes to enhance bone healing or regeneration

Auto-, allo- and xenografts

Autografts still represent the 'gold standard' material for enhancement of bone regeneration because these grafts contain all of the essential components needed to promote bone formation, including osteoprogenitor cells, matrix, and bone morphogenetic proteins. Philip von Walther has been cited as having performed, in 1820, the first clinically successful autogenous bone transfer in a man (17). However ten years earlier, Merrem had already achieved good results with bone-graft experiments in animals (18).

The use of cancellous and cortical bone autografts in veterinary orthopaedic surgery has also become very popular and is well documented (19). Cancellous bone

grafts are typically used to provide live cells and growth factors that stimulate the production of new bone. Because little support is provided by these cancellous grafts, the addition or use of cortical bone is justified when structural support is of major importance (6).

Autografts are still preferred over the use of allo- and xenografts, although the latter two obviate donor morbidity encountered during autograft retrieval, and can serve as an osteoconductive and osteoinductive tool to enhance bone healing (20, 21). On the other hand, both graft substances possess considerably less capacity for osteoinduction and osteoconduction compared to autografts. Their resorption rate is often mismatched compared to the rate of new bone formation, thus increasing the chance for non-integration of the graft. Moreover, the antigenic response elicited by the presence of 'foreign' material increases the likelihood of graft rejection; the likelihood of graft rejection is higher when using pure bone xenografts.

Demineralised bone matrix (DBM) is a good option as an allograft material. By reducing the mineral phase, growth factors become more available, thus increasing the osteoinductive properties (22). However, since there is not any structural strength provided, its primary use is limited to a structurally stable environment. Several excipients such as hydroxyapatite, autografts or even bone marrow aspirate can be combined with DBM to improve its handling characteristics and mechanical properties (20). Demineralised bone matrix is available for human use in a variety of forms including fibres, flex, mouldable gels, putties, as well as an injectable version. Because DBM lacks structural properties, it is recommended only as a gap filler in non-weight bearing areas (23).

Deproteinised bovine bone is the most widely used xenograft substance (22). Heat-treated bovine cortical bone has also been proposed as a xenograft-alternative to bone grafts and synthetic alloplasts. This is because it combines the advantages of allografts, which have a high stiffness and acceptable strength, and synthetic materials which are characterised by an abundant supply and a reduced risk of rejection and disease transfer (24). Other alternative xe-

nografts originate from the exoskeleton of crustaceans (chitosan) (25).

Synthetic and natural bone substitutes

Many synthetic materials are available to the surgeon, including ceramics or ceramic-collagen composites, natural corals, coralline hydroxyapatite, and resorbable polymers in different forms such as sponges, microfibers, foils, porous membranes. They have been experimentally used together with titanium implants to enhance bone healing. Good bone healing properties were reported in studies done on sheep, goats, rats, dogs, rabbits, pigs, and cats (28–36).

Bone marrow stem cells

Bone marrow contains osteoprogenitor stem cells that are able to form bone when combined with various elements incorporated into an osseous matrix (37). Although several investigations have indicated that bone marrow is certainly capable of promoting new-bone formation, techniques for enriching the active component of bone marrow – namely mesenchymal stem cells – are of primary importance, because these cells constitute only 0.01% of all marrow cells (38). Even if osteogenic cells at the site of a fracture are working at full capacity, the defect will not heal if too few cells are present, nor will any drugs directed at enhancing bone formation be effective (39). The use of pure bone marrow has yielded inconsistent results in the promotion of bone formation (40).

Molecules enhancing bone healing

Several growth-promoting substances involved in local regulation of bone healing at fracture sites have been identified. These substances can be divided into two groups, namely the peptide signalling molecules (generally referred to as growth factors), and immunomodulatory cytokines such as interleukin 1 and 6 (41).

