Effect of growth factors on the osteoinductive potential of Hydroxyapatite β-Tricalcium Phosphate (HA-TCP)

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Chapter 1. A REVIEW OF THE OSTEOINDUCTIVE POTENTIAL OF HYDROXYAPATITE β-TRI-CALCIUM PHOSPHATE (HA-TCP), ENAMEL MATRIX DERIVATIVE (EMD) AND PLATELET DERIVED GROWTH FACTOR-BB (PDGF-BB)

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1.1 Introduction

The replacement of missing teeth by osseointegrated dental implants is a commonly utilised treatment option in dentistry. However, successful treatment outcomes are dependent on sufficient bone quantity in the proposed surgical site for implant placement (Buser *et al.*, 2004).

Surgical augmentation of bone defects is commonly performed prior to or during implant placement. Bone augmentation procedures of the maxillary sinus or guided bone regeneration (GBR) procedures of alveolar ridge defects have utilised a variety of bone graft materials in block or particulate form, either alone or in combination with resorbable or non-resorbable barrier membranes.

1.2 Osteogenesis, Osteoconduction, Osteoinduction

Bone graft materials are defined as any implanted material that "promotes new bone formation through osteogenic, osteoinductive or osteoconductive processes" (Bauer and Muschler, 2000). However, these three processes differ in their mechanisms of new bone formation. Osteogenesis occurs when the graft material contains viable osteoprogenitor cells capable of differentiating into osteoblasts to produce new bone. Osteoconduction occurs when the graft material serves as a scaffold, allowing osteogenic cells to infiltrate from the adjacent bone margins, to proliferate and form bone on the surface of the graft material with subsequent replacement or incorporation of the graft material with new bone (Jensen *et al.*, 2006). Finally, osteoinduction occurs when the graft material stimulates undifferentiated mesenchymal stem cells from the surrounding tissue to differentiate into osteogenic cells to form new bone (Urist, 1965, Yuan *et al.*, 2001b, Habibovic *et al.*, 2005b, Yuan *et al.*, 2006b).

During skeletal development and repair, bone formation can occur via the processes of endochondral or intramembranous ossification. Endochondral ossification occurs primarily in long bones while in the flat bones of the skull and also the mandible, bone formation occurs via the process of intramembranous ossification.

During endochondral ossification, undifferentiated mesenchymal stem cells condense at the site of future bone formation. Cells within the condensation centre differentiate into chondrocytes to produce cartilage while those at the periphery differentiate into fibroblast like perichondrial cells to produce perichondrium. During this process, cartilage becomes mineralised as the chondrocytes become hypertrophic and this is followed by vascular invasion of the mineralised cartilage. Subsequently, chondroclasts degrade the mineralised cartilage allowing osteoblast migration and osteoid deposition onto the cartilaginous matrix with replacement of the cartilage precursor with mineralised bone (Chung *et al.*, 2004, Colnot, 2005).

In contrast to endochondral ossification, no cartilage precursor is formed during intramembranous ossification. Cell differentiation occurs within a membranous, condensed plate of mesenchymal cells present in fibrous connective tissue with differentiation of these cells directly into those of an osteoblastic lineage. These cells continue to differentiate and proliferate into osteoblasts that deposit bone matrix resulting in the formation of woven bone (Kronenberg, 2003, Chung *et al.*, 2004).

1.3 Bone graft materials

The ideal bone graft material should be biocompatible, with a physicochemical structure similar to natural bone in order to promote angiogenesis and fibrovascular tissue ingrowth with incorporation of the graft material with the new bone (Klawitter *et al.*, 1976, Daculsi and Passuti, 1990, Chang *et al.*, 2000). Additionally, an ideal bone graft should be osteogenic or osteoinductive resulting in formation of new bone or at a minimum, osteoconductive, promoting direct bone contact and growth along the graft material. Additionally, the ideal graft material should undergo remodelling with a resorption rate similar to the rate of new bone formation resulting in an augmented site consisting of host bone alone (Shetty and Han, 1991).

Four different types of bone graft materials have been commonly used and are classified as autografts, allograft, xenografts or alloplasts.

1.3.1 Autografts

Autografts are harvested from the intended graft recipient and are considered the gold standard bone graft material. Autografts are osteogenic, due to the presence of viable osteogenic cells, osteoinductive, due to the presence of bone matrix proteins such as bone morphogenetic proteins (BMP) and osteoconductive, due to the porous mineralised component of bone (Misch and Dietsh, 1993). However, the amount of graft material that can be harvested is limited and may be associated with increased morbidity and risk of surgical or postoperative complications (Laurie *et al.*, 1984, Younger and Chapman, 1989, Clavero and Lundgren, 2003, Cricchio and Lundgren, 2003).

1.3.2 Allografts

Allografts are grafts that have been harvested from one individual and implanted into another individual of the same species. Donors can be living related persons, living unrelated persons or, more commonly, from cadavers after the removal of viable cells (Friedlaender *et al.*, 1999). Allografts are generally prepared in freeze-dried forms (FDBA) or hydrochloric acid treated to produce demineralised freeze-dried forms (DFDBA) to expose growth factors such as the BMP sequestered in the bone matrix (Urist, 1971). As cells are removed from allografts, they are not osteogenic and the extent of osteoinductive and osteoconductive properties of the allograft may vary depending on graft processing (Becker *et al.*, 1995) as well as the donor age (Schwartz *et al.*, 1998). Although allografts have been used with clinical success in implant dentistry (Simion *et al.*, 1996, Olson *et al.*, 2000), their application may be limited due to high cost and the patient's perceived risks of viral transmission, immunogenicity or other social and religious concerns (Buck *et al.*, 1989, Friedlaender *et al.*, 1999).

1.3.3 Xenografts

Xenografts are grafts harvested from different species, commonly of bovine origin resulting in hydroxyapatite bone mineral after removal of the organic component (Jensen *et al.*, 1996). Xenografts are osteoconductive as they have a mineral content and porosity similar to human bone (Spector, 1994) but do not contain osteogenic cells or osteoinductive agents. Animal and human studies have demonstrated new bone formation in direct contact with demineralised bovine bone matrix (DBBM) particles when used in bone grafting procedures (Fukuta *et al.*, 1992, Yildirim *et al.*, 2000, Zitzmann *et al.*, 2001). Similar to allografts, the use of DBBM may not be acceptable to some patients due to the perceived risk of disease transmission or other social or religious concerns (Sogal and Tofe, 1999, Wenz *et al.*, 2001).

1.3.4 Alloplasts

Alloplasts represent a large group of chemically and structurally diverse materials with varying mechanical and biological properties (Aichelmann-Reidy and Yukna, 1998). These materials include calcium sulfate (De Leonardis and Pecora, 1999), composite polymers (Ashman and Lopinto, 2000), bioactive glass ceramics (Oonishi *et al.*, 1997) as well as the calcium phosphate based ceramics. As alloplasts do not provide any osteogenic cells or osteoinductive proteins, they are considered to be osteoconductive only (Aichelmann-Reidy and Yukna, 1998).

1.4 Calcium Phosphate (CaP) ceramics

Calcium phosphate (CaP) ceramics are a group of polycrystalline ceramics with varying structural and mineral arrangements with hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and the mixture of HA and β-TCP, biphasic calcium phosphate (BCP) investigated with the most interest.

These materials have been widely used as bone graft materials in dentistry (Monroe *et al.*, 1971, Nery and Lynch, 1978, Jarcho, 1981, Jepsen *et al.*, 2008, Lee *et al.*, 2008b) due to their biocompatibility and similarity in composition to bone mineral (Rey, 1990, LeGeros, 2002).

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] is the primary mineral component of bone, while tricalcium phosphate [$Ca_3(PO_4)_2$] is not a natural component of bone but has chemical proportions of calcium and phosphate similar to bone mineral (Jarcho, 1986). These materials are not considered osteogenic or osteoinductive but provide a biocompatible osteoconductive scaffold for new bone formation. A unique property of CaP ceramics is their bioactivity, with formation of carbonate hydroxyapatite [$Ca_{10}(PO_4.HPO_4.CO_3)_6(OH)_2$] on their surfaces prior to bone deposition resulting in formation of a bond at the bone-ceramic interface (Jarcho, 1986).

1.4.1 Preparation of CaP ceramics

Calcium phosphate ceramics are produced by the preparation of calcium deficient apatites (CdA) with varying Ca/P ratios which are compacted and sintered under high pressure (10,000 to 20,000 psi) and high temperatures (≥1000°C) (LeGeros, 1993, Jarcho, 1986).

The physicochemical properties of the ceramic are determined by the Ca/P ratio of the apatite in addition to the sintering pressure and temperature. By controlling these variables, a wide range of calcium phosphate ceramics with different physicochemical properties can be produced. Apatites with a Ca/P ratio of 1.67 result in the formation of hydroxyapatite (HA) while a Ca/P ratio of 1.5 results in the formation of tricalcium phosphate (TCP). When apatites with varying Ca/P ratios are sintered simultaneously, different amounts of HA and TCP are formed in the final CaP ceramic, resulting in the formation of biphasic calcium phosphate (BCP) ceramics (Blokhuis *et al.*, 2000, Bouler *et al.*, 2000, Daculsi *et al.*, 2003).

1.4.2 Biphasic Calcium Phosphate (BCP)

Biphasic calcium phosphate (BCP) ceramics consist of a mixture of β-TCP and HA (HA-TCP) (Daculsi *et al.*, 2003). BCP ceramics were developed to provide the benefits of rapid resorption of β-TCP whilst maintaining the osteoconductive scaffold of the minimally resorbable HA (Hashimoto-Uoshima *et al.*, 1995, Piattelli *et al.*, 1996).

Due to the presence of β-TCP, HA-TCP is considered a resorbable graft material, with greater resorbability associated with increasing ratios of β-TCP to HA (Daculsi *et al.*, 1989, Farina *et al.*, 2008, Jensen *et al.*, 2008). Most animal studies have reported that HA-TCP is osteoconductive, with new bone formation occurring directly against the graft materials (Daculsi *et al.*, 1989, Fleckenstein *et al.*, 2006, Jensen *et al.*, 2007), however, fibrous encapsulation and an absence of osteoconductivity have been reported 18 months after implantation of HA-TCP particles into rat osseous defects (Develioglu *et al.*, 2007).

Others have suggested that HA-TCP may be more osteoconductive than HA due to its greater solubility and greater bone formation detected *in vivo* (Schopper *et al.*, 2005, Jensen *et al.*, 2007, Jensen *et al.*, 2008).

HA-TCP was initially used in periodontics and implant dentistry in the treatment of periodontal osseous defects and maxillary alveolar ridge defects (Nery *et al.*, 1990, Piattelli *et al.*, 1996). More recently, HA-TCP with a 60HA:40TCP ratio has been used in maxillary sinus augmentation resulting in new bone formation and an absence of inflammatory or foreign body reaction (Lee *et al.*, 2008b).

A new particulate HA-TCP ceramic has recently been released (Straumann Bone Ceramic®). The CdA is sintered at 1100-1500°C on a polymer matrix to produce a ceramic with a HA:TCP ratio of 60:40 and crystallinity of 100%. The porous block has a total porosity of 90% and macropore size of 100-500 µm with interconnected pores of 100-150 µm in diameter. This block is then ground and sieved to produce particle sizes ranging from 400-700 µm.

Recent animal studies have reported on the osteoconductive properties of this material (Jensen *et al.*, 2007, Jensen *et al.*, 2008). In membrane covered defects in minipig mandibles, Bone Ceramic® particles were used effectively as an osteoconductive graft material, with similar bone formation when compared to autogenous bone after 24 weeks. Additionally, only limited degradation of the HA-TCP was reported after this time period (Jensen *et al.*, 2007). However, others have reported fibrous encapsulation of this material with minimal graft resorption after nine weeks (Schwarz *et al.*, 2007). In human clinical trials, this material has been used in maxillary sinus

augmentation, either alone or combined with autogenous bone (Artzi *et al.*, 2008, Cordaro *et al.*, 2008). In both studies, biopsies taken 180-270 days after augmentation demonstrated close contact between the HA-TCP particles and new bone. Additionally, some resorption of the graft particles had occurred with a reduced percentage of graft particles demonstrated histomorphometrically (Cordaro *et al.*, 2008).

1.5 Osteoinduction

Osteoinduction has been defined as "the mechanism of cellular differentiation of one tissue towards bone due to the physicochemical effect or contact with another tissue" (Urist *et al.*, 1967). *In vivo*, the osteoinductive properties of bone graft materials can be demonstrated by the formation of ectopic bone after implantation into non-osseous sites lacking osteogenic cells.

The development and clinical application of an osteoinductive bone graft material would be advantageous in the reconstruction of large bone defects, as the concurrent process of osteoinduction within the centre of the defect as well as osteoconduction at the defect margins could result in greater and more rapid bone formation (Yuan *et al.*, 2006a). Several animal studies have suggested that an osteoinductive agent may translate to better bone-healing orthotopically, with enhanced healing in osseous defects when compared to non-osteoinductive or weakly osteoinductive materials (Habibovic *et al.*, 2006b, Yuan *et al.*, 2006a, Habibovic *et al.*, 2008).

The phenomenon of material related osteoinduction was first reported in a study where endochondral bone formation was detected 8 to 16 weeks after intramuscular implantation of demineralised bone matrix into the anterior abdominal wall of rabbits, rats, mice and guinea pigs (Urist, 1965). Later studies suggested that bone morphogenetic proteins (BMP) within the demineralised bone matrix were responsible for inducing the differentiation of resident perivascular mesenchymal cells firstly into chondrocytes and then into osteoblasts (Urist and Strates, 1971, Reddi and Huggins, 1972, Reddi, 1981, Urist *et al.*, 1983). Since then, the osteoinductive potential of native

and recombinant BMP have been demonstrated in a number of animal studies (Cook and Rueger, 1996, Riley *et al.*, 1996) as well as trialled in clinical practice (Jung *et al.*, 2003).

1.5.1 Osteoinduction by CaP ceramics

The presence of BMP was determined to be unnecessary when materials devoid of these proteins such as a polyhydroxylethylmethacrylate sponge were observed to be osteoinductive after implantation into the soft tissues of pigs and rats (Winter and Simpson, 1969). Calcification of the polymeric sponge occurred prior to bone formation, suggesting the importance of *in vivo* calcification in the process of osteoinduction. Since this early finding, others have demonstrated osteoinduction after soft tissue implantation of a variety of materials including porous titanium blocks (Fujibayashi *et al.*, 2004, Kokubo, 2004), bioactive glasses (Yuan *et al.*, 2001a) as well as CaP ceramics.

Although CaP ceramics are usually encapsulated by fibrovascular connective tissue (Piecuch, 1982, Yamasaki and Sakai, 1992, Fellah *et al.*, 2008), the first evidence of CaP ceramic associated osteoinduction was reported after subcutaneous implantation of HA in dogs (Yamasaki, 1990).

In the last two decades, several research groups have reported osteoinduction by CaP ceramics without the addition of osteogenic cells or bone growth factors after implantation into non-osseous sites. It has been suggested that the bioactive properties of CaP ceramics and release of calcium and phosphate ions may enhance the calcification required for material associated osteoinduction (Habibovic *et al.*, 2005a) with this phenomenon independent of the type of CaP ceramic (**Table 1)**

CaP Ceramic	Osteoinduction (Reference)
HA	Heughebaert *et al.*, 1988 Yamasaki and Sakai, 1992 Ripamonti, 1996 Yuan *et al.*, 1998b Yuan *et al.*, 1999 Yuan *et al.*, 2001c Habibovic *et al.*, 2005b, Yuan *et al.*, 2006b
TCP (β-TCP, α-TCP)	Yuan *et al.*, 2001a
BCP (HA-TCP)	Yang *et al.*, 1996 Yang *et al.*, 1997 Yuan *et al.*, 1998a Kurashina *et al.*, 2002 Habibovic *et al.*, 2005b Le Nihouannen *et al.*, 2005 Yuan *et al.*, 2006a, 2006b Habibovic *et al.*, 2008

Table 1: Studies reporting osteoinduction after implantation of CaP ceramics

1.5.2 Osteoinduction by HA-TCP

Precipitation of the biological apatite layer on the HA-TCP surface could provide a suitable chemical environment to induce differentiation of mesenchymal stem cells into osteogenic cell lines (Daculsi *et al.*, 1990, Daculsi *et al.*, 2003) with the greater solubility of HA-TCP (LeGeros *et al.*, 2003, Schopper *et al.*, 2005) resulting in improved osteoinductivity (Yuan *et al.*, 1998a, Habibovic *et al.*, 2005b, Yuan *et al.*, 2006a, 2006b, Habibovic *et al.*, 2008).

After intramuscular and subcutaneous implantation of HA-TCP in dogs and pigs, ectopic bone formation was detected 45 days after intramuscular implantation and 60 days subcutaneously. Fifteen days after implantation, invasion of fibrovascular connective tissue into the ceramic macropores was seen, followed by the appearance of polymorphic mesenchymal cells near the invading vasculature and the ceramic interface after 30 days with some of these cells positive for alkaline phosphatase (ALP) activity. From day 45, osteoblast differentiation and formation of bone matrix in contact with the ceramic surface was observed. Osteoblast differentiation occurred within the cell clusters that had aggregated on the pore inner surface and new bone formation was always closely associated with the presence of blood vessels (Yang *et al.*, 1996, Yang *et al.*, 1997).

More recently, new bone formation within the ceramic macropores as well as bony trabeculae bridging the ceramic particles were identified after intramuscular implantation of an HA-TCP with a 60:40 HA:TCP ratio into sheep for six months. Blood vessels were observed within the macropore structure, as well as associated with new bone formation within the macropores and between the graft particles (Le Nihouannen *et al.*, 2005).

This newly formed bone appears to be stable with the absence of pathology in the surrounding soft tissues four and a half years after implantation and ongoing bone remodelling resulting in the presence of lamellar bone with Haversian like structures (Ye *et al.*, 2007).

However, not all studies have demonstrated osteoinduction after implantation of HA-TCP with an absence of new bone formation and fibrous encapsulation of the ceramic (Fellah *et al.*, 2008). After subcutaneous implantation into rats, demineralised bone powder resulted in ectopic endochondral bone formation however no bone formation was reported in the sites implanted with the HA-TCP ceramic with a 65/35 HA:TCP ratio (Eid *et al.*, 2001).

