The rationale behind novel bone grafting techniques in small animals

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Summary
Autograft is considered ideal for grafting procedures, providing osteoinductive growth factors, osteogenic cells, and an osteoconductive scaffold. Limitations, however, exist regarding donor site morbidity and graft availability. Although allograft provides an osteoconductive matrix with some osteoinductivity, its availability is limited. To achieve optimal bone graft properties, researchers are developing new materials with the goal of designing synthetics as close to autograft as possible while still facilitating their clinical use. However, the constant evolution of internal fixation stimulates the search for growth factors and cells which could stimulate bone healing.

Introduction
Bone grafts have been used in veterinary surgery for several decades. Currently, bone grafting techniques are used in different areas of orthopaedic surgery, including augmentation of fracture healing, strengthening of arthrodesis, periprosthetic augmentation, spinal fusion procedures, and filling in defects after fracture, osteotomy, osteectomy, or tumour resection (1, 2). The use of autologous bone graft is widely considered the gold standard. In fact, autograft possesses all the characteristics necessary for the growth of new bone as it is osteoconductive, osteoinductive, and osteogenic, without causing problems of compatibility and disease transmission (3). Osteoconductivity refers to the situation in which the graft supports the attachment of new osteoblasts and osteogenitor cells, providing an interconnected structure through which new cells can migrate. Osteoinductivity is a process that supports the differentiation of osteogenitor cells into osteoblasts. Osteogenicity refers to the presence of the live cellular components that can ultimately synthesise bone formation. Autologous bone grafts do, however, have several limitations. Harvesting the graft requires an additional surgery at the donor site which may lead to complications (4). These include pain, infection, blood loss, increased surgical time, variable quality of the obtained graft, and limited supply (2, 3). Limited supply is more commonly problematic in small dogs and cats, or when autograft has been previously harvested. For those cases, the humerus may be preferred since greater weights of bone can be harvested compared to the tibia, and the restoration of cancellous bone is more rapid and complete (5). Severe complications such as fracture have also been described (6). Finally, autologous cancellous graft lacks biomechanical strength, precluding its use as a structural graft.

Despite the advantages of using autologous bone graft, its limitations have necessitated the pursuit of alternatives. Investigators have developed several alternatives using the three basic criteria of a successful graft: osteoconductivity, osteoinduction, and osteogenicity. However, many other important features also influence design consideration (Table 1). Some techniques are already available for clinical use whereas others are still in developmental stage. These alternatives are available in a variety of biomaterials or involve factor- and cell-based strategies that can be used alone or in combination with a biomaterial. The purpose of the present review is to provide a critical overview of the novel bone grafting techniques which are currently available and those which are under development. Novel bone grafting techniques offer new approaches to enhance bone healing, but the successful clinical application of bone grafting techniques requires optimal application.

Osteoconductive bone graft substitutes

The osteoconductive materials include a variety of agents such as bone products, ceramics, polymers, and composites. The properties and clinical applications of each material depend upon its composition and physical presentation, as well as other factors such as manufacturing technique, granulometry, and interconnective porosity.