Growth factors exert multiple effects on cells at both local and systemic levels. These factors include bone morphogenetic proteins, transforming growth factor- β , platelet-derived growth factors 1 and 2, osteogenic growth peptide, and a variety of hematopoietic factors such as lymphokines and monokines (42). Recombinant technology has allowed isolation, production and application of these synthesised molecules for osteoinductive and osteoconductive purposes required for healing of bone defects (43).

Urist noted that DBM could induce *de novo* formation of cartilage and bone when implanted in extraskeletal sites (44). Further investigations identified the active component of the DBM as being proteinaceous, and demonstrated that it could be extracted from the bone matrix (45). The proteinaceous and osteoinductive component was named ‘bone morphogenetic protein’ (BMP) and up until now, over 20 types of BMP have been identified, each having a variety of systemic functions (46). Bone morphogenetic protein 2, 4 and 7, and more recently BMP-6 and 9 were demonstrated to have osteoinductive potential (47–50).

Earlier studies used BMP purified from bone whereas current studies now use growth factors produced as recombinant proteins by synthesis from microbiological agents (e.g. *Escherichia coli*) transfected with a growth factor gene (e.g. human BMP-2 gene). The resulting new protein called recombinant human BMP-2 (rhBMP-2) is purified and tested for its biological activity before *in vivo* application. Local application of rhBMP-2 in multiple critical sized defect experiments resulted in production of structurally sound orthotopic bone in rats, sheep, rabbits, and dogs (51–54). Interspecies amino acid sequence homology for rhBMP-2 is 100% in most mammalian species, thus allowing for its use in all species that are commonly treated by veterinarians. However, BMP derived from the animal species has been shown to result in better bone formation in the same species at lower doses, compared with the use of recombinant BMP from another species (47).

Development of an optimal delivery system for BMP use is still of major con-

cern. Bone morphogenetic protein can be administered systemically with possible risk of unintended adverse effects. Gene transfer technology can be used to deliver growth factor genes (cDNA) to specific cells located at the fracture site using a viral or non-viral vector and *in vivo* or *ex vivo* methods (55). These genes are then expressed by cells at the fracture site achieving sustained high concentrations of biologically more-active growth factors compared to *ex vivo* synthesised BMP. As cDNA is a stable molecule with a long shelf-life, storage and manufacturing may be less expensive than synthesising recombinant proteins; this molecule offers positive perspectives for use in gene therapy. Delivery of the BMP genes to the fracture site using gene therapy has been evaluated in laboratory animal models using non-union fractures with promising results (56). Nevertheless, further research is needed to overcome the multiple drawbacks still encountered. Unexpected cartilage formation was observed after single injections of adenovirus carrying BMP-2 in 50% of created femoral defects in rats, and after mesenchymal stem-cell-mediated gene delivery of BMP-2 in an articular fracture model in rats (57, 58).

The final delivery method consists of implanting BMP with a carrier matrix. In this modality, two different BMP are currently available for clinical human applications, rhBMP-2^{a,b} and rhBMP-7^{c,d}. Both are manufactured using a process involving mammalian cell expression. Non-union, open tibial fractures, spinal fusions and certain oral and maxillofacial bone grafting procedures are conditions for which a clinical approval has been granted for the use of BMP (59–61).

Composites

Non-solid bone enhancing substances, such as bone marrow, have been reported to be washed easily out of the fracture site. Many authors have studied the positive ef-

^a InductOs®: Wyeth Europe Ltd., Berkshire, UK

^b INFUSE Bone Graft®: Medtronic, Minneapolis, MN, USA

^c Osigraft®: Howmedica International S. de R.L., Limerick, Ireland

^d OP1 Implant®: Stryker, Kalamazoo, MI, USA

fects of composite grafts formed by combining bone-graft substitutes (e.g. demineralised bone matrix, ceramics) and autologous bone marrow, the combination of which enhances the practical use of the products and possibly its bone regeneration properties (62–66).