1.5.3 Animal model specificity

The implantation of materials into non-osseous sites has been performed in a variety of animal models although the extent and predictability of osteoinduction appears to be dependent on the animal model used. Osteoinduction has commonly been observed after intramuscular implantation in large animals such as dogs, baboons, sheep and goats **(Table 2).**

Animal Model	Osteoinduction reported (Reference)
Dogs	Yamasaki and Sakai, 1992 Klein *et al.*, 1994 Ripamonti, 1996 Yang *et al.*, 1997 Yuan *et al.*, 1998a Yuan *et al.*, 1999 Yuan *et al.*, 2000 Yuan *et al.*, 2001a Yuan *et al.*, 2001c
Baboons	Ripamonti, 1996
Sheep	Gosain *et al.*, 2002 Le Nihouannen *et al.*, 2005
Goats	Yuan *et al.*, 2002 Habibovic *et al.*, 2005a Habibovic *et al.*, 2008

Table 2. Studies demonstrating osteoinduction after implantation of CaP ceramic in large animal models

Osteoinduction has been demonstrated less consistently in smaller animal models such as rabbits (Kurashina *et al.*, 2002, Yuan *et al.*, 2006b) with smaller amounts of bone formation (Ripamonti, 1996) or longer implantation periods required before ectopic bone formation. Similarly, intramuscular or subcutaneous implantation in rodent models have not usually reported ectopic bone formation (Ohgushi *et al.*, 1989a, Goshima *et al.*, 1991, Yang *et al.*, 1996) however the osteoinductive potential of HA-TCP have been demonstrated recently in rodents (Yuan *et al.*, 2006b).

Differences in osteoinductive activity have been attributed to possible differences in the levels of endogenous proteins such as BMP between animal species (Ripamonti, 1996) as well as genetic differences in osteoinductive potential between individuals (Marusic *et al.*, 1999, Habibovic *et al.*, 2005b). The size of the implanted graft may also influence the osteoinductive potential, with greater bone formation generally reported after implantation of larger grafts. Larger grafts provide a mechanically stable surface necessary for bone growth (Szmukler-Moncler *et al.*, 1998) as well as greater surface area for cell migration and biological fluid flow. The lack of osteoinductive activity in smaller animal models may be partly explained by the presence of shear stresses or micromotion or

insufficient surface area in the smaller grafts utilised (Yuan *et al.*, 1998a, Yuan *et al.*, 2001c, Habibovic *et al.*, 2006a).

1.5.4 Structural influences on CaP ceramic associated osteoinduction

Although the process of osteoinduction is not fully understood, the three-dimensional structure and geometry of the biomaterial appears to have a crucial role in influencing osteoinduction (Fujibayashi *et al.*, 2004). Certain structural elements such as macroporous and microporous surfaces appear to be necessary. The presence of macropores may reduce the shear stresses present on the outer surface of the ceramic (Yuan *et al.*, 1998a, Yuan *et al.*, 2001c) as well as allowing development of a sufficient calcium and phosphate ion concentration within the pore during CaP ceramic dissolution (Duan *et al.*, 2005). Animal studies have demonstrated that ectopic bone formation is limited to the macroporous surfaces of the implanted ceramic and is absent around non-porous materials (Ripamonti, 1991, Yamasaki and Sakai, 1992) with maintenance of the macroporous structure after implantation critical for osteoinduction (Kurashina *et al.*, 2002).

Recent studies have indicated that a microporous surface is important for CaP ceramic osteoinduction (Yuan *et al.*, 1998a, Habibovic *et al.*, 2005b, Habibovic *et al.*, 2008) with bone formation occurring after intramuscular implantation in dogs of HA or HA-TCP macroporous ceramics with surface microporosity, whereas bone formation did not occur with dense ceramics (Yuan *et al.*, 1999, Yuan *et al.*, 2002). Similarly, others have also reported increased bone formation associated with increasing microporosity of the HA-TCP ceramic (Habibovic *et al.*, 2005b, Habibovic *et al.*, 2006a, Habibovic *et al.*, 2006b, Habibovic *et al.*, 2008).

These structural elements increase the specific surface area of the material, enhancing surface reactivity and dissolution of the ceramic in body fluid. Greater precipitation of the biological apatite layer may occur on the ceramic surface resulting in increased protein adsorption and cell adhesion to the ceramic (Hing *et al.*, 2005, Li *et al.*, 2008). *In vivo*, when HA ceramics with high and low specific surface areas were implanted intramuscularly into goats, only the HA ceramic with a

higher specific surface area induced bone. Similarly, HA-TCP with a lower specific surface area induced significantly less bone than those with a higher specific surface area (Habibovic *et al.*, 2005b).

A microporous surface may have other cellular effects, such as enhancing cell deposition of extracellular matrix components, with abundant collagen fibrils seen within the β-TCP micropores after intramuscular implantation in dogs (Kondo *et al.*, 2006). Microporosity may also enhance osteogenic cell differentiation. Pluripotent mesenchymal cells demonstrated greater levels of alkaline phosphatase (ALP) activity when cultured on HA-TCP ceramics with a greater microporosity and specific surface area than those cultured on ceramics with reduced microporosity and smaller surface area (Li *et al.*, 2008).

1.5.5 Molecular mechanisms of osteoinduction

The molecular mechanisms of osteoinduction by biomaterials are not completely understood and several hypotheses have been proposed (Barrere *et al.*, 2003, Habibovic *et al.*, 2005b).

It has been proposed that an interconnected macroporous and microporous surface is critical for osteoinduction due to the effect on specific surface area and formation of the biological apatite layer (Habibovic *et al.*, 2005b). As CaP ceramics have a high affinity for proteins (De Groot, 1998) adsorption of endogenous proteins and bone growth factors including BMP (Ripamonti *et al.*, 1992, Reddi, 2000) can occur from the surrounding fluid (Combes and Rey, 2002, Wilson *et al.*, 2005). Coprecipitation of these endogenous proteins above a concentration threshold (Wang *et al.*, 1990) within the biological apatite layer at the ceramic surface may act as a physico-chemical trigger for attachment and differentiation of pluripotent mesenchymal cells into an osteogenic lineage (Habibovic *et al.*, 2005b, Fan *et al.*, 2007) (**Figure 1**). The presence of endogenous BMP such as BMP-3 and BMP-7 at the bone-ceramic interface within the concavities of HA or HA-TCP after intramuscular implantation suggests these ceramics can serve as the substratum for the adsorption and storage of endogenous circulating BMP (Ripamonti, 1991, Yuan *et al.*, 1998b).

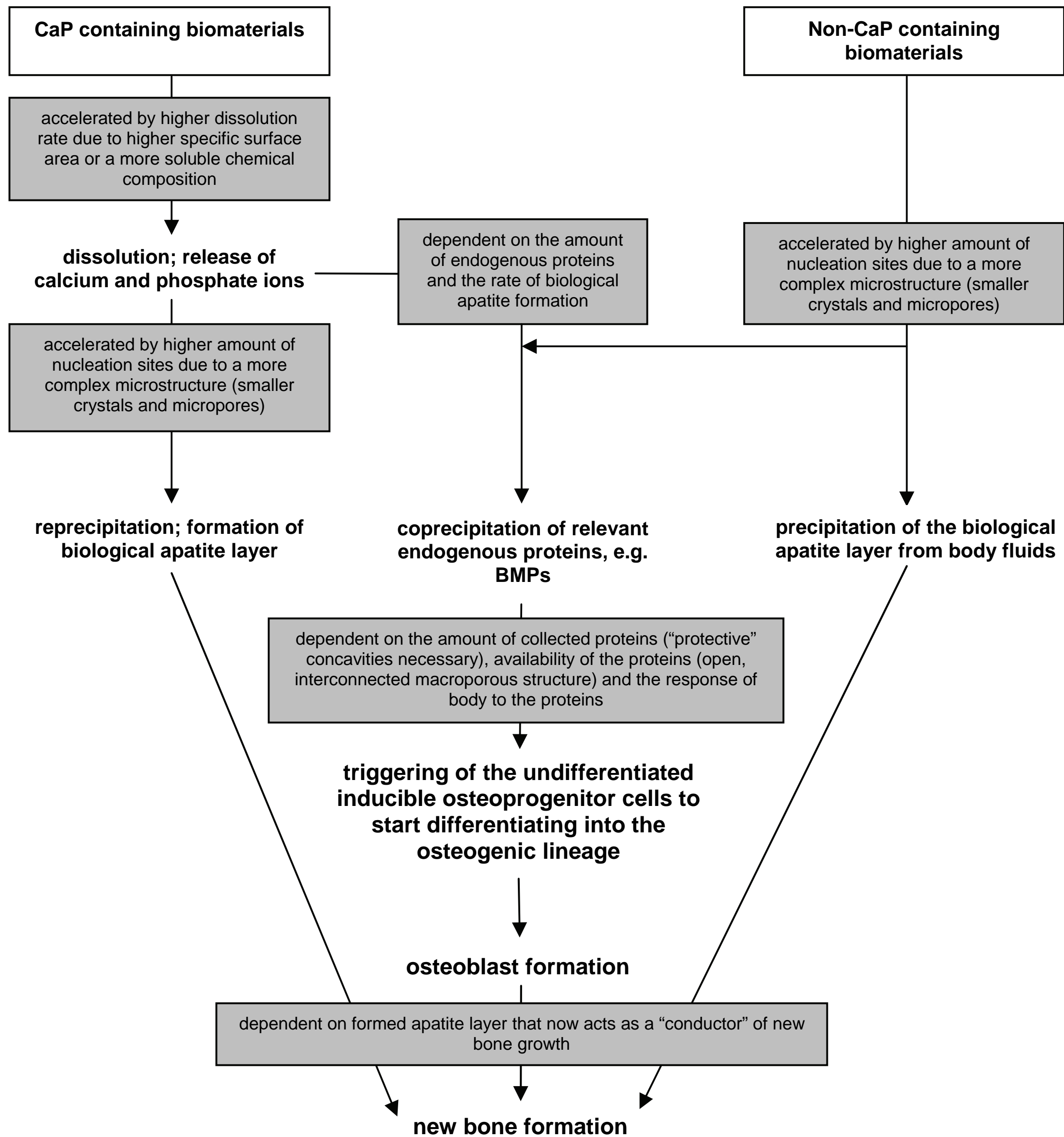

Figure 1. Mechanism of material related osteoinduction as proposed by Habibovic *et al.*, (2005b)

Although endogenous BMP have been suggested to play a role in CaP material osteoinduction (Ripamonti, 1996, Habibovic *et al.*, 2005b), material associated osteoinduction results in intramembranous bone formation (Ripamonti, 1996, Yang *et al.*, 1996) while BMP induced bone formation usually proceeds along an endochondral pathway (Urist, 1965, Reddi, 1981). Additionally, ectopic bone formation associated with CaP ceramics occurs within the macropores and never on the periphery of the graft material, while bone formation after implantation of BMP loaded carriers is

seen on the outside of the carrier and even in distant soft tissue (Yuan *et al.*, 2001b). These observations suggest that there may be other mechanisms regulating material associated osteoinduction.

Some authors have suggested that low oxygen tension within the central region of the graft material may stimulate differentiation of pericytes from blood vessels into osteoblasts, as osteogenic cells have been observed to arise from pericytes adjacent to small blood vessels in connective tissue (Diaz-Flores *et al.*, 1992, Reilly *et al.*, 1998, Collett and Canfield, 2005). Others have also suggested that inflammatory cytokines released by macrophages into the local environment during phagocytosis of ceramic microparticles (Laquerriere *et al.*, 2003, Lu *et al.*, 2004) may stimulate the differentiation of pericytes or circulating stem cells into osteoblasts (Le Nihouannen *et al.*, 2005) **(Figure 2)**.

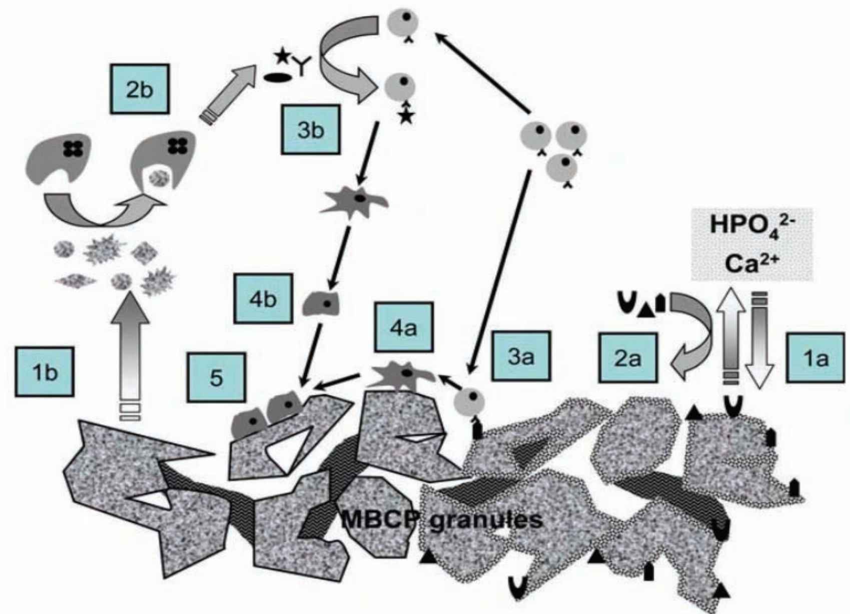

Figure 2. Diagram of possible mechanisms for material associated osteoinduction. Following implantation, the MBCP (Micro-macroporous biphasic calcium phosphate) granules partly dissolve (1a) and a biological apatite precipitates onto the surface of the MBCP concentrating endogenous bone growth factors (2a). Circulating stem cells are recruited to this surface (3a) and differentiate into osteoprogenitors (4a). Microparticles detach from the microporous surface of MBCP (1b) and are phagocytosed by macrophages releasing inflammatory cytokines (2b). The inflammatory cytokines stimulate circulating stem cells (3b) into osteoprogenitors (4b). Osteoblastic cells align and produce bone extracellular matrix on the MBCP (5) (Le Nihouannen *et al.*, 2005).

1.6 Composite grafts

Another method to provide osteogenic or osteoinductive properties to alloplasts, replicating properties traditionally associated with autografts or allografts, is to utilise a tissue engineering strategy with an alloplastic graft material as a carrier delivering osteogenic cells or osteoinductive agents within the defect to stimulate chemotaxis, proliferation and differentiation of mesenchymal stem cells into osteogenic cells.

Without a scaffold or carrier, the *in vivo* application of osteogenic cells or growth factors is ineffective (Urist *et al.*, 1984b, Goshima *et al.*, 1991) due to the short half-life of proteins, the rapid

diffusion of proteins and cells away from the defect site as well as their susceptibility to uptake, catabolism or proteolysis (Hotz and Herr, 1994, Winn *et al.*, 1999) resulting in an insufficient local concentration of the growth factor or number of osteogenic cells (Reddi, 1995, Wozney and Rosen, 1998).

The ideal matrix or scaffold for osteoinductive growth factors or osteogenic cells should display the following properties and characteristics (Bartold *et al.*, 2006, Yuan *et al.*, 2006b, Guillot *et al.*, 2007):

- Biocompatibility
- Biodegradability to enable bone remodelling and replacement
- Interconnective macroporosity and microporosity to facilitate vascularisation, oxygen and nutrient transfer as well as cellular recruitment, adherence and ingrowth
- High mechanical strength and stability to sustain shape and bulk of defect until replaced by host bone
- Ease of handling
- Osteoconductivity to guide bone around or inside the bone graft
- Release of growth factors at the desired concentration over the correct time period
- Maintain cell viability to allow cell differentiation and ECM production within the carrier

CaP ceramics have been suggested as carriers as they demonstrate a number of these qualities. The surfaces of CaP ceramics are chemically stable, display a high adsorption capacity for proteins (Urist *et al.*, 1984a, Hartman *et al.*, 2005) and provide good substratum for the attachment of osteogenic mesenchymal stem cells (MSCs) (Goshima *et al.*, 1991, Toquet *et al.*, 1999). After formation of new bone, the bioactive and osteoconductive properties of porous CaP ceramics can promote rapid ingrowth of newly formed bone along the ceramic surface.

1.6.1 Composite grafts of CaP ceramics and osteogenic cells

CaP ceramics have been used as carriers for multipotent bone marrow MSCs that are capable of differentiating into various mesenchymal tissues including those of osteogenic lineage (Friedenstein *et al.*, 1966, Caplan, 1991).

The combination of porous CaP ceramics (HA, β-TCP, HA-TCP) and bone marrow or bone marrow MSCs have been evaluated *in vivo* with ectopic bone formation in a variety of animal models including rodents (Ohgushi *et al.*, 1990, Goshima *et al.*, 1991, Dong *et al.*, 2002, Harris and Cooper, 2004). Similarly, in animal osseous defects the combination of CaP and MSCs or bone marrow improved bone ingrowth when compared to CaP ceramic alone (Ohgushi *et al.*, 1989b, Kon *et al.*, 2000, Jafarian *et al.*, 2008).

In these studies, new bone formation occurred directly against the ceramic surface within the ceramic pores, progressing from the periphery of the ceramic towards the centre (Ohgushi *et al.*, 1990, Kruyt *et al.*, 2007, Kruyt *et al.*, 2008). Bone formation occurred via an intramembranous pathway, although in sites where insufficient vascularity was present cartilage was observed (Goshima *et al.*, 1991).

1.6.2 Composite grafts of CaP ceramics and growth factors

Growth factors are proteins expressed by a variety of cell types or released during matrix degradation. They regulate different cellular activities during bone healing and their levels are tightly regulated by a number of positive and negative feedback mechanisms (Giannobile, 1996, Schilephake, 2002).

Injury to bone and vasculature results in an acute inflammatory response with platelet activation as well as fibrin deposition. Activated platelets release growth factors such as fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), insulin like growth factor (IGF) and platelet derived growth factor (PDGF). Migrating inflammatory cells, local osteogenic cells and disruption of the extracellular matrix result in further release of IGF-I, PDGF, TGF-α and TGF-β as

well as other pro-inflammatory cytokines including prostaglandins, nitric oxide, kinins, vasoactive amines, complement factors and interleukins (Hansson *et al.*, 1987, Rappolee *et al.*, 1988, Antoniades *et al.*, 1991). The cumulative effect of these chemical mediators is angiogenesis and development of fibrovascular granulation tissue within the osseous defect, with subsequent mesenchymal cell migration, proliferation and osteogenic cell differentiation (Bauer and Muschler, 2000).

The use of growth factors in bone regeneration aims to mimic and enhance this healing cascade, increasing the rate and amount of new bone formation within the bony defect.

Due to the high affinity of CaP ceramics for proteins, a variety of growth factors in solution can be adsorbed onto the ceramic. Along with members of the TGF-β superfamily, BMP has received the most attention in the literature as ectopic bone formation as well as enhanced osseous wound healing have been demonstrated over the CaP ceramic alone (Urist *et al.*, 1987, Ohyama *et al.*, 2004, Jung *et al.*, 2006).