The primarily osteoconductive properties of allogenic cancellous or cortical bone grafts enhance bone healing (7–10). Allogenic bone grafts may be effectively incorporated when used in repair of fractures or bony defects, or in arthrodesis in dogs and cats. Cortical allografts offer additional mechanical support and help reconstitute
and stabilise existing cortical bone. The use of cortical allografts is associated with the early principles dating back to 1958 of the AO (Arbeitsgemeinschaft für Osteosynthese), and include anatomic reduction and rigid internal fixation (11). However, the incorporation of these cortical allografts is typically slow. The osteoinductive properties of allograft vary according to the type and the processing methods used to prepare, sterilise, and store the allograft material. Freeze-drying preparation destroys all osteogenic cells and leaves only limited osteoinductive capability. Allografts can be processed as a powder, cancellous or cortical chips, wedges, pegs, dowels, or struts (2). Small particle allografts as de-mineralised bone matrix are processed to preserve the osteoinductive properties and are therefore discussed later in the text. Incorporation of allograft bone begins with passive osteoconduction, but the incorporation differs according to the type of graft used (12). Cortical strut grafts are incorporated by creeping substitution through the process of intramembranous bone formation from reconstituted periosteum rather than by extension of bone from the cut cortical ends (13). Cortical grafts ending with an exposed medullary canal are incorporated by endochondral ossification. This process involves weakening of the initial structural strength of the cortical graft as it is resorbed. Strength is recovered as formation of new bone occurs. Finally, cancellous allograft chips or powders are incorporated solely by endochondral bone formation along the osteoconductive framework of the graft, which strengthens the construct over time. Allograft bone is an alternative to autologous bone as it avoids morbidity at the donor site, but it presents potential risks including transfer of infectious diseases and immunological rejection (7, 10, 13, 14). Frozen allografts are usually stored at temperatures below -60°C which decreases enzyme degradation and host immune response. The inconvenience of collection, processing, and storage with a limited shelf life make bone banking impractical. However, canine bone allografts have become commercially available in the United States. Sources of commercially available allograft material include cancellous block and dowel, Bergman block, cortical strut, shaft section as well as whole bone. The evolution of internal fixation led to a change of emphasis from mechanical to biological priorities limiting the use of allografts for fracture fixation (15). Intercalary allografts are mostly used for spinal fusion and the treatment of large bone defects including limb-sparing surgery for primary bone cancer. Recent advances in veterinary implants have led to the design of an endoprosthesis. This is an attractive alternative to allografts for limb-sparing surgeries of the distal radius because the outcome is similar and availability is easier than for cortical allografts (16).

Other osteoconductive biomaterials have gained popularity over the last few decades since they have been proven to be biocompatible substrates for bone-cell migration and bone formation, and are readily available. These osteoconductive biomaterials are usually grouped in two main categories. The first category is the ceramic-based bone graft substitutes including calcium sulfate, bioactive glass, and calcium phosphate. Calcium sulfate was one of the first materials investigated as a bone graft substitute and was found to be easy to use, inexpensive, readily available, stable for filling cavities in bone, and it did not inhibit bone healing in dogs (17, 18). However, significant loss of its mechanical properties and fast degradation limit the clinical use of calcium sulfate (Fig. 1A) (12). Bioactive glass, initially designed as a dental graft, has been evaluated for ortho-

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**Table 1**

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<th>Features of the optimal bone graft substitute</th>
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<td>Biocompatible / Safe</td>
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<td>Osteogenic</td>
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<td>Biomechanical strength</td>
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<td>Bioabsorbable</td>
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<td>Easy preparation, handling and delivery</td>
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**Fig. 1**