'Bone tissue engineering' has become a new approach to enhance bone regeneration. In this field, it is believed that by combining a synthetic three-dimensional porous template (scaffold) with an osteogenic potent cell population, it will be possible to develop bone tissue equivalents that can induce total regeneration of a large affected area. This ideal cell population should possess a high osteogenic potential, while the cells should be easily expandable and maintainable in cultures for prolonged periods of time. Mesenchymal stem cells (MSC) are considered highly suitable to fulfil the requirements for such a cell population (67). Mesenchymal stem cells seeded on scaffolds have been used to repair experimentally induced, critical sized bone defects in rats, mice, dogs, and sheep (68–71). In large animal models, a significant advantage in the healing of segmental bone defects was observed after delivery of a MSC-loaded bioceramic scaffold in a mechanically stable environment (70, 71). Finally, angiogenesis in a tissue-engineered device may be induced by incorporating growth factors (e.g., vascular endothelial growth factor), genetically modified cells, or vascular cells (72).

Alternative ways to enhance bone healing

Yasuda reported in 1953 that new bone was formed around a negative electrode (cathode) while bone resorption occurred at the positive electrode (anode) if both electrodes were placed directly on the bone (73). Several forms of electrostimulation currently exist to enhance bone healing including direct current implants, external pulsed electromagnetic field systems, capacitively coupled electrical stimulation, and surface interferential stimulation (74, 75). More than 80% of human non-unions treated with electrostimulation successfully progressed toward a bony union (76).

Extracorporeal shock wave therapy has also been used for the treatment of a number of musculoskeletal conditions, and it has shown promising results in attempts to improve fracture healing and delayed union in general (77). The rationale underlying the explanation of this treatment is the stimulation of bone growth and vessels by the production of nitric oxide (78).

Reports of enhanced bone regeneration techniques in veterinary clinical cases

Enhanced bone regeneration has mainly been applied in human medicine and experimental animals. Apart from a few case reports and a small number of clinical trials, the application of enhanced bone regeneration in veterinary medicine is relatively limited to experimental studies using animal models for human purposes. However, bone grafting and enhanced bone regeneration are an interesting but often underused part of the surgical treatment of many orthopaedic conditions in domestic animals.

Dogs and cats

Autologous cancellous grafts have been used for a variety of indications in dogs and cats including the following: treatment of highly comminuted fractures for stimulation of bone union before implant failure, usage in patients with a poor osteogenic potential (older, debilitated or small and toy-breed patients), non-union fractures, filling of bone defects created by aneurysmal bone cysts or by the performance of surgical curettage of bone following tooth extraction, and to enhance healing following ventral stabilisation procedures in the cervical spine or after joint arthrodesis (79–87).

Frozen allogeneic cancellous bone graft has been commercially available for several years as cancellous bone chips^c. When used

in the primary repair of fractures as well as for carpal and scapulohumeral arthrodesis in dogs, these grafts are effectively incorporated (88). The commercial chips can be mixed with autogenous cancellous bone graft to increase the volume of graft for application into a cortical defect (6). Although a delayed sequence in all aspects of the repair process and some bone resorption has initially been observed, allografts were successfully incorporated in canine ulnar defects after a longer period of time (89).

Cortical and cortico-cancellous bone grafts (auto- and allografts) are primarily used in small animals to provide structural support and osteoconduction in areas devoid of bony column, such as in a highly comminuted fracture or after bone removal required for tumour resection (90, 91). Less frequent indications for the application of these bone grafts are arthrodesis of joints, lengthening of bones, correction of cleft palates, and mal- and non-unions (92–95). Recently, the strength of allogenic cortical bone pins has been evaluated for use as biodegradable fixation devices in fracture fixation (96). A cancellous bovine bone xenograft was also successfully used together with autogenous cancellous bone (at a ratio of 4:1) to fill a curetted osteolytic lesion of the distal radius in one dog (97).