The degree of bone formation appears to be dose dependent with increasing concentrations of BMP (Oda *et al.*, 1997, Yuan *et al.*, 2001b), however a minimum threshold concentration appears to be necessary (Oda *et al.*, 1997). The degree and mechanism of bone formation also appears to be dependent on the three-dimensional geometry of the CaP ceramic. Bone formation was commonly found in the porous structure of the ceramics (Ripamonti *et al.*, 1993, Oda *et al.*, 1997) while no bone formation was seen when BMP was combined with non-porous ceramics (Kuboki *et al.*, 1998). Additionally, the use of a porous BMP loaded CaP carrier with the appropriate three-dimensional architecture may provide sufficient angiogenesis after implantation to promote new bone formation via an intramembranous pathway without a cartilage intermediate (Kuboki *et al.*, 1995, Kuboki *et al.*, 1998).

1.6.3 Adsorption and release kinetics of growth factors on CaP ceramics

An effective composite graft for bone regeneration requires adsorption of the growth factor to the carrier while remaining biologically active, followed by controlled delivery to target cells at concentrations seen during wound healing (Winn *et al.*, 1999, Whitaker *et al.*, 2001). Adsorption of growth factors onto CaP ceramics is rapid (Jiang *et al.*, 1999, Laffargue *et al.*, 2000, Stephan *et al.*, 2000) with adsorption occurring immediately after combination (Ziegler *et al.*, 2002). Proteins are mainly absorbed through electrostatic attractions between the ionic groups on the protein and the ceramic surface (Gorbunoff and Timasheff, 1984, Wassell *et al.*, 1995), therefore growth factors with fewer of these groups may have a lower adsorption to CaP ceramics.

Characteristics of the CaP ceramic will also affect the protein adsorption, with a higher Ca/P ratio (Sharpe *et al.*, 1997) and specific surface area of the ceramic resulting in a greater amount of protein able to be adsorbed (Matsumoto *et al.*, 2004, Zhu *et al.*, 2008). However, *in vitro* protein adsorption may not be representative of an *in vivo* situation as CaP ceramics can interact with a variety of proteins and cells within body fluids which compete with the growth factor for binding sites on the ceramic surface (Sharpe *et al.*, 1997).

A biphasic release kinetic with an initial rapid burst release followed by a sustained release over a longer period (Winn *et al.*, 1999) has been reported *in vitro* and *in vivo* for CaP ceramic adsorbed growth factors however this may vary depending on the binding capability of the protein and available surface area of the ceramic (Guicheux *et al.*, 1998a, Guicheux *et al.*, 1998b, Uludag *et al.*, 1999, Laffargue *et al.*, 2000, Lee *et al.*, 2000, Ziegler *et al.*, 2002, Matsumoto *et al.*, 2004, Bateman *et al.*, 2005, Poehling *et al.*, 2006). A biphasic release kinetic may be beneficial *in vivo* as the initial burst release may augment the initial wound healing response, attracting differentiating cells into the porous ceramic carrier with the slower secondary release providing continued exposure of the growth factor to cells present within the bone defect (Uludag *et al.*, 1999, Ziegler *et al.*, 2002).

In addition to the growth factors previously mentioned, CaP ceramics have also been combined with platelet derived growth factor (PDGF) and Enamel Matrix Derivative (EMD) for use in bone and periodontal regeneration.

1.7 Enamel Matrix Protein Derivative (EMD)

Enamel matrix proteins (EMP) constitute the largest portion of the enamel matrix (Hammarstrom, 1997) and are involved in regulating cell differentiation processes during tooth development (Bartlett *et al.*, 2006). A number of enamel matrix proteins are synthesised and secreted by ameloblasts during amelogenesis including Amelogenin, Ameloblastin, Tuftelin and Enamelin (Bartlett *et al.*, 2006). The major EMP is Amelogenin with this protein having a role in regulating HA crystal deposition and growth during amelogenesis (Zeichner-David *et al.*, 1997).

EMPs have been detected on the developing root surface and are suggested to be released by Hertwig's epithelial root sheath (Lindskog, 1982a, 1982b, Lindskog and Hammarstrom, 1982, Slavkin *et al.*, 1989, Bosshardt and Nanci, 2004) with a role in cementoblast differentiation to produce acellular extrinsic fibre cementum (Hammarstrom, 1997).

Amelogenin expression may also have a role in inducing mesenchymal stem cell recruitment during bone development and remodelling. Amelogenin mRNA and protein expression were detected from mesenchymal cells such as osteocytes, osteoblasts and bone marrow progenitor cells in rats, dogs and humans suggesting a possible role in osteogenic activity (Haze *et al.*, 2007).

1.7.1 Emdogain®

A commercial preparation of enamel matrix protein derivative (EMD) has been developed (Emdogain®), derived from enamel buds of developing porcine teeth and contained within a viscous propylene glycol alginate (PGA) carrier. EMD comprises 90% amelogenin and 10% of non-amelogenin enamel matrix proteins such as enamelins, tuftelin, amelin, enamelin and ameloblastin and other proteins such as albumin (Maycock *et al.*, 2002).

After coating the tooth root in the periodontal defect, EMD precipitates onto the root surface (Gestrelius *et al.*, 1997a) and remains detectable for one to four weeks *in vivo* (Gestrelius *et al.*, 1997a, Sculean *et al.*, 2002c, Cornelini *et al.*, 2004). It has been used in periodontal regeneration by mimicking the cellular and signalling events that occur during periodontal development, promoting mesenchymal cell differentiation into cementoblasts to form acellular cementum, periodontal ligament fibroblasts to form periodontal ligament and osteoblasts to form alveolar bone after exposure to EMD (Hammarstrom, 1997).

1.7.2 Molecular and cellular effects of EMD

Studies have demonstrated that the clinical use of EMD in periodontal regeneration does not result in any adverse immunological or antibody-mediated reactions in humans (Zetterstrom *et al.*, 1997, Nikolopoulos *et al.*, 2002). No specific growth factors were identified in early analysis of EMD preparations (Gestrelius *et al.*, 1997b). However, TGF-β or a TGF-β like molecule was later identified as a principal bioactive factor in EMD (Kawase *et al.*, 2002). More recently, members of the TGF-β superfamily such as the BMP or BMP-like molecules including BMP-2, BMP-4 and BMP-6 have also been identified within EMD or enamel extracts (Iwata *et al.*, 2002, Suzuki *et al.*, 2005, Narukawa *et al.*, 2007). Similarly, EMD has also been suggested to stimulate macrophage release of osteoinductive growth factors such as BMP-2 and BMP-4 (Fujishiro *et al.*, 2008).

The cellular effects of EMD are poorly understood. However, it seems that the effects of EMPs go beyond that of amelogenesis and cementogenesis with *in vitro* studies demonstrating the effects of EMD on a variety of different cell types.

In a number of *in vitro* studies, proteins contained in EMD were shown to act as multipurpose growth factors able to affect different periodontal cell types including epithelial cells (Kawase *et al.*, 2000, Lyngstadaas *et al.*, 2001), gingival fibroblasts (Haase and Bartold, 2001, Rincon *et al.*, 2005), periodontal ligament fibroblasts (Gestrelius *et al.*, 1997b, Lyngstadaas *et al.*, 2001), cementoblasts (Tokiyasu *et al.*, 2000, Swanson *et al.*, 2006) as well as osteoblasts.

In vitro, EMD has been reported to enhance DNA synthesis (Kawase *et al.*, 2002, Zeldich *et al.*, 2007), cell proliferation (Keila *et al.*, 2004, Zeldich *et al.*, 2007) and cell attachment of human gingival fibroblasts (Haase and Bartold, 2001, Rincon *et al.*, 2005). EMD is mitogenic for PDL fibroblasts resulting in increased cell proliferation (Gestrelius *et al.*, 1997b, Lyngstadaas *et al.*, 2001) as well as increased RNA and DNA synthesis (Brett *et al.*, 2002, Rincon *et al.*, 2005) with this effect appearing to be dose dependent (Davenport *et al.*, 2003, Palioto *et al.*, 2004, Rodrigues *et al.*, 2007) with EMD negatively affecting cell viability at higher concentrations (Davenport *et al.*, 2003). EMD also enhances PDL fibroblast migration (Hoang *et al.*, 2000, Rodrigues *et al.*, 2007) while its effect on PDL fibroblast cell adhesion has been mixed with EMD having no effect (Gestrelius *et al.*, 1997b, Palioto *et al.*, 2004) or an inhibitory effect (Rodrigues *et al.*, 2007) while others have reported an increase in attachment rate (Lyngstadaas *et al.*, 2001, Suzuki *et al.*, 2001).

EMD also increases the cellular activity of PDL fibroblasts with increased cell metabolism (Lyngstadaas *et al.*, 2001) and upregulation of gene expression related to nucleic acid metabolism, protein metabolism and signal transduction (Barkana *et al.*, 2007) as well as increased extracellular matrix protein production (Gestrelius *et al.*, 1997b, Haase and Bartold, 2001, Rodrigues *et al.*, 2007).

In contrast, the effect of EMD on epithelial cells appears to be inhibitory with a cytostatic effect on DNA synthesis and cell proliferation (Kawase *et al.*, 2000, Lyngstadaas *et al.*, 2001). However, this may depend on the epithelial source with EMD demonstrating a stimulatory effect on DNA synthesis and cell attachment of epithelial cells harvested from epithelial cell rests of Malassez (Rincon *et al.*, 2005).

1.7.3 Effect of EMD on osteoblastic cells

A multitude of cellular effects have been reported after exposure of osteogenic cells to EMD. EMD has been demonstrated to enhance cell viability *in vitro* with a dose dependent effect on mature primary osteoblast survival (Jiang *et al.*, 2001) with the positive effect on cell survival possibly due to an inhibitory effect of EMD on cell apoptosis. A reduction in TNF-α induced

apoptosis of MC3T3-E1 cells was reported after 24 hours when 100 μg/ml of EMD was included in the cell culture (He *et al.*, 2005).

EMD may enhance cell attachment and motility of osteoblastic cells. EMD improved alveolar bone cell attachment to EMD treated culture plates (Rincon *et al.*, 2005) as well as the motility of different osteoblastic cell lines placed on untreated glass surfaces over 24 hours, however this effect was less than the ECM components type I collagen and laminin (Klein *et al.*, 2007).

A number of studies have suggested positive proliferative effects of EMD on osteoblast progenitors as well as differentiated osteoblasts. Reports on the effect of EMD on bone marrow MSC proliferation have been mixed with some reporting enhanced cell proliferation (Keila *et al.*, 2004) in a dose dependent manner (Guida *et al.*, 2007) while others have reported no effect on proliferation (Gurpinar *et al.*, 2003, van den Dolder *et al.*, 2006). The proliferative effect of EMD on the osteoblastic progenitor cell line MC3T3 was reported by a number of authors (Tokiyasu *et al.*, 2000, He *et al.*, 2004b, 2004a, Jiang *et al.*, 2006). These cells behave as immature cells committed to the osteoblast lineage and when cultured in the presence of ascorbic acid and other hormones, cytokines and growth factors are stimulated to differentiate along the osteoblast pathway (Franceschi *et al.*, 1994). Enhanced cell proliferation still occurred in the presence of a porous membrane, suggesting that the cellular effects of EMD do not require direct cell contact but are mediated by soluble molecules released from EMD (He *et al.*, 2004a). In phenotypically mature human osteoblasts, 30-100μg/ml EMD significantly stimulated human osteoblast cell growth and proliferation (Mizutani *et al.*, 2003, Galli *et al.*, 2006), however, EMD did not have a proliferative effect on alveolar bone cells (Rincon *et al.*, 2005).

The effect of EMD on alkaline phosphatase (ALP) expression activity in osteogenic cells is inconsistent although increased ALP expression and *in vitro* mineralisation after EMD exposure have been reported in a subset of PDL fibroblasts (Nagano *et al.*, 2004, Lossdorfer *et al.*, 2007, Rodrigues *et al.*, 2007) that are capable of differentiating into a osteoblast-like or cementoblast-like phenotype (McCulloch and Melcher, 1983, Liu *et al.*, 1997). The *in vitro* effect of EMD on bone marrow MSCs

differentiation have varied with reports of a two fold increase in ALP activity and increased mineralised nodule formation in bone marrow (Keila *et al.*, 2004) while others report no effect on the differentiation of these cells (van den Dolder *et al.*, 2006) despite simultaneously enhancing cell proliferation (Guida *et al.*, 2007).

In other osteoblastic cell lines, EMD enhanced ALP expression of MC3T3-E1 osteoblastic cells under differentiating conditions (He *et al.*, 2004b, Jiang *et al.*, 2006) and in KUSA/A1 mouse osteoblast cells, EMD stimulated ALP activity and mineralised nodule formation in a dose dependent manner with the addition of 12.5 to 50µg/ml EMD (Yoneda *et al.*, 2003). In contrast, foetal rat calvarial cells treated with 10, 50 or 100 µg/ml EMD *in vitro* significantly inhibited ALP activity and mineralised nodule formation in a dose dependent manner after five to 17 days (Hama *et al.*, 2008).

In human osteoblastic cell cultures, 50 and 100 µg/ml of EMD enhanced ALP activity and increased mineralised nodule formation after two and three weeks of treatment (Galli *et al.*, 2006) while human primary osteoblasts cultured in conjunction with 50 µg/ml EMD enhanced osteoblast maturation with a two fold increase in ALP activity (Reseland *et al.*, 2006). However, an earlier study reported that 30 µg/ml EMD decreased ALP expression and cell differentiation in these cells despite having a positive effect on cell proliferation (Mizutani *et al.*, 2003).

The inconsistent reports of the effect of EMD on osteoblast proliferation and differentiation may be due to differences in osteoblastic cell types or stage of osteoblast maturation. EMD had differing effects when cultured with two different mouse osteoblastic cell lines (ST2 and KUSA/A1). In ST2 cells capable of differentiating into adipocytes or osteoblasts, EMD did not enhance cell growth while in KUSA/A1 cells, which are highly committed to an osteogenic lineage, cell growth was enhanced by EMD in a concentration dependent manner (Yoneda *et al.*, 2003).

EMD may stimulate cell proliferation during the early stages of osteoblast maturation but have a predominantly cell differentiation effect when applied to mature cell lines (Schwartz *et al.*, 2000). The addition of EMD to pre-osteoblastic cells (2T9 cells which exhibit osteogenesis in response to BMP-2) enhanced cell proliferation but had no effect on their differentiation or ALP activity. In

contrast, EMD decreased cell proliferation and increased ALP activity as well as osteocalcin production in cultures of osteoblast like cells (MG63 human osteoblast-like osteosarcoma cells). Interestingly, when EMD was cultured with mature normal human osteoblasts (NHOst cells), EMD had a dual effect, enhancing cell proliferation and differentiation with an increase in ALP activity (Schwartz *et al.*, 2000).

1.7.4 Effect of EMD on pluripotent mesenchymal stem cell differentiation

EMD may stimulate the differentiation of pluripotent mesenchymal cells into osteoblastic and/or chondroblastic lineages (Ohyama *et al.*, 2002). A pluripotent mesenchymal cell line (C2C12) was cultured in differentiation medium with or without the addition of EMD. Without the addition of EMD, C2C12 cells altered their phenotype to myoblasts while cells cultured in the presence of EMD were strongly inhibited from myoblast development but increased ALP activity two- to four-fold. EMD stimulated cells also increased the mRNA expression of osteocalcin and type X collagen suggesting that EMD stimulated osteoblastic differentiation of these cells. In a later study, a pluripotent mouse fibroblastic cell line (C3H10T1/2) cultured with 10 to 100 μg/ml EMD for up to seven days demonstrated increased mRNA levels of osteogenesis- and chondrogenesis-related transcription factors as well as Runx2 and Sox9 protein expression (Narukawa *et al.*, 2007). The authors suggested that expression of these transcription factors were mediated by a BMP-6 like molecule present in EMD.

1.7.5 Osteoinductive potential of EMD

Early evidence of the osteoinductive properties of enamel proteins were demonstrated after soft tissue implantation of demineralised enamel (Urist, 1971). A number of studies have subsequently investigated the osteoinductive potential of EMD and formation of ectopic bone in non-osseous sites. Intramuscular implantation of 2 mg or 4 mg of EMD alone or in conjunction with inactive DFDBA into mouse calf muscle did not demonstrate osteoinduction after 56 days while

implants that contained active DFBDA resulted in ectopic bone formation (Boyan *et al.*, 2000). The addition of 2 mg of EMD to active DFDBA did not provide an additive effect of active DFDBA alone, however, the addition of 4 mg to DFDBA resulted in enhanced bone induction, area of new bone, and cortical bone suggesting a minimum threshold concentration of EMD was required (Boyan *et al.*, 2000). In other reports, implantation of 1 mg EMD within a collagen carrier into the hind thigh muscle of rats for two weeks did not demonstrate ectopic bone formation (Yoneda *et al.*, 2003) and the implantation of EMD or PGA with undemineralised dentine matrix into the rectus abdominis muscles of rats confirmed that neither EMD nor PGA had osteoinductive properties with an absence of ectopic bone formation after 21 days (Koike *et al.*, 2005).

When EMD was combined with a graft material, no osteoinductive activity was observed after implantation of 30 mg/0.3 ml EMD and DBBM into the pectoralis muscle of rats (Donos *et al.*, 2006). More recently, subcutaneous implantation of 0.25, 0.5 or 0.8 mg EMD combined with a CaP/poly (D,L-lactic-coglycolic acid) carrier in rats also did not result in osteoinduction with a highly vascularised loose connective tissue surrounding the implant observed histologically after four weeks (Plachokova *et al.*, 2008). These studies have demonstrated that EMD does not provide the inductive factors or the three dimensional scaffold required for differentiation of mesenchymal progenitor cells into an osteoblastic lineage.

1.7.6 Osteopromotive effects of EMD

Although the osteoinductive properties of EMD have not been demonstrated, it may exhibit a number of osteopromotive effects in addition to its direct effects on osteogenic cells. EMD has also been demonstrated to regulate expression of molecules that control bone mineralisation (Ganss *et al.*, 1999) such as bone sialoprotein (He *et al.*, 2004b, Shimizu *et al.*, 2004), osteopontin (Yoneda *et al.*, 2003) and osteocalcin (Reseland *et al.*, 2006). Furthermore, EMD may enhance wound healing by stimulating ECM protein production and remodelling (Gestrelius *et al.*, 1997b, Yoneda *et al.*, 2003, He *et al.*, 2004b, Goda *et al.*, 2008) as well as stimulating growth factors and cytokines critical

to wound healing including transforming growth factor-β1 (Yoneda *et al.*, 2003), insulin-like growth factor-I (Mizutani *et al.*, 2003), fibroblast growth factor-2 (Mizutani *et al.*, 2003), interleukin-6 (Lee *et al.*, 2008a) and prostaglandins (Mizutani *et al.*, 2003, Takayanagi *et al.*, 2006). EMD may also directly enhance angiogenesis (Yuan *et al.*, 2003, Mirastschijski *et al.*, 2004) by enhancing endothelial cell chemotaxis and proliferation (Yuan *et al.*, 2003, Schlueter *et al.*, 2007) as well as indirectly by stimulating secretion of pro-angiogenic vascular endothelial growth factor (VEGF) (Mirastschijski *et al.*, 2004).