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* Veterinary Transplant Services, Kent, WA, USA
* Osteoset® Bone Graft Substitute: Wright Medical Technology, Arlington, TN, USA
paedic surgery, but its use remains limited by its brittle nature, radio-opacity, and very slow resorption; the last two of which also prevent radiographic evaluation of bone healing (Fig. 1B) (9). Calcium phosphate is less radio-opaque, undergoes faster resorption than bioactive glass, and has variable rates of osteointegration based on its crystalline size and stoichiometry (12). Several types of calcium phosphate materials exist, including tricalcium phosphate, hydroxyapatite, and combinations of the two. Pore size and porosity are important characteristics of bone graft substitutes since osteoid formation requires a minimum pore size of 100 μm, with pore sizes of 300 to 500 μm reported to be ideal for osseous ingrowth (19). Thermochemical treatment of coral with ammonium phosphate results in tricalcium phosphate (TCP) with a structure and porosity similar to cancellous bone. Synthetic β-tricalcium phosphate has been used to fill gaps associated with open corrective osteotomies in Dachshunds, and was found to integrate with the surrounding bone within two months (20). Calcium phosphate materials are available in a variety of products, including blocks, granules, powders, and cements (21, 22). Interlocking granules of TCP can also be used as void filler. These 4 mm granules have been developed with an interlocking shape to form a matrix for bone ingrowth while limiting granule migration, but use of these granules should be limited to non-load sustaining situations. The use of Jax TCP yielded mixed results in a critical sized femoral defect model as bony union was improved compared to the control group, however delayed bone formation was observed compared to the autograft group (21). One reason for the considerable variation observed in the degree of bone healing could be due to the variability of the pore size (21). Cements are some of the more recent products (Fig. 1C). The addition of an aqueous solution allows the calcium to dissolve, which is then followed by a precipitation reaction in which the calcium phosphate crystals form and the cement hardens (23). The primary advantages of cement formulation are its abilities to custom-fill defects to increase compressive strength and to be applied using a minimally invasive approach (12, 23, 24). Few clinical studies have been done in human orthopaedic surgery to evaluate the effectiveness of calcium phosphate cement. According to a prospective study on distal radial fractures, patients treated with cement augmentation have superior functional outcomes two years after surgery (25). In a randomised prospective study, femoral neck fractures treated with cannulated screws augmented with calcium phosphate cement had less postoperative displacement than those treated with cannulated screws alone (25). Clinical studies in veterinary medicine are lacking, but experimental studies on screw augmentation in dogs and cats had promising results (23, 24). Ceramic-based bone graft substitutes can be used alone or as a carrier of osteogenic or osteoinductive materials, however, the surgeon should be aware that these products do not provide a substitute for rigid construct fixation. Cement has also been injected in humans percutaneously under fluoroscopic guidance. This may be of interest in small animals for delayed union or biological internal fixation (27, 28). In fact, one of the objectives of biological internal fixation remains maximal preservation of the blood supply to the fractured bone, using either open indirect reduction techniques or minimally invasive plate osteosynthesis (29–31). One main disadvantage of the use of cement is the risk of extrusion beyond the boundaries of the fracture, potentially damaging the surrounding tissue.

The second category of osteoconductive materials includes polymer-based bone graft substitutes. Polyesters are available with different physical, mechanical, and chemical properties. The polymers used in orthopaedic surgery are divided into natural and synthetic, degradable and nondegradable. They are less commonly used than the ceramic-based substitutes. The most recently released bioscaffold on the veterinary market consists of collagen-co-polymerised with a polysaccharide, creating a configuration mimicking tertiary embryonic connective tissue. This hydrogel is radiolucent and can be used as a powder, or mixed with saline or blood prior to injection or implantation within the surgical site. This agent is devoid of any biomechanical strength, but upregulates the gene expression of specific growth factors as transforming growth factor-β (TGF-β) and vascular endothelial growth factors (VEGF) (32).

Osteoinductive bone graft substitutes

Osteoinductive bone graft substitutes stimulate the osteogenic differentiation of local undifferentiated cells. Osteoinductive bone graft substitutes lack structural properties compared with osteoconductive agents. They are therefore especially attractive in cases with compromised healing capacities, such as nonunions. This technique is derived from the pioneering work by Urist in 1965, when it was shown that ectopic implantation of demineralised bone induced bone formation in rodents (33). Since then, it has become evident that a group of proteins is responsible for osteoinduction.

One strategy is to obtain an optimal mixture of growth factors to stimulate bone regeneration. Demineralised bone matrix (DBM) is a type of bone allograft processed to preserve its osteoinductive properties. The allogenic bone is chemically sterilised (typically combined with a demineralisation process) to give rise to a demineralised matrix consisting of type I collagen and non-collagenous proteins (3). Demineralised bone matrix provides osteoinduction by the inherent growth factors liberated during demineralisation, and osteoconduction with the collagen I network naturally present in bone. Numerous DBM formulations exist based on refinements of the manufacturing process. Human products are available as freeze-dried powder, granules, gel, putty, or strips. They have also been developed as com-
Bone morphogenetic protein-signalling pathway (Modified from [41]).