Use of DBM as a substitute or adjunct for autogenous cancellous bone graft has been described in a retrospective and case-matched study of 75 dogs that had undergone orthopaedic procedures (comminuted fractures, tibial plateau levelling osteotomies where correction for tibial rotation created an osteotomy gap, arthrodeses, open corrective osteotomies). Mean (\pm standard deviation) healing time for orthopaedic surgeries with DBM augmentation was 15 ± 6.97 weeks, and the complication rate was 19 % (14 dogs). Dogs with a tibial plateau levelling osteotomy gap filled with DBM were allowed to return to normal exercise two weeks earlier than dogs with a well-apposed tibial plateau levelling osteotomy site. Radiographic healing, duration of exercise restriction, and timing of destabilisation were similar in dogs undergoing carpal and tarsal arthrodesis, regardless of whether they received DBM, autogenous graft, or both (23). The

^c Osteoallograft®: Veterinary Transplant Service, Kent, WA, USA

use of DBM has been evaluated experimentally in cats for human purposes (98). An experimental study with dogs was conducted in which DBM gel^f was used alone or in combination with autograft material to see if it would enhance spinal fusion. The gel formulation of DBM had better handling properties and it was able to spread into the irregular contours of the surgical defects. The mixture of autograft with DBM diminished the required quantities of the autograft material, and it appeared to facilitate a more rapid incorporation of the autograft, as it induced an excellent repair response (99).

Despite the extensive and frequent use of ceramics, natural corals, coralline hydroxyapatite, and resorbable polymers in combination with titanium implants in human medicine and dentistry, the current application in veterinary medicine is almost exclusively restricted to experimental procedures (100–102). Clinically, different ceramics have been used successfully in dogs or cats for different indications, including excision of a tumour using calcium phosphate, after arthrodeses with β -tricalcium phosphate or hydroxyapatite, non-unions treated with β -tricalcium phosphate, long-bone fractures or chronic osteomyelitis and osteochondrosis using dentine hydroxyapatite and β -tricalcium phosphate, and alveolar supplementation following canine extraction (12, 103–108).

Bone marrow has been used to enhance bone regeneration in skeletal long-bone defects and non-unions in dogs (37, 109). Clinically, supplementation of ceramic substances with bone marrow improves the handling characteristics of the graft material and accelerates radiographic healing (109). Bone marrow graft added to macroporous biphasic calcium phosphate is an appropriate material in dogs to fill bone defects in irradiated tissue, and can be used after bone removal for oncologic resections (110).

Six non-union fractures (five radius-ulna, one tibia) and four fractures (one femur, one metatarsal bone, two radius-ulna), all of which were associated with critical-sized bone defects as a result of

bone resorption were treated by percutaneous injection of autologous bone marrow derived stromal cells. Complete bone healing was achieved in seven out of 10 cases. The failure of the therapy in three dogs was attributed to resorption of an extremely large segment of the bone, excessive instability and chronicity of the disease (111). Despite multiple positive animal experiments and the successful application in man, reports of clinical use of BMP in veterinary patients are rare. One report described the successful treatment of a four-year-old Pomeranian dog with a two-year history of a femoral non-union fracture with a revision surgery and adjunctive use of rhBMP-2 (112). Additionally, the use of nonglycosylated BMP-2 in a fibrin matrix delivery vehicle was reported for the management of long-bone atrophic non-unions in five cats and three dogs with a complication-free outcome in six of the eight cases. The implant was administered through a stab incision into the fracture gap (113). Four dogs with delayed- or non-unions after a long-bone fracture, osteotomy or arthrodesis were treated with either minimally invasive, fluoroscopically guided, percutaneous administration or direct surgical application of rhBMP-2. A rapid radiographic union was noticed in all dogs with an excellent long-term outcome. Adverse effects included transient worsening of lameness after percutaneous administration of rhBMP-2 (114). A rhBMP-2 solution impregnated on a commercial collagen sponge^a was placed along the diaphysis of an atrophic radius in an Italian Greyhound dog with a history of recurring fractures. Two months after rhBMP-2 treatment, new mineralised bone was present, which significantly increased the diameter of the radius and allowed the removal of the external skeletal fixation system (115). Finally, rhBMP-2 delivered from an absorbable collagen sponge containing β -tricalcium phosphate and hydroxyapatite was also clinically and successfully used in dogs as a graft substitute in reconstruction of large mandibular defects (116–118).