A number of investigators have investigated the *in vivo* effect of EMD on bone formation with a recent systematic review suggesting that EMD promotes osteogenic differentiation of pluripotent mesenchymal cells resulting in greater bone formation *in vivo* (Rathe *et al.*, 2009). In rat osseous defects, a higher volume of trabeculae bone was seen seven days after application of EMD compared with PGA (Kawana *et al.*, 2001). Similarly, greater stromal cell accumulation as well as greater bone formation were observed after EMD/PGA were applied to bony defects compared to PGA alone at 60 days postoperatively (Sawae *et al.*, 2002). In a rat skull defect, EMD within a collagen carrier enhanced bone repair resulting in greater mineralised bone as well as greater radiopacity when compared to defects treated with the collagen carrier alone after two weeks (Yoneda *et al.*, 2003).

However, no osteopromotive effects were seen in tibial bone defects in rabbits (Cornelini *et al.*, 2004) or in implant osteotomy sites in rabbits after the addition of 0.5ml EMD immediately prior to placement of commercially pure titanium implants (Franke Stenport and Johansson, 2003). Similarly, no additive effect of EMD was demonstrated when combined with a GBR technique with a resorbable collagen membrane in critical sized rat calvarial defects. Defects treated with EMD alone healed in a similar manner to untreated defects with incomplete healing while all the defects treated by GBR resulted in complete bony healing with EMD providing no additive effect (Donos *et al.*, 2004). Similar results were reported later with EMD having no effect on bone formation beyond the

borders of natural bone when combined with an ePTFE membrane capsule on the lateral aspect of a rat mandibular ramus (Donos *et al.*, 2005).

1.7.7 Clinical use of EMD

Animal and human histological studies have demonstrated that administration of EMD in periodontal fenestration or intrabony defects successfully promotes formation of new cementum, periodontal ligament and alveolar bone (Hammarstrom *et al.*, 1997, Heijl, 1997, Sculean *et al.*, 2000a, Sculean *et al.*, 2000b, Yukna and Mellonig, 2000). The use of EMD in the treatment of periodontal intrabony defects has been found to significantly reduce probing pocket depth as well as enhance clinical attachment gain and alveolar bone growth (Heijl *et al.*, 1997, Sculean *et al.*, 1999, Tonetti *et al.*, 2002, Heden and Wennstrom, 2006, Sculean *et al.*, 2008a) with a recent meta-analysis of the literature suggesting that use of EMD in the treatment of intrabony defects provided significantly better clinical outcomes than periodontal flap surgery alone (Esposito *et al.*, 2005).

1.7.8 Composite grafts containing EMD

Because of its gel like consistency, EMD alone possesses poor space making potential, limiting its use in regeneration of large defects (Mellonig, 1999, Kuru *et al.*, 2006, Rathe *et al.*, 2009). In unsupportive osseous defects, the combination of EMD with a bone graft material may maintain space and wound stability for the regenerative process and bioactive properties of EMD.

EMD has been combined with a variety of bone substitutes including xenografts such as DBBM (Scheyer *et al.*, 2002, Sculean *et al.*, 2002b) as well as alloplasts such as bioactive glass (Sculean *et al.*, 2002a, Sculean *et al.*, 2005, Sculean *et al.*, 2007) and CaP ceramics, with most used in the treatment of periodontal intrabony defects.

The addition of EMD to DBBM has shown no clinical (Scheyer *et al.*, 2002, Sculean *et al.*, 2002b) or histological (Sculean *et al.*, 2003) benefit over DBBM alone, however the composite graft has demonstrated improved clinical outcomes over EMD alone in the treatment of periodontal

intrabony defects (Lekovic *et al.*, 2000, Velasquez-Plata *et al.*, 2002, Zucchelli *et al.*, 2003). Similarly, no additive effect of EMD and DBBM to GBR was reported in critical sized calvarial defects in rats (Donos *et al.*, 2004). In a follow up study, the addition of EMD to DBBM did not enhance the amount of bone formation when placed under a ePTFE capsule on the lateral aspect of the rat mandibular ramus (Donos *et al.*, 2005).

Alloplasts such as bioactive glasses have been suggested as a potential carrier for EMD with mouse preosteoblasts cultured in contact with bioactive glass and EMD exhibiting significantly higher total protein production and osteocalcin expression *in vitro* than bioactive glass alone (Hattar *et al.*, 2005). Although the addition of EMD to bioactive glass lead to enhanced bone mineralisation around the bioactive glass particles histologically, this did not enhance the clinical outcome in the treatment of human periodontal intrabony defects (Sculean *et al.*, 2002a, Sculean *et al.*, 2005, Sculean *et al.*, 2007).

EMD has also been combined with CaP ceramics including β-TCP and HA-TCP. Although the release kinetics of EMD when combined with a CaP scaffold are not well documented, a biphasic release kinetic was observed from the CaP carrier with approximately 10% of EMD released during the first five hours as a result of release of EMD from the surface of the scaffold followed by a sustained release of approximately 2% during the first week (Plachokova *et al.*, 2008). Thereafter, release of EMD increased rapidly as a result of scaffold degradation, with 60% of the EMD released into the medium by day 28.

No additional benefits were reported when EMD and β-TCP were combined, with similar clinical outcomes to EMD alone in the treatment of periodontal intrabony defects (Bokan *et al.*, 2006). The combination of EMD and HA-TCP (Bone Ceramic®) is commercially available (Emdogain Plus®) with similar clinical outcomes to EMD alone six months after treatment of wide periodontal intrabony defects (Jepsen *et al.*, 2008). Histological examination of this composite graft material nine months after treatment of periodontal intrabony defects suggest only limited osteoconductivity with

limited new bone formation and fibrous connective tissue encapsulation of the ceramic particles (Sculean *et al.*, 2008b).

1.8 Platelet Derived Growth Factor (PDGF)

Platelet derived growth factor (PDGF) plays a role in embryonic development (Schatteman *et al.*, 1992, Shinbrot *et al.*, 1994) as well as during soft and hard tissue wound healing (Ross *et al.*, 1986). It is released from the bone matrix, activated platelets and macrophages at the site of tissue injury (Bolander, 1992, Andrew *et al.*, 1995, Fujii *et al.*, 1999).

The PDGF family consists of dimeric glycoproteins with two disulphide bonded polypeptide chains. Each of these chains is referred to as A, B, C or D chains (Alvarez *et al.*, 2006) which associate to form the homodimers PDGF-AA, BB, CC, DD with the A and B chains also able to form the heterodimer PDGF-AB (Fredriksson *et al.*, 2004). A number of cell types express PDGF including osteoblasts, fibroblasts, keratinocytes, skeletal and smooth muscle cells, neural cells, vascular endothelial cells, macrophages and platelets (Heldin and Westermark, 1999). Cells of the osteoblast lineage express the PDGF-A gene and to a lesser extent the PDGF-B gene (Zhang *et al.*, 1991, Andrew *et al.*, 1995) with an autocrine effect of PDGF-AA but not PDGF-BB reported (Rydziel *et al.*, 1994, Andrew *et al.*, 1995, Rydziel and Canalis, 1996, Yang *et al.*, 2000).

The PDGF receptor consists of two polypeptide chains (PDGF-Rα and PDGF-Rβ) encoded by two genes (Matsui *et al.*, 1989, Stephenson *et al.*, 1991) that combine to form three dimeric isoforms (PDGF-Rαα, PDGF-Rββ and PDGF-Rαβ). The PDGF-Rαα binds PDGF-AA, AB, BB and CC, while PDGF-Rαβ binds PDGF-AB, BB, CC and DD and the PDGFR-ββ binds PDGF-BB and PDGF-DD (Fredriksson *et al.*, 2004). Upon binding to its specific cell surface receptor, PDGF stimulates a signal transduction pathway leading to mRNA transcription and protein production.

Of the different PDGF isoforms, PDGF BB is biologically most potent (Hock and Canalis, 1994) and this has been attributed to its ability to bind to all PDGF receptor isoforms (Canalis *et al.*,

1992, Pfeilschifter *et al.*, 1992) as well as displaying a higher binding affinity to the PDGF-Rβ receptor (Hart and Bowen-Pope, 1990).

PDGF receptors are found on a number of different cell types including platelets, fibroblasts, myoblasts, vascular smooth muscle cells, capillary endothelial cells, pericytes, neural cells, myeloid haematopoietic cells and macrophages (Alvarez *et al.*, 2006). The levels of α and β receptor chain expression can vary considerably, determining the cellular response to PDGF stimulation (Heldin and Westermark, 1999). In addition, the level of PDGF receptor expression on cells is not constant, with levels increasing during inflammation (Rubin *et al.*, 1988) or after stimulation by other growth factors or cytokines (Schollmann *et al.*, 1992).

1.8.1 Effect of PDGF in wound healing

Following injury, formation of a blood clot occurs with platelets adhering and aggregating to the injured vessel wall. Platelet degranulation releases varying amounts of PDGF-AB, PDGF-AA, PDGF-BB and PDGF-CC from α-granules (Singer and Clark, 1999, Hollinger *et al.*, 2008). Within the injured site, endothelial cells as well as fibroblasts also release PDGF within the wound site (Harlan *et al.*, 1986, Paulsson *et al.*, 1987). The net result of PDGF release is a proangiogenic, proliferative and mitogenic effect resulting in the formation of a fibrovascular tissue to begin the process of tissue repair or regeneration (Grotendorst *et al.*, 1985).

PDGF is chemotactic for neutrophils, monocytes and macrophages (Deuel *et al.*, 1982) as well as for fibroblasts (Seppa *et al.*, 1982) with the PDGF-BB isoform the most potent (Siegbahn *et al.*, 1990). PDGF also enhances fibroblast synthesis of ECM components including fibronectin (Blatti *et al.*, 1988), collagen (Grotendorst *et al.*, 1985, Thomopoulos *et al.*, 2005) and proteoglycans (Heldin *et al.*, 1989, Schonherr *et al.*, 1991) as well as stimulating tissue and wound remodelling through fibroblast collagenase production (Bauer *et al.*, 1985).

The effects of PDGF on bone healing are similar to those seen in soft tissue healing and are summarised in **Figure 3.** After injury, release of PDGF from platelets or bone matrix results in

angiogenesis, chemotaxis, and mitogenesis of mesenchymal progenitor cells. PDGF can enhance angiogenesis directly or indirectly via upregulation of other growth factors such as VEGF. The net effect of PDGF results in an increased number of osteoprogenitor cells within the bone defect that will respond to other differentiating factors such as the bone morphogenetic proteins (BMP).

NOTE:
This figure is included on page 34 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3 Role of PDGF in bone healing (Adapted from Lynch *et al.*, (2008))

1.8.2 Effect of PDGF on fibroblasts

PDGF exerts chemotactic and mitogenic activity on a variety of mesenchymal derived cells, including fibroblasts and PDL fibroblasts (Piche and Graves, 1989, Bartold *et al.*, 1992, Bartold and Raben, 1996). In oral tissues, a number of *in vitro* studies have reported that PDGF has strong proliferative and mitogenic effects on gingival fibroblasts (Bartold, 1993, Dennison *et al.*, 1994, Marcopoulou *et al.*, 2003) as well as chemotactic, proliferation and mitogenic effects on PDL fibroblasts (Oates *et al.*, 1993, Bartold and Raben, 1996, Chong *et al.*, 2006). This effect appears to be time and dose dependent (Oates *et al.*, 1993, Ojima *et al.*, 2003) with PDGF-BB having a greater effect than PDGF-AA or PDGF-AB (Boyan *et al.*, 1994).

PDGF-BB modulates adhesion and attachment of PDL fibroblasts with enhanced attachment and adherence of human PDL fibroblasts to periodontally involved root surfaces (Gamal and Mailhot,

2000). Similarly, PDGF-BB had a positive effect on adhesion and growth of cultured fibroblasts to periodontally diseased surfaces with the density and shape of PDL cells seen after PDGF treatment similar to the healthy controls (Belal *et al.*, 2006).

PDGF has been reported to enhance synthesis of ECM components by both gingival and PDL fibroblasts with increased collagen synthesis as well as modulation of the production of proteoglycans such as hyaluronate and versican (Matsuda *et al.*, 1992, Bartold and Raben, 1996, Haase *et al.*, 1998, Ojima *et al.*, 2003).

1.8.3 Effect of PDGF on osteoblasts

PDGF has an important role in bone healing and repair with increased gene expression of PDGF-A and B chains and PDGF receptors seen during normal fracture healing (Fujii *et al.*, 1999). PDGF is chemotactic for osteogenic cells with PDGF-BB demonstrating greater potency than PDGF-AA (Lind *et al.*, 1995). PDGF-BB stimulates chemotactic activity in the MC3T3-E1 osteoblast cell line (Tsukamoto *et al.*, 1991, Mehrotra *et al.*, 2004) with maximal chemotaxis reported at a concentration of 25 ng/ml (Tsukamoto *et al.*, 1991). However, the chemotactic effect may be dependent on the stage of differentiation with PDGF being more chemotactic for undifferentiated cell than osteoblast rich populations (Hughes *et al.*, 1992). This suggests that PDGF may enhance early wound healing by increasing the number of undifferentiated osteoprogenitor cells within the bone defect rather than the number of differentiated osteoblasts seen in the later stages of bone healing.

The mitogenic effects of PDGF on osteoblasts and osteoprogenitor cells have also been reported with rhPDGF-BB, PDGF-AA and PDGF-AB stimulating DNA synthesis and cell proliferation in foetal rat osteoblasts (Centrella *et al.*, 1991) as well as in human osteoblasts (Zhang *et al.*, 1991) with PDGF-BB a more potent mitogen than PDGF-AA (Hock and Canalis, 1994). PDGF-BB was also found to enhance DNA synthesis in rat calvarial osteoblast like cells (Canalis *et al.*, 1989, Hsieh and Graves, 1998), in bovine osteoblast like cells (Giannobile *et al.*, 1997) as well as in MC3T3-E1 osteoblasts (Mehrotra *et al.*, 2004).

Both PDGF-BB and PDGF-AA have been demonstrated to enhance cell proliferation of osteoprogenitor and osteoblast cell cultures (Strayhorn *et al.*, 1999, Yang *et al.*, 2000). A 2 to 2.5 fold proliferative effect on foetal rat calvarial osteoblasts was observed with PDGF-AA and PDGF-BB with a more potent effect associated with PDGF-BB (Hock and Canalis, 1994). PDGF-BB also has a greater effect on osteoprogenitors, increasing replication by 2.5 fold compared to 1.5 fold with PDGF-AA. However, PDGF-BB did not stimulate cell proliferation of MC3T3-E1 preosteoblasts after a 48 hour culture period (Kim *et al.*, 2007). It is possible that differences in the mitogenic effect of PDGF may be dependent on the stage of osteoblastic differentiation with a reduced response to PDGF in well differentiated cells compared to less differentiated cells (Abdennagy *et al.*, 1992, Yu *et al.*, 1997).

Although the chemotactic, proliferative and mitogenic effects of PDGF on osteoblastic cells are well documented, PDGF may have an inhibitory effect on cell differentiation and expression of an osteoblast like phenotype, reducing ALP activity and mineralisation *in vitro* (Giannobile *et al.*, 1997). The application of rhPDGF-BB, PDGF-AB and PDGF-AA to osteoblast enriched cultures from foetal rat bone reduced ALP activity with PDGF-BB having the greatest inhibitory effect on differentiation (Centrella *et al.*, 1991). Similarly, the inhibition of differentiation of foetal rat calvarial osteoblasts into mature osteoblasts as well as a reduction in bone matrix formation were observed after culture with PDGF-BB and PDGF-AA for 24-72 hours, with PDGF-BB a more potent inhibitor than PDGF-AA (Hock and Canalis, 1994).

In cell culture studies, prolonged PDGF exposure to osteoprogenitors may inhibit osteoblast differentiation. Cells cultured with 10 or 20 ng/ml PDGF for 8 days reduced the expression of osteopontin and osteocalcin genes associated with osteoblast differentiation and matrix mineralisation (Strayhorn *et al.*, 1999) while continuous PDGF treatment suppressed osteoblast differentiation as demonstrated by the inhibition of ALP, type I collagen, and osteocalcin expression as well as inhibiting mineralised nodule formation *in vitro* (Yu *et al.*, 1997). Similarly, in cultures of foetal rat osteoblastic cells exposed to a longer duration, continuous exposure to PDGF resulted in a

dose dependent decrease in ALP activity and mineralised nodule formation while differentiating osteoblasts treated with multiple, brief exposures to PDGF demonstrated 50% enhanced mineralised nodule area (Hsieh and Graves, 1998). More recently, prolonged incubation of osteoprogenitors with PDGF-BB over three consecutive days were attributed to a reduction in osteoblast differentiation and a negative effect on *in vivo* bone regeneration. In contrast, PDGF-BB had a short duration effect when applied directly to the surgical defects resulting in increased proliferation of the resident osteoblastic cells but did not affect the differentiation of these cells (Marzouk *et al.*, 2008).

From the literature, it appears that the main effect of PDGF on bone healing is mediated through its chemotactic and mitogenic ability at the site of injury (Kieswetter *et al.*, 1997). Exposure to PDGF inhibits osteoblast differentiation transiently while enhancing osteoblast progenitor proliferation and chemotaxis resulting in a total increase in the number of progenitor cells, which can differentiate when stimulated by other growth factors and signalling proteins.

PDGF may also have an anabolic effect on bone healing by enhancing matrix protein synthesis from osteogenic cells. PDGF had a dose dependent stimulatory effect on bone matrix apposition in foetal rat calvaria with a two fold increase after 48 hours (Pfeilschifter *et al.*, 1990). ECM protein synthesis was also enhanced with the addition of PDGF-BB, PDGF-AB or PDGF-AA to fetal rat bone osteoblast cultures with an increase in the rate of collagen and non-collagen protein synthesis in differentiated and undifferentiated bone cells (Centrella *et al.*, 1989, Centrella *et al.*, 1991, Giannobile *et al.*, 1997).

PDGF is proangiogenic with all members of the PDGF family, including PDGF-BB displaying potent angiogenic activity *in vivo* (Risau *et al.*, 1992, Cao *et al.*, 2002). PDGF-BB also displays potent mitogenic and chemotactic effects on pericytes and vascular smooth muscle cells (vSMCs) (Westermark and Heldin, 1993, Lindahl *et al.*, 1997, Hellstrom *et al.*, 1999) with lack of PDGF-BB signalling resulting in pericyte loss as well as capillary dilation and rupture (Lindahl *et al.*, 1997, Richardson *et al.*, 2001). The effect of PDGF on endothelial cells (EC) has also been reported

(Castellon *et al.*, 2002) and expression and release of PDGF by cultured EC has been documented (DiCorleto and Bowen-Pope, 1983, Collins *et al.*, 1985, Collins *et al.*, 1987).