In dogs undergoing carpal and tarsal arthrodesis regardless of whether they received DBM, autologous bone graft, or both (1). Demineralised bone matrix has been commercialised for canine-use since 1996, and has been used recently more routinely in canine orthopaedic surgery (Fig. 2) (1, 35). Sources of DBM material that are commercially available for dogs include DBM® alone, or DBM mixed with cancellous allograft chips (chips sieved to <4 mm, <2.5 mm, or to <0.7 mm). Although optimism is visible at the osteotomy gap (arrow).

Platelet concentrates also contain a mixture of growth factors, and have recently been investigated for their effects on bone healing. Platelets provide initial haemostasis, and release mediators to help modulate the inflammatory response and cellular functions involved in bone repair. Multiple growth factors are contained in the alpha granules of platelets, such as platelet-derived growth factor, TGF-β, VEGF, fibroblast growth factor-β (FGF-β), and insulin-like growth factor. These factors are known to play important roles in bone repair (36). Platelet concentrates have been shown to promote osteoblast proliferation and differentiation (37). Systems to obtain platelet-rich plasma are available for small animals. A newer product was recently commercialised to extract platelet concentrate from 9 mL of patient blood within a few minutes. However, the role of platelet concentrate as a promoter of bone healing remains controversial (36, 38). Clinical evidence is lacking to support the use of platelet-rich plasma in the treatment of long-bone defects and nonunions (28, 36).

Another osteoinductive strategy is the production of a single purified molecule, which could ultimately be confirmed as the agent of choice for stimulation of bone healing and regeneration. The bone morphogenetic proteins (BMP) have received the most attention of all the growth factors evaluated for clinical use because of their potent ability to induce new bone formation. At least 20 BMP have been identified (39). They are low-molecular-weight glycoproteins and, with the exception of BMP-1, belong to the TGF-β superfamily of growth and differentiation factors (40). Different BMP are important at various stages of the differentiation process. Their biological functions are mainly related to bone and cartilage formation, although some do not have any known role in bone and cartilage (40, 41). The BMP exert their effect through the activation of the transmembrane heteromeric receptor complex, which then phosphorylates the transcription factors Smad, and thus activating the expression of target genes (Fig. 3) (42). The skeletal hallmark of the BMP is their ability to enhance osteoinduction and stimulate de novo bone formation. As chemotactic agents, they influence migration of progenitor and stem cells to the site of need. They also stimulate angiogenesis and stem cell proliferation, and induce, as dif-
ferentiating factors, the maturation of stem cells into chondrocytes, osteoblasts, and osteocytes (43).