The application of bone tissue engineering in dogs and cats to enhance bone regeneration has been up to now limited to experimental studies (119).

The use of electric current to enhance bone regeneration has not yet gained widespread use in dogs and cats. No clinical studies have been published, although the majority of the original research was done in small animals (120). Although the use of extracorporeal shock wave therapy in dogs and cats is gaining in popularity, there have not yet been any studies that objectively evaluated the efficacy associated with the application of this technique to musculoskeletal tissue (121).

Ruminants

Although the number of dogs used for orthopaedic research still outnumbers that of sheep and goats, the number of small ruminants used for bone research has substantially increased over the last decade (122). In order to verify the practicability of bone enhancing products in more realistic clinical situations, large animal models were developed. Most studies use large segmental long-bone defects to investigate the wide scale of different bone substitutes that enhance bone healing. The studies differ, especially with regard to animal model (sheep, goat), bone treated (femur, tibia, mandible), as well as chemical composition, geometry and resorbability of the used bone-enhancing product (123). Small ruminants have also been used as model for dentistry related research, as well as craniofacial, spinal, and joint research (124–128). In contrast however, there were not any clinical reports available on the use of bone enhancing materials in sheep and goats.

The use of bone grafts in cattle is also limited, mostly because of pure economic considerations. Autogenous cancellous bone grafts were successfully used for treatment of osteolytic defects in the phalanges of cattle (129). Septic physisitis of the metacarpal or metatarsal bones were treated in young animals using homologous cancellous bone grafts (130).

Horses

Autogenous cancellous bone graft techniques have been described in horses for enhancing the treatment of primary frac-

^f Grafton Demineralised Bone Matrix Flowable Gel: Osteotech, NJ, USA

ture repair, delayed or non-union fractures, bone cysts, osteomyelitis, joint arthrodesis, cystic lesion repair, and cervical vertebral interbody fusion (76, 131–140). Autogenous cortical bone grafts have also been used occasionally in horses (141). Autologous cortico-cancellous rib grafts have been used for correction of wry nose, and a cortico-cancellous graft was used to fill a mandibular bone cyst after surgical debridement (142, 143).

Although rarely used in horses, a full cortical allograft was successfully used to repair a metatarsal fracture in addition to external coaptation in a foal (144, 145). Xenografts are also rarely applied in horses, mainly because of technical difficulties and costs, although successful incorporation of bovine xenografts has been recorded in a cervical spinal fusion procedure (140, 141).

The use of β -tricalcium phosphate has been reported in circular metacarpal-metatarsal defects in horses for the purpose of enhancing bone healing (146). It was concluded that β -tricalcium phosphate was effective as a synthetic bone-grafting material in horses. However, no additional advantage was gained by the use of β -tricalcium phosphate because control defects healed in a fashion similar to defects implanted with autogenous cancellous bone combined with β -tricalcium phosphate on a 50/50 weight basis.

It has been shown that stem cells derived from sternal bone marrow aspirates or subcutaneous adipose tissue from foals and horses show potential for use in tissue engineering applications (147, 148). Yet, no clinical applications of bone marrow or stem cells to enhance bone healing in horses have been described so far. Controlled, well-designed studies of the basic biological characteristics and properties of these cells are needed to stimulate this new equine research field. Stem cell research in the horse has exciting perspectives that will most likely benefit the health of horses (149).