PDGF may also stimulate angiogenesis indirectly, by upregulating release of growth factors such as VEGF (Guo *et al.*, 2003) from host cells including osteoblasts (Bouletreau *et al.*, 2002). This was reported *in vivo* with an increased concentration of VEGF detected in wound fluid after application of rhPDGF-BB to periodontal osseous defects (Cooke *et al.*, 2006). Others have also suggested a cooperative effect between PDGF-BB and Fibroblast growth factor-2 with PDGF-BB upregulating FGF receptor promoter activity and expression *in vitro* in human vSMCs (Millette *et al.*, 2005, Nissen *et al.*, 2007).

PDGF may also play a role in bone resorption and remodelling by increasing the number of osteoclasts within the bone defect (Cochran *et al.*, 1993, Hock and Canalis, 1994) as well as collagenase activity (Canalis *et al.*, 1989, Varghese *et al.*, 1996) with increases in bone resorption and turnover *in vivo* as measured by carboxyterminal telopeptide of type I collagen (ICTP) after application of 0.3mg/ml rhPDGF-BB in periodontal osseous defects (Cooke *et al.*, 2006, Sarment *et al.*, 2006).

1.8.4 *In vivo* effects of PDGF

PDGF has been used safely for the treatment of chronic cutaneous foot ulcers in diabetic patients (Knight *et al.*, 1998, Smiell, 1998) and has also been released for use in treatment of periodontal or orthopaedic osseous defects.

Animal studies have demonstrated that PDGF enhances the rate and degree of bone healing (Nash *et al.*, 1994). Most of the studies have focussed on the more potent PDGF-BB isoform and identification of the human PDGF-BB gene has allowed production of quantities of recombinant human form of PDGF-BB (rhPDGF-BB) via genetic transfer into bacterial or fungal cells greater than that obtainable through platelet concentration alone.

Lynch and co-workers first introduced PDGF to periodontology and implant dentistry by applying 3 mg of recombinant PDGF-BB and IGF-I in a methylcellulose gel carrier to naturally occurring periodontal defects in beagle dogs (Lynch *et al.*, 1989, Lynch *et al.*, 1991b). The half-life of locally applied PDGF-BB was reported to be 4.2 hours with 96% of the proteins cleared after 96 hours. The authors observed increased cellular activity, periodontal regeneration as well as new bone formation in treated sites. Similar findings were reported after application of 10 µg PDGF-BB or the combination of 10 µg each of PDGF-BB and IGF-I in a methylcellulose gel vehicle during periodontal surgery to experimental periodontitis defects in monkeys. Improved wound healing and bone fill were seen after four and 12 weeks with the greatest improvements seen in the combined PDGF-BB/IGF-I group (Giannobile *et al.*, 1996).

In a study of peri-implant osseous defects in dogs, direct application of rhPDGF-BB in combination with IGF produced two to three times more new bone at earlier time points (Lynch *et al.*, 1991a) while similarly, the application of 5 µg/ml each of PDGF/IGF-I around implants placed in extraction sockets in dogs resulted in a significantly greater bone to implant contact compared to the untreated control group after 3 weeks (Stefani *et al.*, 2000).

Human trials utilising recombinant human PDGF have also reported statistically significant alveolar bone formation nine months post treatment (Howell *et al.*, 1997). Two doses of rhPDGF-BB and rhIGF-I (50 µg/ml of each or 150 µg/ml of each) were combined in a methylcellulose gel carrier and placed in periodontal intraosseous defects. The application of 150 µg/ml of rhPDGF-BB and rhIGF-I resulted in a 1.33 mm greater new vertical bone formation and 24% greater defect fill compared to sites with no growth factors after 6-9 months of healing.

1.8.5 Composite grafts containing PDGF

When administered systemically, PDGF has a short biological half-life with a plasma clearance half-life of 2-10 minutes after intravenous administration into baboons and rats (Bowen-Pope *et al.*, 1984, Cohen *et al.*, 1990). From a clinical perspective, these studies suggest that

maintenance of plasma and tissue concentrations of PDGF at levels equivalent to those showing activity *in vitro* would be difficult to achieve following systemic administration.

In contrast, when PDGF is applied locally to periodontal osseous defects, the half-life of PDGF is approximately four hours (Lynch *et al.*, 1991b) suggesting that local delivery of the growth factor is more suitable for obtaining a sufficient local concentration required to enhance bone regeneration.

The use of rhPDGF-BB appears to be most effective after short term administration, replicating the events that would occur during initial clot formation. Multiple, brief exposures to PDGF would enhance bone formation *in vivo*, while prolonged exposure to PDGF would inhibit differentiation of osteoblasts and mineralisation and instead promote fibrosis and granulation tissue formation (Hsieh and Graves, 1998).

Regulation of the spatial and temporal levels of PDGF *in vivo* with the combination of an appropriate carrier is likely to have a major influence on its biological effect. The use of a graft material in conjunction with PDGF may allow for a sufficient concentration of PDGF to be delivered to the local bone defect. As most growth factors exhibit a biphasic release kinetic from graft material, an initial high concentration of PDGF released locally would mimic that found during clot formation followed by a lower concentration of PDGF during early bone healing.

rhPDGF-BB has been approved for clinical application in the surgical treatment of periodontal intrabony defects and has been combined with a variety of graft materials including allografts (Nevins *et al.*, 2007), xenografts such as DBBM and alloplasts.

PDGF-BB is adsorbed to DBBM in a concentration dependent manner, with the greatest adsorption seen at lower PDGF-BB concentrations and maximal PDGF-BB adsorption after 15 minutes (Jiang *et al.*, 1999). The release of PDGF-BB from DBBM demonstrated a biphasic release pattern, with 18% rapidly released in the first hour, approximately 43% released after 48 hours and stabilising thereafter with 50% of the PDGF-BB released after 10 days. *In vitro*, proliferation of cultured osteoblastic cells was significantly enhanced on PDGF-BB treated DBBM compared to DBBM alone, suggesting that addition of PDGF-BB enhances the bone regenerative properties of

this graft material (Jiang *et al.*, 1999). PDGF-BB adsorbed to a DBBM-collagen matrix graft material demonstrated a similar manner of adsorption as reported in the previous study although the slower release of PDGF may be due to the addition of collagen matrix to the DBBM with 6.5% released after one hour and approximately 30% of the adsorbed protein released after 10 days (Stephan *et al.*, 2000). Similar to the previous study, PDGF-BB treated matrix enhanced proliferation of cultured osteoblastic cells *in vitro* compared to the matrix alone. However, *in vivo* studies have reported that the addition of PDGF-BB to DBBM was not beneficial in enhancing new bone formation over DBBM alone when a composite graft of rhPDGF-BB and DBBM was placed under Teflon capsules in rats for five months (Lioubavina-Hack *et al.*, 2005).

The use of this composite graft material has also been evaluated *in vivo* for surgical reconstruction of large alveolar defects (Simion *et al.*, 2006). A rhPDGF-BB infused block of DBBM was placed in a canine mandibular defect and stabilised using two implants with or without an overlying resorbable membrane and compared with a buffer treated DBBM graft with or without a membrane. Radiographic and histological analysis demonstrated that the greatest bone regeneration occurred in the rhPDGF-BB treated graft block without the collagen membrane. Bone formation progressed from both coronal and apical surfaces of the treated graft, indicating that osteoblasts were attracted into the graft from both the superior (coronal) periosteal surface and inferior medullary spaces. The finding that greatest bone formation occurred without a barrier membrane demonstrates that the additive effects of PDGF were dependent on direct access to a rich supply of osteogenic and angiogenic cells within the periosteum.

In a recent human case report, the same authors reported on vertical ridge augmentation utilising the combination of rhPDGF-BB and DBBM followed by implant placement five months after augmentation. Histological examination demonstrated new bone formation through the whole bovine bone block trabeculae with the xenograft particles embedded in bone with evidence of new bone formation and ongoing bone remodelling (Simion *et al.*, 2007).

1.8.6 Clinical results with PDGF-BB and CaP ceramics

The combination of the CaP ceramic β-TCP and rhPDGF-BB is commercially available (Gem 21S®). In an early study, PDGF-BB was combined with a chitosan and TCP sponge in the rat calvarial defect model and evaluated histologically after two and four weeks (Lee *et al.*, 2000). Histologically, this composite graft promotes osseous healing of calvarial defects with no connective tissue encapsulation of the TCP graft material. A biphasic release kinetic was demonstrated with a high initial burst release in the first day, slowing down for the next six days and then maintaining a slower release for the remaining 14 days with an effective therapeutic concentration maintained throughout the study period (Lee *et al.*, 2000). More recently, PDGF-BB was adsorbed to β-TCP in a concentration and time-dependent manner with rapid adsorption occurring between one and five minutes after subcutaneous implantation of PDGF-BB and β-TCP into mice (Bateman *et al.*, 2005). *In vitro* release studies demonstrated burst release kinetics with 16% of PDGF-BB released after one hour, approximately 30% released after 24 hours and 45% of the adsorbed PDGF-BB released after ten days. *In vivo* release was found to occur more rapidly than *in vitro* release with over 90% of the PDGF-BB released from the β-TCP after 6 days. In addition, PDGF-BB had a stimulatory effect on osteoblasts *in vitro* with the osteoblastic cells incubated with PDGF-BB treated matrices demonstrating attachment within the ceramic pores as well as greater cell proliferation than the control matrices alone (Bateman *et al.*, 2005).

In a large multi-centre study, the use of this composite graft material has been evaluated for the treatment of periodontal osseous defects (Nevins *et al.*, 2005). The combination of 0.3 mg/ml or 1.0 mg/ml of rhPDGF-BB with β-TCP resulted in greater CAL gain over β-TCP alone at three months although this was not statistically different after six months. Radiographic evaluation of the rhPDGF/β-TCP treated sites demonstrated significantly greater bone growth and bone fill at six months compared to β-TCP alone with ongoing bone formation and maturation after 24 months (McGuire *et al.*, 2006). Histologically, the use of this material has demonstrated periodontal

regeneration six months after surgical treatment of human intraosseous periodontal defects (Ridgway *et al.*, 2008).

Commercially available 0.3 mg/ml rhPDGF and β-TCP (Gem21S®) has also been used in guided bone regeneration (GBR). In a recent case report, β-TCP/rhPDGF-BB was utilised in a simultaneous GBR technique at the time of dental implant placement (Byun and Wang, 2008). Autogenous bone was used as an inner graft surface directly adjacent to the implant surface with an outer graft material of β-TCP/rhPDGF-BB and a collagen barrier membrane tented over these materials. Complete bone healing at the defect site was demonstrated when second stage surgery was performed after 5 months healing, suggesting that the combination of rhPDGF-BB and β-TCP may be a suitable material for guided bone regeneration.

Only limited data exist evaluating the combination of HA-TCP and rhPDGF although the use of particulate Bone Ceramic® loaded with 0.3 mg/ml rhPDGF-BB in conjunction with a collagen membrane on initial bone formation in lateral ridge augmentation has been recently reported in dogs (Schwarz *et al.*, 2009). Three weeks after augmentation, the sites with the addition of rhPDGF-BB demonstrated greater augmented area and mineralised tissue formation as well as greater transglutaminase II antigen reactivity in augmented sites than the control when used as a bone graft material. As transglutaminase II is an enzyme that has been demonstrated to be directly involved in the process of angiogenesis (Haroon *et al.*, 1999), the combination of HA-TCP and rhPDGF-BB may enhance initial bone healing in lateral ridge augmentation.

1.9 Conclusions

There has been ongoing research for an alloplastic bone graft material with the osteogenic, osteoinductive and osteoconductive properties of autogenous bone. CaP ceramics have been evaluated and are a suitable bone graft materials for clinical use due to their excellent biocompatibility and osteoconductivity. The combination of HA and TCP to produce HA-TCP provides the advantages of each of these individual ceramics and appears to offer better

osteoconductivity and bioactivity when compared to HA and TCP alone. However, use of these graft materials alone do not provide osteogenic or osteoinductive properties although osteoinduction has been reported when CaP ceramics with an appropriate geometry and architecture are implanted into certain animal models. Structural modifications that provide osteoinductive properties would be considered advantageous clinically in the augmentation of large osseous defects.

The next step in bone regeneration and engineering is tissue engineering with the production of composite grafts incorporating growth factors or osteogenic cells to induce or enhance the normal osseous healing process. The combination of these agents with a graft material may offer benefits over use of the agent alone, in regards to adsorption, release kinetics, resistance to diffusion and proteolysis as well as provide space making ability in large bone defects. CaP ceramics including HA-TCP appear to be ideal carriers for a number of growth factors and cells with osteoinduction demonstrated when combined with the BMP as well as bone marrow MSCs. Other studies have also demonstrated enhanced bone healing when these materials have been combined with a number of growth factors.

The potential of combining HA-TCP with growth factors such as PDGF or EMD appears promising. EMD stimulates a variety of different cell types directly and indirectly through a number of different pathways including those involved in wound healing and bone regeneration although the current evidence suggests that EMD is not osteoinductive but rather osteopromotive. PDGF is an important growth factor in bone healing and affects a number of different cell lines during early wound healing. Most of the current literature focuses on the more potent isoform PDGF-BB and suggests that PDGF-BB is mitogenic, chemotactic and proliferative for poorly differentiated osteoblastic cell populations with reduced effects on well differentiated osteoblasts. However, osteoinductivity by PDGF has not been demonstrated. The combination of a CaP ceramic and PDGF, in particular rhPDGF-BB may enhance bone growth and regeneration. rhPDGF-BB with a β-TCP is currently commercially available, however the use of a HA-TCP may be a more suitable

carrier due to its lower solubility and prolonged maintenance of an osteoconductive scaffold for new bone formation.

Given that BCP ceramics with the appropriate three-dimensional architecture and geometry have demonstrated osteoinductivity, the combination of this graft material with EMD or PDGF-BB could be an important development in bone tissue engineering in replicating the osteoinductive and osteoconductive properties of autogenous bone grafts in the surgical treatment of large bone defects.

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Chapter 2. THE EFFECT OF GROWTH FACTORS ON THE OSTEOINDUCTIVE POTENTIAL OF HYDROXYAPATITE TRI-CALCIUM PHOSPHATE (HA-TCP)

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2.1 Abstract

Objective: The aim of this study was to determine whether Hydroxyapatite β-Tricalcium Phosphate (HA-TCP) either alone or combined with Enamel Matrix Derivative (EMD) or recombinant human Platelet Derived Growth Factor-BB (rhPDGF-BB) is osteoinductive when implanted into a non-osseous site.

Methods: Twenty CD-1 adult male mice underwent intramuscular implantation into both hindlimbs of an empty gelatine capsule or a gelatine capsule containing one of the following: 10 mg of uncoated particulate HA-TCP, (Straumann Bone Ceramic®, HA-TCP), EMD coated HA-TCP, (Emdogain®, HA-TCP + EMD) or rhPDGF-BB coated HA-TCP (HA-TCP + PDGF). Ten animals were sacrificed at four and eight weeks with five specimens from each group retrieved at each time point. The area of graft placement was radiographed and after graft retrieval, a semi-quantitative histological examination was performed with the aim of assessing the inflammatory changes, reparative processes and osteoinduction within the graft site.

Results: At both 4 and 8 weeks, histological analysis failed to demonstrate any osteoinductive activity in any of the specimens from the three experimental groups. A minimal chronic inflammatory response and foreign body reaction was seen in the experimental groups which reduced over time. The particles were embedded within fibrous connective tissue and were encapsulated by a dense cellular layer consisting of active fibroblasts and occasional macrophages with the thickness of this layer decreasing over time. At 4 weeks, a greater density of the fibrous connective tissue was demonstrated in the HA-TCP + EMD group (P<0.001) while a greater thickness in the capsule thickness was seen in the HA-TCP group (P=0.022) although no differences were seen after 8 weeks. Greater neovascularisation was seen in the HA-TCP + PDGF group after 8 weeks (P=0.043) while greater amounts of adipose tissue surrounding the particles were detected in the HA-TCP + PDGF group at 4 weeks (P=0.002) and in the HA-TCP + EMD group at eight weeks (P=0.002).

Conclusions: The results of this study suggest that the use of commercially available HA-TCP alone or in combination with EMD or rhPDGF-BB is biocompatible but not osteoinductive in the murine model.

2.2 Introduction

The use of osseointegrated dental implants for the replacement of missing teeth is a commonly utilised treatment option however successful treatment outcomes are dependent on sufficient bone quantity at the proposed surgical site (Buser *et al.*, 2004).

Surgical augmentation of osseous defects have traditionally utilised autografts, allograft or xenograft bone graft materials with autogenous bone considered the gold standard as it is osteogenic, osteoinductive and osteoconductive. However, the amount of bone that can be harvested is limited and may be associated with an increased risk of surgical complications and postoperative morbidity (Clavero and Lundgren, 2003, Cricchio and Lundgren, 2003). Alternatives such as osteoinductive and osteoconductive allografts (Schwartz *et al.*, 1998) as well as xenografts (Simion *et al.*, 1996, Olson *et al.*, 2000, Buser *et al.*, 2008) are available although social and religious issues may restrict their use in some patients. In addition, as xenografts lack osteogenic cells or osteoinductive proteins, they are considered osteoconductive only and therefore limited to smaller osseous defects.

Some of these issues have driven the development of synthetic alloplasts including biphasic calcium phosphate (BCP), a member of the family of calcium phosphate (CaP) ceramics (Aichelmann-Reidy and Yukna, 1998, LeGeros, 2002). However, similar to xenografts, alloplasts do not provide any osteogenic cells or osteoinductive proteins and are considered osteoconductive only (Aichelmann-Reidy and Yukna, 1998).

The biphasic calcium phosphate (BCP) ceramics are made up of varying ratios of hydroxyapatite (HA) to beta-tricalcium phosphate (β-TCP) to form hydroxyapatite β-tricalcium phosphate (HA-TCP). HA-TCP was developed as a resorbable graft material to combine the rapid resorption of β-TCP while maintaining the osteoconductive scaffold of the minimally resorbable HA (Hashimoto-Uoshima *et al.*, 1995, Piattelli *et al.*, 1996). As with other CaP ceramics, HA-TCP is considered a bioactive bone graft material, with the ability to form a direct bond against host bone (Daculsi *et al.*, 1989, Hashimoto-Uoshima *et al.*, 1995, Jensen *et al.*, 2007)

HA-TCP has been previously used in periodontics and implant dentistry (Nery *et al.*, 1990, Piattelli *et al.*, 1996, Lee *et al.*, 2008) with a new particulate HA-TCP ceramic recently released (Straumann Bone Ceramic®). *In vivo* studies have reported close contact between newly formed bone and Bone Ceramic® particles in animal osseous defects (Jensen *et al.*, 2007, Jensen *et al.*, 2008) as well as in human clinical trials with the use of this material in the treatment of periodontal defects (Jepsen *et al.*, 2008, Sculean *et al.*, 2008b) and maxillary sinus augmentation (Artzi *et al.*, 2008, Cordaro *et al.*, 2008).