Although several BMP have been shown to be osteoinductive, only two recombinant human (rh) BMP have been currently developed for clinical applications in human: rhBMP-2 and rhBMP-7 (osteogenic protein-1 [rhOP-1]). Their clinical use in veterinary medicine is off-label and remains limited to the local delivery with a carrier matrix by surgical implantation or direct administration (44, 45). The limited half life of BMP necessitates the use of carrier matrix to increase local retention. An absorbable collagen sponge (ACS) is most commonly used as a carrier but ceramic- and polymer-based bone graft substitutes could be used as well. The ACS has recently brought considerable enthusiasm because of its excellent safety and pre-existing approval for application in human surgery. The sponge is usually immersed in a BMP solution for up to an hour before implantation because BMP retention is a function of soaking time (43). The quantity of rhBMP-2 or –7 needed clinically for osteoinduction represents several times the amount of corresponding endogenous BMP derived from fracture sites or from implantation of autologous bone graft (46). This difference probably reflects the fact that the response to rhBMP is related to locally pre-existent cofactors, osteoblasts, and osteoprogenitor cells (43). The use of BMP has been widely studied in experimental, preclinical and clinical veterinary and human studies showing that BMP hold promise in orthopaedic surgery (42, 47). However, the optimal method of application for BMP in small animals is still unknown despite numerous reports of its use in dogs and cats (44, 45, 48–51). The efficacy of rhBMP-7 (rhOP-1) was first reported by Cook et al in a large segmental defect model in dogs (52). The application of rhBMP-7 has been limited in small animal since this first report; whereas rhBMP-2 has been used in experimental studies as well as for clinical cases. The application of rhBMP-2 in an ACS at a concentration of 0.2 mg/mL was found to be superior to a concentration of 0.05 mg/mL using force plate analysis and radiographic evidence of bone healing (53). In another study, the concentration of rhBMP-2 delivered in an ACS was found optimal at 0.2 mg/mL when compared to 0.4 mg/mL for acceleration of bone healing (54). However, the recommended rhBMP-2 concentration delivered in an ACS in humans was found to be 1.5 mg/mL in a large randomised prospective study on 450 open tibial fractures (55). The discrepancy between these optimal concentrations (0.2 mg/mL in dogs versus 1.5 mg/mL in human) may be explained by differences between species or by the absence of correlation between the concentration in the carrier and the total dose and amount used. In the large clinical study in humans, the optimal concentration of 1.5 mg/mL corresponded to a total amount of 12 mg per patient (55). Dose and total amount of BMP-2 used in experimental and clinical cases vary in veterinary medicine; and comparison between studies remains difficult. In dogs, the dose of BMP-2 used for clinical cases ranges from 0.017 mg/kg to 2.1 mg/kg with a total amount ranging from 0.20 to 7.5 mg (44, 48, 49). However, potential adverse effects of BMP have been described despite their unquestionable benefit. Excessive bony overgrowth can affect function, as for spinal cord impingement after vertebral stabilisation (43). The use of BMP is not recommended for use in skeletally immature patients or during pregnancy (43). Finally, transient worsening of lameness was observed in a few cases after injection of rhBMP-2. Suspected aetiologies included discomfort associated with percutaneous injection or transient swelling, and increased vascularisation associated with stimulated bone formation (44). Soft tissue swelling has also been reported in humans and can be associated with significant patient morbidity when rhBMP-2 was applied off-label at the level of the cervical spine, potentially requiring the need for a tracheostomy (56). A publication on InFuse® describing improved tibial fracture healing with rhBMP-2 was withdrawn from the Journal of Bone and Joint Surgery in March 2009 after several problems were found with the study (57).

The use of other single growth factors to stimulate bone healing, including somatotropin, FGF-β, and TGF-β has been evaluated. Further studies are required to determine their potential to increase bone healing before clinical application since their effects seem less conclusive than BMP (58, 59).

**Osteogenic bone graft substitutes**

Another promising application for fracture healing relies in the use of cells. The presence of osteoprogenitor cells is the critical component of all bone formation. Osteogenesis begins with mesenchymal stem cells (MSC) that give rise to progenitor cells. These progenitors advance to pre-osteoblasts and then to osteoblasts (Fig. 4). Eventually, osteoblasts provide matrix

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1. InFuse®: Medtronic Sofamor Danek, Memphis, TN, USA
2. Osteogenic protein-1 / OP-1®: Stryker Biotech, Hopkinton, MA, USA

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Fig. 4 Induction of an osteogenic phenotype of mesenchymal stem cells harvested from canine adipose tissue cultured in osteogenic media, as demonstrated by alkaline phosphatase (A) and by Alizarin Red (B) staining at day 5 (10 x).
for new bone tissue as well as bone-lining cells and osteocytes.

The term connective tissue progenitor was defined as the entire heterogeneous population of stem and progenitor cells that are capable of differentiating into one or more connective tissue phenotypes, including bone (60). The tissue progenitors include true quiescent, multipotent stem cells as well as cells that lack self-renewal capabilities and exhibit intrinsic commitment to various stages of diverse lineages (61). A high concentration of connective tissue progenitors can be obtained from bone marrow. Use of bone marrow aspirate is therefore a strategy to apply connective tissue progenitors to enhance bone growth and repair (12). Connective tissue progenitors derived from bone marrow were first used to enhance bone healing in 1980 (62). This procedure can be done intra-operatively with ease and a low morbidity rate. In a study on a canine tibial nonunion model, gaps were filled with bone marrow aspirate, DBM, a composite graft of bone marrow aspirate and DBM, or autologous bone graft after application of an external fixator (63). Use of the combination of DBM and marrow yielded results that were superior than single-agent groups and similar to autologous bone graft group (63). Muschler et al reported the use of a selective cell-retention method for enriching allograft⁶ in a canine spine fusion model. The selective cell process allowed a three- to four-fold increase in concentration of connective tissue progenitors (64). Radiographic score, quantitative computed tomography, and mechanical testing showed the superiority of the selective-retention-enriched bone matrix and bone-marrow clot compared to non-enriched bone matrix and bone-marrow clot (64).