Some experimental studies have evaluated the effect of BMP on bone healing in horses. The injection of rhBMP-2 and calcium phosphate into surgically induced osteotomies and ostectomies of the accessory metatarsal bones accelerated early bone healing in an equine model (150). Ishihara

et al evaluated healing of equine metatarsal osteotomies and ostectomies in response to percutaneous injection of adenoviral (Ad) BMP-2, Ad-BMP-6, or β -galactosidase protein vector control administered 14 days after surgery (151). This study demonstrated a greater relative potency of Ad-BMP-2 over Ad-BMP-6 in accelerating osteotomy healing when administered in this regimen, although both genes were effective at increasing bone at both osteotomy and ostectomy sites (151). Adequate gene transfer may be achieved by use of an adenovirus vector in equine cells. High vector doses can be used in equine cells because of relative resistance to cytotoxic effects in those cells. Greater permissiveness and sustained expression of transgenes in bone marrow derived mesenchymal stem cells make them a preferential cell target for gene therapy in horses (152).

Of the clinical reports currently available, few demonstrate the positive effect of rhBMP-2 in horses in enhancing bone healing. Recombinant human BMP-2 was used in a delayed union of a comminuted first-phalangeal fracture in an adult Hanoverian mare while non-glycosylated rhBMP-2 was applied after arthrodesis in a young Warmblood with severe osteoarthritis of the pastern joint (141, 153).

Bone tissue engineering holds great promise for its therapeutic use in horses, but so far no experimental or clinical studies have been described in literature.

Electrostimulation has generally yielded unfavourable results in horses and thus requires further evaluation (154–156). On the other hand, the use of shock-wave therapy in horses has roughly mirrored its use in humans. Shock-wave therapy has been proven to stimulate bone remodelling in horses, especially in stress fractures (157).

Current research focuses and future perspectives

Currently available therapeutic options to enhance bone regeneration (bone grafting and protein-based therapy) do not provide satisfactory solutions to overcome the problem of the healing of major ‘critical sized’ bone defects. Bone tissue engineering is an emerging field that could become a

main therapeutic strategy in orthopaedics in the coming years. Engineered adult stem cells combined with biodegradable scaffolds and growth factors can be implanted into target sites with or without an *ex vivo* culture period. Current ongoing research is mainly focused on stem cell-, growth factor-, gene- and biomaterial-based therapies to achieve a viable alternative to current solutions offered by modern medicine for bone-loss repair. Finally, intensive research is being performed to achieve adequate angiogenesis in transplanted bone grafts.

The use of adult stem cells for bone regeneration has gained much attention. In order to form bone *in vivo*, autologous MSC can be seeded onto ceramic scaffolds and implanted in various models of bone loss (non-union defects) or increased bone formation (spinal fusion). Those cell-loaded ceramics succeeded in producing bone formation in rats, dogs, and sheep in different experimental set-ups using critical sized defects (71, 158, 159). This method was also successful in monkeys in a spinal fusion model (160). The unique population of multipotential cells has been isolated from various sources, including bone marrow, adipose, umbilical cord blood, periosteal, and muscle tissues. Bone marrow stromal cells were used in the early days of tissue engineering. However, the harvesting can be painful and might be associated with increased morbidity (161). Therefore, alternative sources for MSC need to be identified; ‘waste material’ such as adipose tissue, femoral head, umbilical cord blood and placenta may be potentially useful sources as they are easily accessible without large negative issues (162, 163).

Mesenchymal stem cells appear to be immunologically privileged, which was illustrated by a study using mismatched allogeneic stem cells that demonstrated bone regeneration without inciting an immunologic response. These results warrant further research into the possibilities of banking allogeneic MSC for bone regeneration purposes (119).

The genetic engineering of adult stem cells with potent osteogenic genes has been used successfully to stimulate fracture repair and rapid bone formation *in vivo* in mice and rats. The efficiency of using stem cell-based gene therapy for bone formation

has been demonstrated in many studies (164). It was hypothesised that these genetically modified cells exert both autocrine and paracrine effects on host stem cells, leading to an enhanced osteogenic effect.