CaP ceramics such as HA-TCP are generally considered to be osteoconductive but not osteoinductive, i.e. they do not possess the ability to form bone when implanted into non-osseous sites (Urist, 1965). However, osteoinductivity of HA-TCP has been reported in several animal studies after intramuscular or subcutaneous implantation (Yang *et al.*, 1997, Habibovic *et al.*, 2005a, Le Nihouannen *et al.*, 2005, Yuan *et al.*, 2006b, Habibovic *et al.*, 2008).

Clinically, the use of an osteoinductive bone graft material would be advantageous in the reconstruction of large bone defects with osteoinduction and osteoconduction occurring concurrently. This would result in greater and more rapid bone formation (Oda *et al.*, 1997, Habibovic *et al.*, 2006b, Yuan *et al.*, 2006a, Habibovic *et al.*, 2008, Jung *et al.*, 2008) in osseous defects when compared to non-osteoinductive or weakly osteoinductive materials.

The process of material induced osteoinduction appears to be dependent on the physico-structural properties of HA-TCP materials with the three-dimensional structure and geometry of the biomaterial having a crucial role in influencing osteoinduction. Certain structural elements such as a macroporous and microporous surface which increases the ceramic specific surface area and enhances the formation of the biological apatite layer (Hing *et al.*, 2005, Li *et al.*, 2008) are necessary for ectopic bone formation (Ripamonti, 1991, Yamasaki and Sakai, 1992, Yuan *et al.*, 1998a, Habibovic *et al.*, 2005b, Habibovic *et al.*, 2008). It has been suggested that precipitation of a biological apatite layer on the surface of the HA-TCP after implantation could provide a suitable chemical environment to induce differentiation of mesenchymal stem cells into osteogenic cell lines

(Daculsi *et al.*, 1990, Daculsi *et al.*, 2003). Coprecipitation of endogenous proteins such as bone morphogenetic proteins (BMP) (Ripamonti, 1991, Yuan *et al.*, 1998b) within the biological apatite layer above a concentration threshold (Wang *et al.*, 1990) may act as a trigger for differentiation of stem cells into an osteogenic lineage (Habibovic *et al.*, 2005b, Fan *et al.*, 2007). Other authors have suggested that inflammatory cytokines released by macrophages in the local environment during phagocytosis of ceramic microparticles (Laquerriere *et al.*, 2003, Lu *et al.*, 2004) or the low oxygen tension within the central region of the graft material may stimulate differentiation of pericytes from blood vessels into osteoblasts (Le Nihouannen *et al.*, 2005) as osteogenic cells have been observed to arise from these cells within connective tissue (Diaz-Flores *et al.*, 1992, Reilly *et al.*, 1998, Collett and Canfield, 2005).

Another method of providing osteogenic or osteoinductive properties to alloplasts, thereby replicating the properties associated with autografts or allografts is to adopt a tissue engineering strategy by utilising an osteoconductive alloplast matrix to deliver osteogenic or osteoinductive agents within the defect. Porous calcium phosphate ceramics have been suggested as carriers as their surfaces are chemically stable and display a high adsorption capacity for proteins (Urist *et al.*, 1984a, Hartman *et al.*, 2005) as well as providing a good substratum for the attachment of osteogenic mesenchymal stem cells (MSCs) (Goshima *et al.*, 1991, Toquet *et al.*, 1999). In addition, the adsorption of growth factors onto CaP ceramics is rapid (Laffargue *et al.*, 2000) and a biphasic release kinetic has been reported both *in vivo* and *in vitro* with an initial rapid burst release followed by a sustained release over a longer period (Winn *et al.*, 1999). Clinically, this may be beneficial as the initial burst release of growth factor may augment the initial wound healing response, attracting differentiating cells into the porous ceramic matrix while the slower secondary release from the implant could provide continued exposure for these cells in the bone defect (Uludag *et al.*, 1999, Ziegler *et al.*, 2002).

Animal studies have demonstrated osteogenesis after soft tissue implantation of bone marrow MSCs loaded onto porous calcium phosphate ceramics (Goshima *et al.*, 1991, Dong *et al.*, 2002,

Harris and Cooper, 2004, Kruyt *et al.*, 2007, Jafarian *et al.*, 2008). In addition, the combination of CaP ceramics with osteoinductive proteins such as the bone morphogenetic proteins (BMP) have been reported in the literature with ectopic bone formation as well as enhanced osseous wound healing over a CaP ceramic alone (Urist *et al.*, 1987, Oda *et al.*, 1997, Jung *et al.*, 2008)

At present, two commercially available growth factors, enamel matrix protein derivative (Emdogain®) and a recombinant human platelet-derived growth factor-BB (rhPDGF-BB) marketed as GEM 21S® have been combined with CaP ceramics for use in periodontal and bone regeneration.

2.2.1 Enamel Matrix Derivative (EMD, Emdogain®)

Emdogain® is an enamel matrix protein derivative (EMD) derived from enamel buds of developing porcine teeth. It is comprised of 90% amelogenin and 10% non-amelogenin proteins contained within a viscous propylene glycol alginate (PGA) carrier (Maycock *et al.*, 2002). Enamel matrix proteins are thought to be released by Hertwig's epithelial root sheath and have been detected on the developing root surface during cementogenesis (Lindskog, 1982a, 1982b, Lindskog and Hammarstrom, 1982, Slavkin *et al.*, 1989, Bosshardt and Nanci, 2004) however amelogenin expression may also have a role in inducing mesenchymal stem cell recruitment during bone development and remodelling (Haze *et al.*, 2007).

In early studies no specific growth factors were identified in EMD preparations (Gestrelius *et al.*, 1997), however, TGF-β or a TGF-β like molecule was later identified as a bioactive factor in EMD (Kawase *et al.*, 2002). In addition, members of the TGF-β superfamily such as the osteoinductive BMP or BMP-like molecules including BMP-2, BMP-4 and BMP-6 have also been identified within EMD or enamel extracts (Iwata *et al.*, 2002, Suzuki *et al.*, 2005, Narukawa *et al.*, 2007). Similarly, EMD has also been suggested to stimulate macrophage release of osteoinductive growth factors such as BMP-2 and BMP-4 (Fujishiro *et al.*, 2008).

In a number of *in vitro* studies, EMD has demonstrated chemotactic, mitogenic and proliferative effects on a variety of different cell types including gingival fibroblasts (Haase and

Bartold, 2001, Rincon *et al*., 2005, Zeldich *et al*., 2007), periodontal ligament fibroblasts (Gestrelius *et al*., 1997, Lyngstadaas *et al*., 2001, Rodrigues *et al*., 2007) and cementoblasts (Tokiyasu *et al*., 2000, Swanson *et al*., 2006). In contrast, the effect of EMD on epithelial cells appears to be inhibitory with a cytostatic effect on DNA synthesis and cell proliferation (Kawase *et al*., 2000, Lyngstadaas *et al*., 2001).

EMD has also demonstrated chemotactic, mitogenic and proliferative effects on osteoblasts and osteoprogenitor cells (Rincon *et al*., 2005, Jiang *et al*., 2006, Guida *et al*., 2007) however, the reports on the effects of EMD on osteoblast differentiation have been inconsistent with some reporting enhanced differentiation as measured by an increase in alkaline phosphatase (ALP) activity (He *et al*., 2004, Jiang *et al*., 2006) while others have reported reduced ALP activity (Guida *et al*., 2007, Hama *et al*., 2008). These differences may be explained by the differing effect of EMD on different cell types (Yoneda *et al*., 2003), or different stages of osteoblast maturation with no effect on differentiation on immature cell lines while enhancing differentiation in mature osteoblast like cells (Schwartz *et al*., 2000).

Recent data suggests that EMD may stimulate pluripotent mesenchymal cell differentiation and proliferation into an osteoblast lineage (Ohyama *et al*., 2002). Without the addition of EMD, these cells altered their phenotype to myoblasts whilst EMD cultured cells seemed to undergo osteoblastic differentiation with increased ALP activity and expression of molecules critical to bone metabolism such as osteocalcin and type X collagen. Recently, a BMP-6 like molecule present in EMD was reported to be responsible for increased mRNA levels of osteogenesis- and chondrogenesis-related transcription factors when cultured with a pluripotent mouse fibroblastic cell line (Narukawa *et al*., 2007).

EMD has also been demonstrated to regulate expression of molecules that control bone mineralisation (Ganss *et al*., 1999) such as bone sialoprotein (He *et al*., 2004, Shimizu *et al*., 2004), osteopontin (Yoneda *et al*., 2003) and osteocalcin (Reseland *et al*., 2006). Furthermore, EMD may enhance wound healing by directly enhancing angiogenesis (Yuan *et al*., 2003, Mirastschijski *et al*.,

2004) with enhanced endothelial cell chemotaxis and proliferation (Yuan *et al.*, 2003, Schlueter *et al.*, 2007) as well as indirectly by stimulating secretion of another important angiogenic growth factor, vascular endothelial growth factor (VEGF) (Mirastschijski *et al.*, 2004).

The clinical application of EMD in periodontal regeneration aims to mimic the cellular and signalling events that occur during periodontal development (Hammarstrom, 1997) with differentiation of mesenchymal cells into cementoblasts, periodontal ligament fibroblasts and osteoblasts after exposure to EMD.

Animal and human histological evidence of periodontal regeneration of periodontal fenestration or intrabony defects after administration of EMD has been demonstrated with formation of new cementum, periodontal ligament and alveolar bone (Hammarstrom *et al.*, 1997, Heijl, 1997, Sculean *et al.*, 2000a, Sculean *et al.*, 2000b, Yukna and Mellonig, 2000) while clinical studies have demonstrated enhanced reduction of probing pocket depth, clinical attachment gain and alveolar bone growth (Heijl *et al.*, 1997, Sculean *et al.*, 1999, Tonetti *et al.*, 2002, Heden and Wennstrom, 2006, Sculean *et al.*, 2008a). A recent meta-analysis of the literature has concluded that the use of EMD in the treatment of intrabony defects provided significantly better clinical outcomes than periodontal flap surgery alone (Esposito *et al.*, 2005).

2.2.2 Platelet derived growth factor-BB (PDGF-BB)

Platelet derived growth factor (PDGF) plays a role in embryonic development (Schatteman *et al.*, 1992, Shinbrot *et al.*, 1994) as well as during soft and hard tissue wound healing (Ross *et al.*, 1986). It is released from the bone matrix, activated platelets and macrophages at the site of tissue injury (Bolander, 1992, Andrew *et al.*, 1995, Fujii *et al.*, 1999) with the proangiogenic, proliferative and mitogenic effect of PDGF resulting in the formation of fibrovascular tissue (Grotendorst *et al.*, 1985). The PDGF protein is a dimeric glycoprotein made up of two disulphide bonded polypeptide chains (A, B, C, D) which associate to form the four homodimer isoforms PDGF-AA, BB, CC, DD and one heterodimer isoform PDGF-AB (Fredriksson *et al.*, 2004) with the PDGF-BB form the biologically

most potent (Hock and Canalis, 1994). The greater potency of PDGF-BB has been attributed to the ability of PDGF-BB to bind to all isoforms of the PDGF receptor (Canalis *et al.*, 1992, Pfeilschifter *et al.*, 1992) as well as a higher binding affinity to the PDGF-Rβ receptor (Hart and Bowen-Pope, 1990).

PDGF is proangiogenic by enhancing chemotaxis, proliferation and differentiation of endothelial cells and vascular smooth muscle cells (Leveen *et al.*, 1994, Hellstrom *et al.*, 1999, Castellon *et al.*, 2002). PDGF may also enhance angiogenesis indirectly by increasing the expression of other angiogenic growth factors such as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF) (Bouletreau *et al.*, 2002, Guo *et al.*, 2003, Millette *et al.*, 2005).

PDGF appears to be a potent chemotactic stimulator of inflammatory cells (Deuel *et al.*, 1982) as well as having chemotactic, proliferative and mitogenic effect on fibroblasts (Seppa *et al.*, 1982, Piche and Graves, 1989, Bartold *et al.*, 1992, Bartold, 1993, Bartold and Raben, 1996, Lin *et al.*, 2006) with the PDGF-BB isoform the most potent (Siegbahn *et al.*, 1990, Boyan *et al.*, 1994).

PDGF has strong chemotactic effects on osteogenic cells (Tsukamoto *et al.*, 1991, Mehrotra *et al.*, 2004) with PDGF-BB again demonstrating the greatest potency (Lind *et al.*, 1995). The chemotactic effect of PDGF may be dependent on the stage of osteoblast differentiation with greater chemotaxis for undifferentiated osteoprogenitors compared to differentiated osteoblasts (Hughes *et al.*, 1992) suggesting that PDGF may enhance early wound healing by increasing the number of undifferentiated osteoprogenitor cells within the bone defect rather than the later stages of bone healing with differentiated osteoblasts.

The mitogenic effects of PDGF-AA, BB and AB on osteoblasts and undifferentiated osteoprogenitor cells have been reported (Canalis *et al.*, 1989, Centrella *et al.*, 1991, Zhang *et al.*, 1991, Hsieh and Graves, 1998, Mehrotra *et al.*, 2004) with PDGF-BB the most potent mitogen (Hock and Canalis, 1994). Similar to its chemotactic effects, the effect of PDGF may depend on the stage of osteoblastic differentiation with a greater response in less differentiated cells compared to differentiated cells (Abdennagy *et al.*, 1992, Yu *et al.*, 1997).

Although PDGF may demonstrate chemotactic, proliferative and mitogenic effects, it may have an inhibitory effect on osteoblast differentiation as indicated by a reduction of ALP activity and mineralisation *in vitro* (Giannobile *et al.*, 1997) with PDGF-BB having the greatest inhibitory effect on differentiation (Centrella *et al.*, 1991, Hock and Canalis, 1994). This effect may depend on the length of PDGF exposure with continuous PDGF stimulation reducing osteoblast differentiation (Yu *et al.*, 1997, Strayhorn *et al.*, 1999) while multiple, brief exposures to PDGF enhances osteoblast differentiation and mineralisation (Hsieh and Graves, 1998, Marzouk *et al.*, 2008).

The application of recombinant forms of PDGF to osseous healing have demonstrated enhanced bone formation in animal osseous defects (Nash *et al.*, 1994). Most studies have focussed on the more potent PDGF-BB isoform and Lynch *et al.*, (Lynch *et al.*, 1989, Lynch *et al.*, 1991b) first described its use in periodontics and implant dentistry with increased cellular activity, bone and periodontal regeneration after the application of a combination of recombinant PDGF-BB and IGF-I (Lynch *et al.*, 1989, Lynch *et al.*, 1991b, Giannobile *et al.*, 1996, Howell *et al.*, 1997). Similarly, in peri-implant osseous defects in dogs, the application of PDGF resulted in greater bone repair and formation than untreated control groups (Lynch *et al.*, 1991a, Stefani *et al.*, 2000).

2.2.3 Combination of CaP ceramics with EMD or rhPDGF-BB

The application of growth factors without a scaffold or carrier is partly ineffective *in vivo* due to their short half-life, rapid diffusion away from the defect site as well as the susceptibility of these proteins to uptake, catabolism or proteolysis (Hotz and Herr, 1994, Winn *et al.*, 1999) resulting in an insufficient local concentration of the growth factor necessary for bone formation (Urist *et al.*, 1984b, Reddi, 1995, Wozney and Rosen, 1998). In addition, inclusion of a bone graft material may provide support of the overlying flap as the fluid like consistency of growth factors limits their use alone due to collapse of the overlying surgical flap (Mellonig, 1999, Kuru *et al.*, 2006).

EMD has been combined with a variety of bone graft materials including DBBM (Sculean *et al.*, 2002b, Velasquez-Plata *et al.*, 2002, Sculean *et al.*, 2003, Zucchelli *et al.*, 2003), bioactive glass

(Sculean *et al.*, 2002a, Sculean *et al.*, 2005, Sculean *et al.*, 2007) as well as CaP ceramics such as β-TCP and HA-TCP. Clinical and human histological studies have reported no additional benefit of EMD when combined with DBBM or bioactive glass compared to these bone graft materials alone (Scheyer *et al.*, 2002, Sculean *et al.*, 2002b, Sculean *et al.*, 2005, Sculean *et al.*, 2007) however, the addition of EMD to DBBM appeared to improve the clinical benefit when compared to EMD alone (Lekovic *et al.*, 2000, Zucchelli *et al.*, 2003).

Limited data exist on the combination of EMD and CaP ceramics although the addition of EMD to β-TCP does not seem to provide any additional benefit over EMD alone (Bokan *et al.*, 2006). Although the release kinetics of EMD when combined with a CaP scaffold is not well documented, a biphasic release kinetic of EMD from a CaP carrier with an initial burst release during the first 5 hours followed by a slower sustained release has been observed *in vitro* (Plachokova *et al.*, 2008). The combination of EMD and HA-TCP (Bone Ceramic®) is commercially available (Emdogain Plus®) and demonstrated similar clinical outcomes six months after treatment when compared to the use of EMD alone in the treatment of wide periodontal intrabony defects (Jepsen *et al.*, 2008). The recent histological findings of EMD combined with Bone Ceramic® (Emdogain Plus®) in the treatment of periodontal intrabony defects suggest only limited osteoconductivity (Sculean *et al.*, 2008b) with limited new bone formation and fibrous encapsulation of the ceramic particles nine months after treatment.

A combination of the CaP ceramic β-TCP and rhPDGF-BB is commercially available (Gem 21S®). In an early study, PDGF-BB was combined with the natural polymer chitosan and TCP sponge in the rat calvarial defect model and evaluated histologically after two and four weeks (Lee *et al.*, 2000). The release of PDGF-BB followed a biphasic release kinetic and histologically, the composite graft promoted osseous healing of calvarial defects with no connective tissue encapsulation of the TCP graft material. More recently, the subcutaneous implantation of PDGF-BB and β-TCP into mice indicated that PDGF-BB was adsorbed to β-TCP in a concentration and time-dependent manner with rapid adsorption occurring between one and five minutes (Bateman *et al.*,

2005). *In vitro* release studies demonstrated biphasic release kinetics with *in vivo* release occurring more rapidly than *in vitro* release. *In vitro*, PDGF-BB had a stimulatory effect on osteoblasts with the osteoblastic cells incubated with PDGF-BB treated matrices demonstrating attachment within the ceramic pores as well as greater cell proliferation than the control matrices alone (Bateman *et al.*, 2005).