All tissues vary substantially with respect to cellularity and prevalence of connective tissue progenitor cells. Bone marrow aspirate is the best characterised source of connective progenitor cells. However, adipose tissue is a promising source of connective tissue progenitors because of its abundance and ease of access with minimal donor site morbidity (65). The prevalence of connective tissue progenitor cells is higher in fat despite its low cellularity compared to bone marrow. The isolation process leads to a mix of adipose-derived MSC, haematopoietic stem cells, pre-endothelial cells, fibroblasts, pericytes, and endothelial cells (66). However, differences in biological potential among progenitor cells derive from various tissues may have important practical implications with regard to the selection of the cell source, but bone marrow will remain the tissue of choice until more is known about the use of adipose derived cells in clinical application.

Mesenchymal stem cells are multipotent cells that can be isolated from bone marrow and adipose tissue as well as muscle, synovium, periosteum, and tissues from endodermal or ectodermal lineage (67). Another cell-based approach for enhancement of bone regeneration is the use of MSC selected by culture expansion. Expansion of cells is the optimal way to select a more homogeneous population. In vitro, clones of cells that divide the most rapidly and have the greatest ability for continued proliferation have a competitive advantage. In vitro expansion produces therefore a strong selective pressure favouring these traits allowing isolation of MSC. Different strategies are possible for the therapeutic application of these isolated MSC. The cells can be implanted where the new tissue is needed or can home into areas of damage after injection into the systemic circulation. Their osteogenic efficacy can be enhanced by the use of biomaterial to selectively concentrate and retain cells in the area of damage (64). Delivering stem cells in an appropriate carrier fulfilling the tenants for bone regeneration is an active area of research (68). Finally, MSC lack certain haematopoietic cell surface markers and possess other characteristics suggesting that they can elude T-cell-mediated cell rejection and therefore are immune-privileged (69). The suggestion that MSC are sufficiently non-immunogenic raised the possibility that allogenic MSC could be utilised as effectively as autologous MSC in bone regeneration.

Conclusion

The successful clinical application of novel bone grafting techniques in small animal depends on the clarification of the optimal application, development of better carrier for growth factors and cells, and further understanding of the local mechanisms for bone repair. This rapidly evolving technology challenges the clinicians to formulate an informed opinion on emerging therapies. The wide range of therapeutic materials and treatments available complicates the decision-making process for orthopaedic surgeons, but provides more freedom to optimise treatment modality for individual cases. Understanding which factor(s) are deficient in a given patient determines the criteria for selecting a therapeutic strategy. If grafting is the answer, a good knowledge of the grafting agents available will guide one to the ultimate choice in matching the need to the product.

Novel bone grafting techniques offer new approaches to enhance bone healing in the management of fractures, nonunions, bone defects, and arthrodesis. However, basic surgical principles such as biomechanical stability and eradication of infections remain essential to provide an ideal environment for bone healing. Techniques are evolving constantly to follow the general trend of fracture fixation with a recent emphasis on biological fixation.

Optimising combinations of biomaterials, locally and systemically active stimuli, and cells will remain a complex process characterised by a highly interdependent set of variables with an almost infinite range of possible combinations. Experimental and clinical studies are needed in small animal surgery, as well as in human medicine, to allow initial evaluation of new biomaterials, growth factors, cell therapy, and any combination of those (27).

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⁶ Cellect™ Selective retention device: DePuy Spine Inc., Raynham, MA, USA


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