The use of direct gene delivery is also promising for *in vivo* bone repair. Several viral and non-viral methods have been used to achieve substantial bone tissue formation in various sites in animal models. To advance these strategies into clinical settings, it will be mandatory to overcome specific hurdles, such as control over transgene expression, viral vector toxicity, and prolonged culture periods of therapeutic stem cells (164).

Growth factors will most likely be a major part of any successful strategy to create synthetic bone. It is important to consider the apparently endless combinations of growth factors that might be used, and the numerous methods of delivery such as gene therapy (165).

Several biomaterials for tissue engineering and regeneration are supplemented by either cells or genes, and are designed to improve the complicated biological event of tissue repair. Ideally, the scaffold should have the following characteristics: be highly porous with an interconnected pore network for cell growth and flow transport of nutrients and metabolic waste; be biocompatible and bioresorbable with controllable degradation and resorption rates to match tissue replacement; have surface chemistry suitable for cell attachment, proliferation and differentiation; and have mechanical properties to match those of the tissues at the site of implantation (166, 167). To date, the ideal 'tissue-engineered bone substitute' has not yet been found. Researchers in different fields, including organic chemistry, must continue to design and fabricate a synthetic scaffold to transform the ultimate dream of a 'tissue-engineered bone substitute' into reality.

Incorporation of arginine-glycine-aspartate sequences into the biomaterial has been an attempt to mimic the extracellular matrix, modulate cell adhesion, and induce cell migration. An important issue is to select suitable peptide sequences, and optimise both the density and distribution of such molecules on the scaffold surface for specific cell functions (168).

Two alternative routes of bone repair using biomaterials are presently under investigation: tissue engineering by preformed scaffolds and *in situ* scaffold formation using injectable materials (169). The use of preformed scaffolds in bone tissue engineering is based on *in vitro* seeding of the three-dimensional scaffolds with osteogenic cells. The cell constructs are cultured in bioreactors and implanted afterwards into the place of injury. With the growing popularity of non-invasive arthroscopic procedures, and the requirement to bridge large and irregular bone defects, injectable materials that harden *in situ* are particularly promising for bone regeneration. Several injectable materials have been investigated as osteogenic bone substitutes, although none has delivered satisfying results. Prior to injection, the material may be a solution, a paste, micro- or nanoparticles, beads or thread-like material. They can be cell-free systems or cell and/or bioactive molecule suspension systems (168).

Nanotechnology can also be applied to improve the characteristics of biomaterials. This research focuses primarily on molecular manufacturing of functional materials on the nanoscale level by building the material atom by atom (170).

Despite some success in the arena of bone tissue engineering, the limitations of these techniques, materials, and strategies are evident in the clinical arena. The need for angiogenesis in the induction of bone formation is a critical concept. Combination therapies of stem cells and polymeric growth factor release scaffolds tailored to promote angiogenesis and osteogenesis are under evaluation and development to actively stimulate bone regeneration. An understanding of the cellular and molecular interactions of blood vessels and bone cells will enhance and aid the successful development of future vascularised bone scaffold constructs, enabling survival and integration of bioengineered bone with the host tissue (5). Researchers are currently trying to stimulate angiogenesis in three-dimensional scaffolds by adding vascular endothelial growth factor, fibroblast growth factor-2, copper or human umbilical vein endothelial cells (72, 171).

Conclusion

In order to reduce the disadvantages of cancellous bone autografts and cortical bone allografts in orthopaedic surgery, different bone enhancing products have been tested for human and veterinary applications. Most of these bone substitutes are still in an experimental phase making their clinical use limited to experimental animal set-ups as preclinical trials before human application and some isolated veterinary case reports. Several clinical orthopaedic problems in animals could be successfully treated using bone marrow, DBM, xenografts, ceramics, and BMP, used alone or in several combinations. In the future, there will be much more in the surgeons' arsenal of strategies to enhance bone regeneration, including the application of stem cell and gene therapy options as well as osteoinductive scaffolds.

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