In a large multi-centre study, the use of this composite graft material has been evaluated for the treatment of periodontal osseous defects (Nevins *et al.*, 2005). The combination of 0.3 mg/ml or 1.0 mg/ml of rhPDGF-BB with β-TCP resulted in greater CAL gain over β-TCP alone at three months although this was not statistically different after six months. Radiographic evaluation of the rhPDGF/β-TCP treated sites demonstrated significantly greater bone growth and bone fill at six months compared to β-TCP alone with ongoing bone formation and maturation after 24 months (McGuire *et al.*, 2006). Histologically, the use of this material has demonstrated periodontal regeneration six months after surgical treatment of human intraosseous periodontal defects (Ridgway *et al.*, 2008).

Gem21S® has also been used in guided bone regeneration (GBR) utilising a simultaneous GBR technique at the time of dental implant placement (Byun and Wang, 2008). Autogenous bone was placed as an inner graft layer directly onto the implant surface and covered with an outer layer of β-TCP/rhPDGF-BB contained by a collagen barrier membrane. Complete bone healing at the defect site was demonstrated when second stage surgery was performed after 5 months healing, suggesting that the combination of rhPDGF-BB and β-TCP may be a suitable material for guided bone regeneration.

Only limited data exists evaluating the combination of HA-TCP and rhPDGF-BB although the use of Bone Ceramic® combined with 0.3 mg/ml rhPDGF-BB on initial bone formation during GBR of a canine alveolar ridge defect was evaluated (Schwarz *et al.*, 2009). Three weeks after augmentation, the sites with the addition of rhPDGF-BB demonstrated greater augmented area and mineralised tissue formation as well as greater transglutaminase II antigen reactivity in augmented

sites than the control. As transglutaminase II is an enzyme that has been demonstrated to be directly involved in the process of angiogenesis (Haroon *et al*., 1999), the combination of HA-TCP and rhPDGF-BB may enhance initial bone healing in lateral ridge augmentation.

To date, there have been no studies reporting the osteoinductive properties of HA-TCP when combined with EMD (Emdogain®) or rhPDGF-BB in the concentration found in the commercially available Gem 21S®. Given that EMD is involved in a mesenchymal cell differentiation and that rhPDGF-BB is a potent mitogenic and proliferative stimulator of osteoblasts, it is possible these growth factors may demonstrate osteoinductive properties when combined with HA-TCP. In this study, our aim was to demonstrate whether HA-TCP is osteoinductive when implanted into non-osseous sites as well as determine whether an enamel matrix protein derivative (Emdogain®, EMD) or recombinant human platelet derived growth factor-BB (rhPDGF-BB) provides or increases the osteoinductive potential of HA-TCP.

2.3 Materials and Methods

2.3.1 Ethics approval

Ethics approval was granted by the Animal Ethics Committee of the Institute of Medical and Veterinary Science (IMVS)/Central Northern Adelaide Health Service under project number 155/07 for the period 6/12/2007 to 30/6/2009.

2.3.2 Animals

Twenty male Swiss/CD-1 mice of six to eight weeks of age with a minimum weight of 30g were used in this study. Animals were stabilised for a minimum of two days prior to commencement of the experimental procedure.

2.3.3 Preparation of HA-TCP ceramic

A commercially available, fully synthetic particulate HA-TCP bone graft substitute of medical grade purity with a HA/TCP ratio of 60/40 was purchased (Straumann Bone Ceramic® – BIORA AB, Straumann, Malmö, Sweden, Lot numbers: F1203, F5860). This material has a 100% crystalline HA component, a particle size of 400-700 µm and 90% porosity with interconnected pores of 100-500 µm in diameter. Ten milligrams (10 mg) of particulate HA-TCP was weighed out (Mettler Analytical Balance AE 260 DeltaRange, Mettler-Toledo Inc, Columbus, Ohio, USA) and placed into thirty gelatine half capsules (Size 5 White Opaque Gelatin Capsules, Capsugel, Pfizer Australia, West Ryde, NSW, Australia). Ten capsules were left empty for the control group. All capsules were sterilised by exposure to ultraviolet light for a minimum of 24 hours prior to implantation.

2.3.4 Preparation of growth factors

rhPDGF-BB: On the day of implantation, 500 µg of unconstituted recombinant human platelet derived growth factor-BB (rhPDGF-BB – PeproTech, Rocky Hill, New Jersey, USA) was reconstituted in 1.67 ml of sterile saline in accordance to the manufacturer's instruction to produce a rhPDGF-BB concentration of 0.3 mg/ml and stored at 4°C until used. This concentration is the same as a commercially available rhPDGF-BB used in conjunction with β-TCP (Gem21S® - Osteohealth, Shirley, New York, USA) in bone regenerative therapy.

Enamel matrix protein derivative (EMD): A commercially available enamel matrix protein derivative with a concentration of 30 mg/ml in a propyl glycol alginate (PGA) carrier was purchased (Emdogain® - BIORA AB, Straumann, Malmö, Sweden, Lot number: F3752) and was opened at the time of implantation.

2.3.5 Implantation procedure

The implantation surgery was performed after administration of inhalation anaesthesia with 2% v/v isoflurane with O_2 flow rate set at 2 L/min. Following administration of inhalation anaesthesia,

the animals were placed in a rodent stereotactic device and inhalation anaesthesia was maintained using a nose cone fitted to the stereotactic frame.

Both hindlimbs were secured and the implantation sites were disinfected with alcohol swabs. A medial incision in both left and right legs was made through the full thickness of the skin over the muscles in the same axis as the femur and tibia. An intramuscular pocket was created in the quadriceps muscle using blunt dissection. Immediately prior to implantation, the open end of the gelatine half capsule was compressed together to partially seal the capsule. One implant was inserted into each pocket and the incision closed with surgical staples. The same procedure was applied bilaterally, giving two implants per animal. The wounds were swabbed with povidine-iodine and the animals closely monitored until they fully recovered from the anaesthetic **(Appendix One: Surgical Protocol)**.

The mice were maintained postoperatively on 0.3 mg/ml of fluoroquinolone in 125 ml of H_2O for 1 week with the solution changed daily. All animals were weighed and reviewed weekly until sacrifice. A commercially available diet (Rat and Mouse Breeder Cubes, Specialty Feeds, Glen Forest, WA, Australia) and water were provided *ad libitum* for 4 and 8 weeks prior to sacrifice.

2.3.6 Experimental groups

Twenty mice were divided into four groups of five mice with implants placed into the left and right quadriceps muscles of each mouse. Mice within each group were identified with individual tail markings. **Table 1** summarises the allocation of graft material and growth factor for each site per group at each time period.

The control group had an empty gelatine capsule implanted while the groups in which growth factors were combined with HA-TCP, 28 μl of 30 mg/ml enamel matrix protein derivative (EMD-Emdogain®) or 10 μl of 0.3 mg/ml recombinant human platelet derived growth factor-BB (rhPDGF-BB) was dispensed immediately prior to implantation into a half capsule containing the HA-TCP and mixed with the dispensing pipette tip. This is the prescribed mixing ratio recommended by the

manufacturer to produce Emdogain PLUS® (Emdogain® + Bone Ceramic®) and Gem-21S® (rhPDGF-BB + β-TCP) preparations except that HA-TCP (Straumann Bone Ceramic®) was used in place of β-TCP.

Group No.	No. of Mice	Experimental Period	Left Thigh	Right Thigh
1	5	4 weeks	Control (Gelatine capsule)	HA-TCP + EMD
2	5	8 weeks	Control (Gelatine capsule)	HA-TCP + EMD
3	5	4 weeks	HA-TCP	HA-TCP + rhPDGF-BB
4	5	8 weeks	HA-TCP	HA-TCP + rhPDGF-BB

Table 1: Allocation of graft materials and growth factors to surgical sites

2.3.7 Retrieval surgery

After 4 weeks, the five mice from Group 1 and the five mice from Group 3 were euthanised using CO_2 inhalation. This was repeated after 8 weeks with the five mice from Group 2 and the five mice from Group 4.

Samples were retrieved by removal of the hindquarters of each mouse followed by removal of skin and fur and then placed immediately in 10% buffered formalin for one week **(Appendix Two: Retrieval protocol)**. Following fixation, the specimens were rinsed in physiological buffered saline (PBS) and the sections were decalcified in 5% formic acid for two weeks with the solution changed weekly. After decalcification, all specimens were placed in 70% ethanol prior to sectioning.

2.3.8 Radiographic evaluation

Digital radiographs of the limbs were taken after fixation to determine the location of the implanted particles and repeated after decalcification to confirm complete decalcification of the specimen.

Standardised radiographic techniques were used to ensure uniform exposure and assessment. The x-ray unit used was a dental radiography unit with settings of 60kV and 7mA

(Siemens SR60/70 7L, Siemens Australia, Bayswater, VIC, Australia) and images visualised and recorded with Sidexis® dental imaging software (Version 5.5, Sirona Australia, Chatswood, NSW, Australia) **(Appendix Three: Radiographic Protocol)**.

2.3.9 Histological evaluation

Using the digital radiographs for guidance, the specimens were sectioned transversely in the area of the implanted particles and then processed for paraffin embedding. The specimens were oriented such that the cut surfaces were viewed when the paraffin blocks were sectioned. Sections of 7μm were cut along the transverse axis of the femur bone **(Appendix Four: Histological Preparation Protocol)** and these were stained with stained with haematoxylin and eosin (H and E) as well as Perl's stain or von Kossa's stain. Some unstained sections were also prepared. All specimens were assessed under light microscopy.

Images were viewed at 40X, 100X and 200X magnification under a light microscope (Olympus BH-2 Research microscope, Olympus Australia, Mount Waverly, VIC, Australia) connected to a 2 megapixel digital CMOS colour camera (Altra20, Soft Imaging System, Gulfview Heights, SA, Australia). Digital images of sections at 200X magnification were obtained (AnalySIS FIVE, Olympus Australia, Mount Waverly, VIC, Australia) and analysed with a separate computer image analysis program (ImageJ version 1.41o, National Institutes of Health, USA).

Three sections of the implanted area were examined for each animal where possible. A semi-quantitative histological examination based on that used by Garraway *et al*., (1998) was performed with the aim of assessing the inflammatory changes, reparative processes and presence of osteoinduction after implantation of uncoated particulate HA-TCP or when combined with the growth factors EMD or rhPDGF-BB.

Acute Inflammation

Acute inflammation was recorded if a predominantly polymorphonuclear (PMN) leucocyte cell infiltrate was detected around the implanted material. The extent of the infiltrate was indicated by the following categories:

- Score 0: No polymorphonuclear leucocytes (PMNs)
- Score 1: PMNs ≤25% of cells around implanted material
- Score 2: PMNs 26-50% of cells around implanted material
- Score 3: PMNs 51-75% of cells around implanted material
- Score 4: PMNs 76-100% of cells around implanted material

Chronic Inflammation

Chronic inflammation was recorded when the cell infiltrate consisted predominantly of plasma cells, monocytes/macrophages or lymphocytes. The extent of the infiltrate was indicated by the following categories:

- Score 0: No chronic inflammation
- Score 1: Chronic inflammatory cells ≤25% of cells around implanted material
- Score 2: Chronic inflammatory cells 26-50% of cells around implanted material
- Score 3: Chronic inflammatory cells 51-75% of cells around implanted material
- Score 4: Chronic inflammatory cells 76-100% of cells around implanted material

Resorption/Foreign Body Reaction

The degree and extent of foreign body reaction or resorption of the implanted material was determined by detection of multinucleated cells such as foreign-body giant cells or osteoclasts around the implanted material. The extent of resorption and organisation was indicated by the following categories:

- Score 0: No evidence of resorption/foreign body reaction

- Score 1: Resorptive cells ≤25% of cells around implanted material
- Score 2: Resorptive cells 26-50% of cells around implanted material
- Score 3: Resorptive cells 51-75% of cells around implanted material
- Score 4: Resorptive cells 76-100% of cells around implanted material

Fibrosis - Distribution

The distribution of fibrosis was reported when a fibrous network produced by fibroblasts was seen around implanted materials. The extent of fibrosis was indicated by the following categories:

- Score 0: No evidence of fibrosis
- Score 1: Fibroblasts and collagen distributed ≤25% around implanted material
- Score 2: Fibroblasts and collagen distributed 26-50% around implanted material
- Score 3: Fibroblasts and collagen distributed 51-75% around implanted material
- Score 4: Fibroblasts and collagen distributed 76-100% around implanted material

Fibrosis - Density

The density of the fibrous network was reported when the density of fibroblasts and connective tissue fibres were subjectively assessed and classified in the following categories:

- Score 0: No evidence of fibrous connective tissue
- Score 1: Loose fibrous connective tissue with few fibroblasts or widely separated collagen fibres
- Score 2: Mildly dense fibrous connective tissue with low numbers of fibroblasts or loosely spaced collagen fibres
- Score 3: Moderately dense fibrous connective tissue with moderate numbers of fibroblasts or minimally separated collagen fibres
- Score 4: Very dense fibrous connective tissue with high numbers of fibroblasts and densely packed collagen fibres

Vascularity - Distribution

The distribution of vasculature was determined by the presence of blood vessels around the implanted particles. The extent of vascularisation was indicated by the following categories:

- Score 0: No evidence of vascular structures
- Score 1: Vascular structures distributed ≤25% around implanted material
- Score 2: Vascular structures distributed 26-50% around implanted material
- Score 3: Vascular structures distributed 51-75% around implanted material
- Score 4: Vascular structures distributed 76-100% around implanted material

Vascularity - Area

The total vasculature area was measured and recorded as a percentage of the total area examined. In addition, this was classified in the following categories:

- Score 0: No evidence of vasculature
- Score 1: Vasculature comprising ≤1% of total area measured
- Score 2: Vasculature comprising 1-1.99% of total area measured
- Score 3: Vasculature comprising 2-2.99% of total area measured
- Score 4: Vasculature comprising ≥3% of total area measured

Adipose Tissue - Distribution

The distribution of adipose tissue was determined by the extent of adipose tissue and adipocytes detected around the implanted particles and recorded under the following categories:

- Score 1: Adipose tissue distributed ≤25% around implanted material
- Score 2: Adipose tissue distributed 26-50% around implanted material
- Score 3: Adipose tissue distributed 51-75% around implanted material
- Score 4: Adipose tissue distributed 76-100% around implanted material

Adipose Tissue - Area

The total area of adipose tissue was measured and recorded as a percentage of the total area examined. In addition, this was classified in the following categories:

- Score 0: No evidence of adipose tissue
- Score 1: Adipose tissue comprising ≤10% of total area measured
- Score 2: Adipose tissue comprising 10-19.99% of total area measured
- Score 3: Adipose tissue comprising 20-29.99% of total area measured
- Score 4: Adipose tissue comprising ≥30% of total area measured

Capsule Thickness

The thickness of the cell layer encapsulating the particle was measured and recorded in the following categories:

- Score 0: No lining cells present around implanted material
- Score 1: Lining cell thickness 1-5 cells thick around implanted material
- Score 2: Lining cell thickness 6-10 cells thick around implanted material
- Score 3: Lining cell thickness 11-16 cells thick around implanted material
- Score 4: Lining cell thickness 16-20 cells thick around implanted material

Osteoinduction

Osteoinduction was reported if matrix resembling the osteoid matrix of woven bone or presence of osteocytes was detected adjacent to implanted materials. The extent of osteoinduction was indicated by the following categories.

- Score 0: No evidence of new bone formation
- Score 1: Osteoid formation detected ≤25% around implanted material
- Score 2: Osteoid formation detected 26-50% around implanted material
- Score 3: Osteoid formation detected 51-75% around implanted material

- Score 4: Osteoid formation detected 76-100% around implanted material

The mean and median score of each semi-quantitative histological category were calculated for each experimental group and at each time period.

2.3.10 Statistical analyses

The mean and median values were tabulated and statistical analyses performed. The non-parametric Kruskal-Wallis test was used to determine any statistical differences between the three experimental groups and the Mann-Whitney test for differences between two groups. Statistical analyses were performed using a statistical and graphing package (GraphPad Prism 5.0). Values of $P<0.05$ were considered statistically significant.

2.4 Results

2.4.1 Surgical and postoperative complications

All animals survived the surgery and the majority had an uneventful postoperative recovery. Three mice (one in 8 week Control/HA-TCP + EMD, two in 4 week HA-TCP/HA-TCP + PDGF) developed wound dehiscence in the first 48 hours postoperatively due to loss of the surgical staples and required additional sutures for wound closure. One mouse (8 week HA-TCP/HA-TCP + PDGF) lost its surgical staple at two weeks postoperatively resulting in wound breakdown and required further suturing. A further mouse (8 week Control/HA-TCP + EMD) had late wound irritation at 4 weeks postoperatively as a result of a loosening staple. These two animals were placed on the antibiotic fluoroquinolone for a further week. All animals eventually recovered and none were lost during the experimental period.

The mice were initially housed in their groups with five mice per cage but were separated into individual cages after one week due to fighting. Mouse weights increased and were maintained

throughout the experimental period. By the time of graft retrieval at four and eight weeks, all implantation sites had healed completely.

2.4.2 Radiographic evaluation

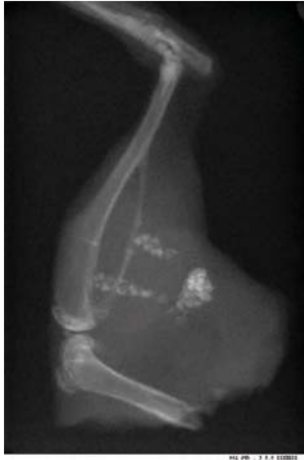
Radiographs were taken of the excised hind limb of each mouse after fixation at 4 and 8 weeks to determine the graft location and assess the degree of graft dispersion. Radiographic analysis of ectopic bone formation radiographically was not possible due to the radiopaque nature of the HA-TCP material. As expected, no radiographic image was visible in the control groups and specimens from this group have been excluded from the radiographic analysis.

After decalcification, samples from all groups demonstrated no radiopaque particles within the hind limb soft tissues.

4 Week Groups

HA-TCP

Radiopaque graft particles were visible within the soft tissue of the hind limb with four samples demonstrating a central well delineated radiopaque area with varying amounts of particle dispersion **(Figure 1A)** while the remaining sample demonstrated less graft material that was widely dispersed through the soft tissue of the specimen **(Figure 1B)**.



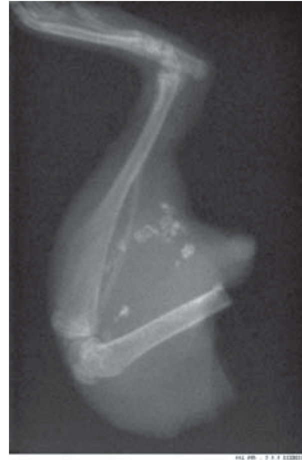
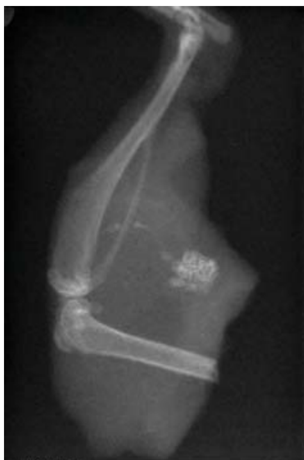


Figure 1A Figure 1B

Figure 1: 4 Week radiographs of hind limb with well defined radiopaque graft material (Figure 1A) and dispersed graft material (Figure 1B) in HA-TCP group

HA-TCP + PDGF

All five samples demonstrated well defined radiopaque areas with four exhibiting minimal graft dispersion **(Figure 2A)** and the remaining sample exhibiting a moderate degree of graft dispersion **(Figure 2B).** The graft particles in samples from this group were more confined with minimal graft dispersion when compared to the other two groups.



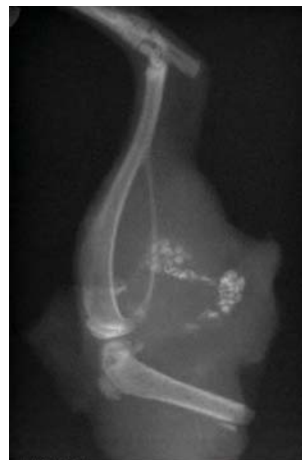


Figure 2A Figure 2B

Figure 2: 4 Week radiographs of hind limb with well defined radiopaque graft material (Figure 2A) and dispersed graft material (Figure 2B) in HA-TCP + PDGF group

HA-TCP + EMD

Three of the five samples demonstrated well defined radiopaque areas with minimal graft dispersion in one sample (Figure 3A) while the other two were moderately dispersed. The other two samples demonstrated greater dispersion of the graft material throughout the soft tissue of the specimen (Figure 3B).

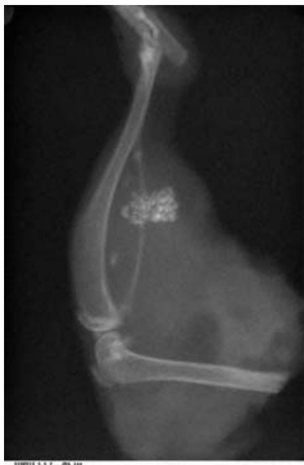


Figure 3A

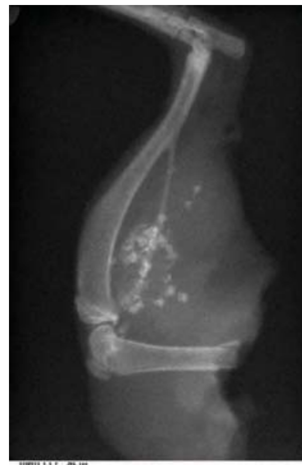


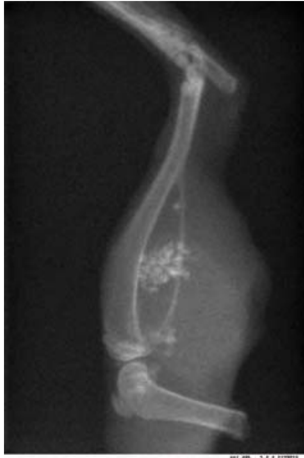
Figure 3B

Figure 3: 4 Week radiographs of hind limb with well defined radiopaque graft material (Figure 3A) and dispersed graft material (Figure 3B) in HA-TCP + EMD group

8 Week Groups

HA-TCP

Three of the five samples demonstrated well defined radiopaque areas (Figure 4A) although varying degrees of particle dispersion were evident in this group. Of the remaining specimens, the grafter material in one specimen was widely dispersed in the soft tissue (Figure 4B) while the other demonstrated less radiopaque material dispersed within the local area of implantation.



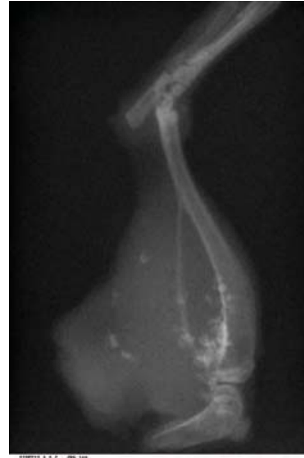
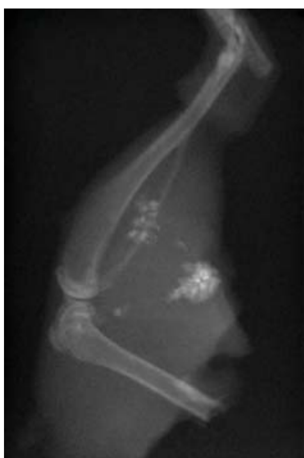


Figure 4A Figure 4B

Figure 4: 8 Week radiographs of hind limb with well defined radiopaque graft material (Figure 4A) and dispersed graft material (Figure 4B) in HA-TCP group

HA-TCP and PDGF

Three of the five samples demonstrated well defined radiopaque areas (Figure 5A) although these were less well contained than those in the four week group with varying degrees of graft dispersion evident. Of the remaining two samples, one demonstrated wide dispersion of the graft material in the soft tissue (Figure 5B) while the other contained less radiopaque graft material dispersed within the local area of implantation.



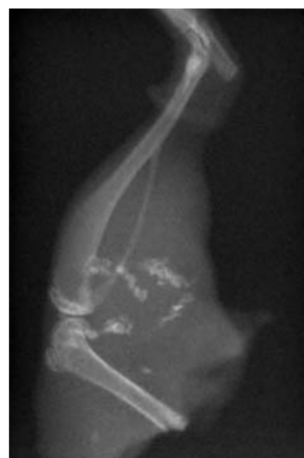


Figure 5A Figure 5B

Figure 5: 8 Week radiographs of hind limb with well defined radiopaque graft material (Figure 5A) and dispersed graft material (Figure 5B) in HA-TCP + PDGF group

HA-TCP + EMD

Four of the five samples demonstrated well defined radiopaque areas with varying degrees of particle dispersion in the local area of implantation (Figure 6A). The dispersion of the graft particles in these samples was generally less than the uncoated HA-TCP samples. However, the remaining sample in the HA-TCP + EMD group demonstrated widespread scattering of the graft material within the soft tissue (Figure 6B).

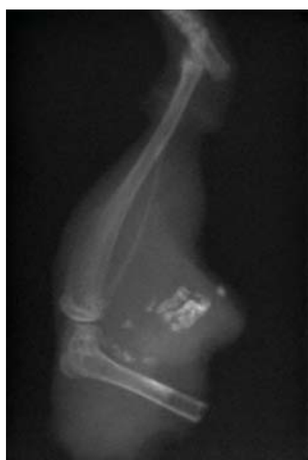


Figure 6A

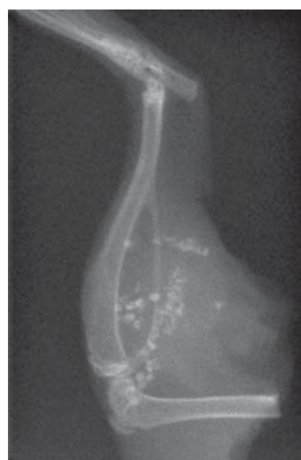


Figure 6B

Figure 6: 8 Week radiographs of hind limb with well defined radiopaque graft material (Figure 6A) and dispersed graft material (Figure 6B) in HA-TCP + EMD group

2.4.3 Histological evaluation

All animals survived the four and eight week experimental period. No histological evidence of the implanted gelatine capsule was present in the control group and this group was not included in the histological evaluation. Implanted graft particles could not be detected in the tissues analysed from one animal of the 4 week HA-TCP group. As a result this sample was left out of the analyses. In addition, minimal graft material was detected in one animal from the 8 week HA-TCP + PDGF group and only one section from this animal was included for analysis.

In all specimens, a black material was present around the implant site. This was more commonly identified in the specimens containing HA-TCP than the control group due to the greater

ease in identifying the implantation site with the radiopaque HA-TCP. This black material aggregated within the cell layer adjacent to the graft particles as well as within the surrounding fibrous connective tissue. On closer examination, the material appeared to be granular and was located both intracellularly and extracellularly within the soft connective tissue. The material identity and source was unknown.

In order to identify this material, unstained sections as well as sections stained with von Kossa's and Perl's stain were analysed.

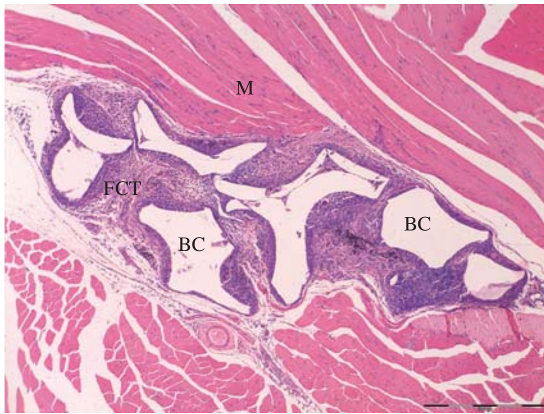
2.4.4 Qualitative analysis - Haematoxylin and Eosin

4 Week Groups

HA-TCP

The implantation site was identified by the presence of irregularly shaped voids representing the decalcified ceramic particles, which were encapsulated by a cellular layer and fibrous connective tissue network separating the particles from the surrounding skeletal muscle tissue. In all samples, a black particulate matter was evident around the implanted particles. This had a clumped appearance and was densest in the cell layer lining the particle void as well as the immediate fibrous connective tissue surrounding the particles. More scattered black material was present in areas further away from the implant site. This material was rarely seen in muscle and appeared to be located both intracellularly and extracellularly. In areas of skeletal muscle close to the area of implantation, a reactive change in the myocytes was demonstrated with enlarged nuclei located in the centre of the muscle fibre while cells distant to the implant area demonstrated peripherally located nuclei. A dense cellular layer of one to 15 cells thick was demonstrated immediately lining the particle void, with vasculature seen within this layer. Due to the densely packed nature of this cell layer, identification of individual cell types was difficult although these cells had a mesenchymal cell appearance resembling active fibroblasts with plump nuclei and granular cytoplasm. Macrophages and isolated multinucleated giant cells were also seen within this cell layer. Surrounding this cellular layer was a

fibrous connective tissue layer consisting of mature spindle shaped fibroblasts with condensed and elongated nuclei with vasculature also seen within this fibrous connective tissue layer. The density of the fibrous connective tissue between particles appeared to be greater than that surrounding the graft area or adjacent to muscle. Within the fibrous connective tissue, a negligible acute inflammatory response was identified with isolated polymorphonuclear leucocytes seen only in association with blood vessels. A low level chronic inflammatory response was seen with macrophages and lymphocytes identified within the surrounding connective tissue while minimal multinucleated cells were seen (Figure 7A and 7B). In sections obtained from one animal, the graft particles were surrounded by large amounts of adipose tissue with adipocytes demonstrating a small cytoplasm and peripherally displaced nucleus scattered within loose, collagenous supporting tissue. In sites where adipose tissue predominated, the cell capsule layer as well as surrounding fibrous connective tissue was thinner. However this remained relatively dense between particles. In all samples, no osteoblastic activity or bone matrix synthesis was detected and there was no evidence of intramuscular bone or cartilage formation.



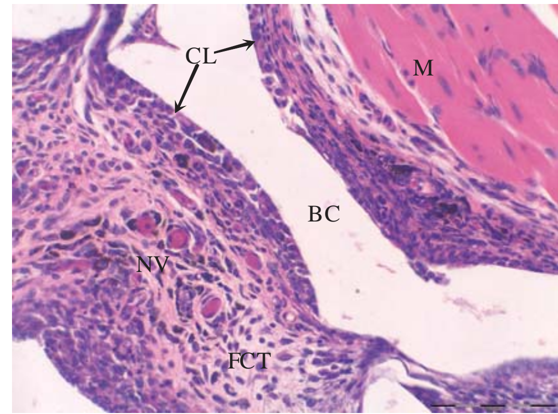


Figure 7A

Figure 7B

Figures 7A: Photomicrograph of H and E section from HA-TCP group at 4 weeks at 40x magnification (Bar = 500μm)

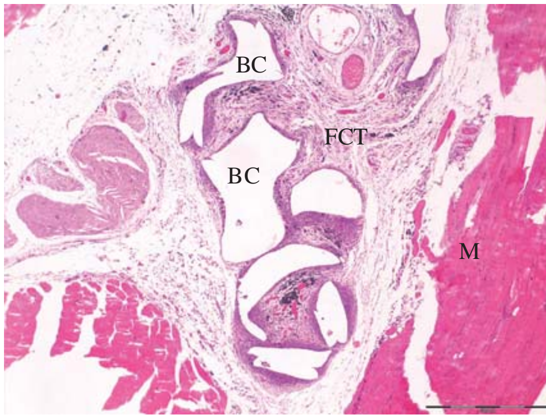
Figure 7B: Photomicrograph of H and E section from HA-TCP group at 4 weeks at 200x magnification (Bar = 100μm)

(BC=Bone Ceramic particle, CL=Cellular capsule layer, FCT=Fibrous connective tissue, M=Muscle, NV=Neovascularisation)

HA-TCP + PDGF

Specimens exhibited regularly shaped voids similar to the HA-TCP group surrounded by a cellular capsule and fibrous connective tissue with nearby skeletal muscle. The graft particles in sections obtained from three animals were surrounded by large amounts of adipose tissue or fibro-adipose tissue. In most of the specimens, a black granular material was evident around the implanted particles with a similar density and distribution as the other groups. Similar to the other groups, a reactive change in the muscle was evident in the area immediately adjacent to the implanted site as well as the presence of a highly cellular layer surrounding the particle void. This cell layer thickness appeared to be thinner than the HA-TCP group and ranged from one to 10 cells thick. Surrounding this layer was a connective tissue layer that walled off the particles from the surrounding muscle or adipose tissue consisting of mature fibroblasts with spindle shaped nuclei and dense collagen fibres although the density of the connective tissue varied between specimens with

some specimens demonstrating very dense connective tissue while others had much looser tissue. However, in all specimens the fibrous connective tissue located between particles appeared to be denser than that interposed between the particles and muscle. New blood vessels were seen within this fibrous connective tissue layer with several samples demonstrating marked vascularity within the interparticulate connective tissue (Figure 8A and 8B). Blood vessels were associated with increased density of the interparticulate connective tissue. The degree of neovascularisation within this fibrous connective tissue varied among animals with two animals demonstrating high levels of vascularity while the other three animals exhibited lower levels. Samples where the particles were surrounded by large amounts of adipose tissue and fibro-adipose tissue (Figure 8C) were associated with a reduced density of the fibrous connective tissue, reduced vasculature and a thinner cellular layer surrounding the particle. In all samples, there was minimal acute inflammatory response with the absence of or minimal presence of polymorphonuclear leucocytes seen in close proximity to the blood vessels. Low levels of macrophages and lymphocytes were demonstrated within the connective tissue and minimal multinucleated cells were seen within the connective tissue. In all samples, no osteoblastic activity or bone matrix synthesis was detected and there was no evidence of intramuscular bone or cartilage formation.



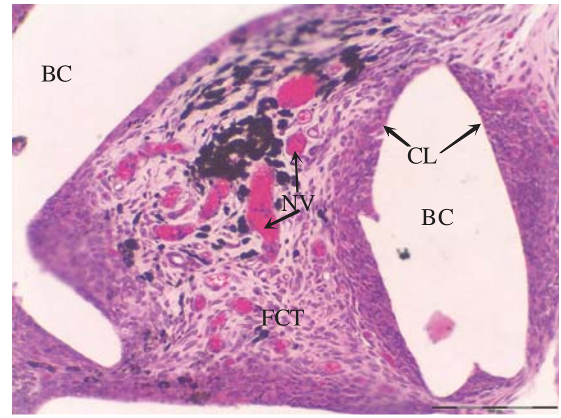


Figure 8A

Figure 8B

Figure 8A: Photomicrograph of H and E section from HA-TCP + PDGF group at 4 weeks at 40x (Bar = 500μm)

Figure 8B: Photomicrograph of H and E section from HA-TCP + PDGF group at 4 weeks at 200x (Bar = 100μm)

(BC=Bone Ceramic particle, CL=Cellular capsule layer, FCT=Fibrous connective tissue M=Muscle, NV=Neovascularisation)

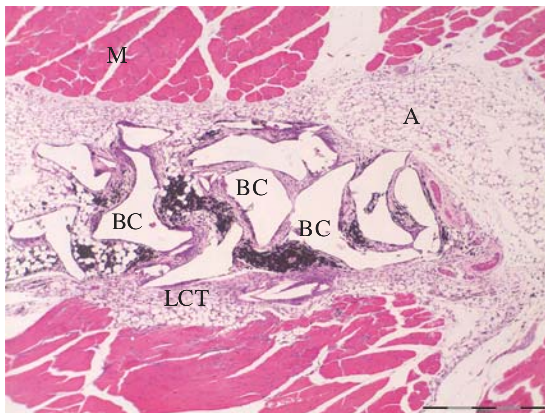


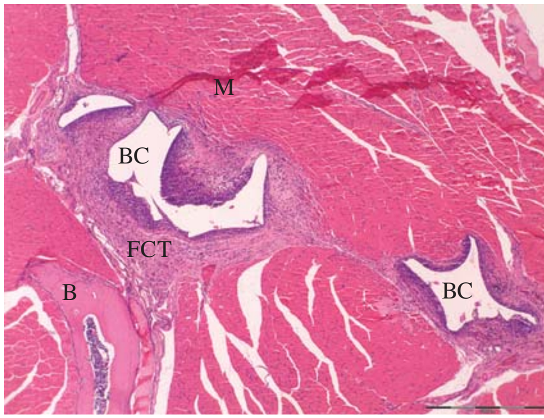
Figure 8C: Photomicrograph of H and E section from HA-TCP + PDGF group at 4 weeks at 40x magnification demonstrating adipose tissue surrounding graft particles (Bar = 500μm)

(A=Adipose Tissue, BC=Bone Ceramic particle, LCT=Loose Connective Tissue, M=Muscle)

HA-TCP + EMD

Irregular shaped voids were present surrounded by fibrous connective tissue and muscle with the black particulate matter evident around most of the implanted particles with a similar density and

distribution as the other groups. Although adipose tissue was detected around samples from two animals, this was less common and to a lesser degree than the HATCP + PDGF group. Similar to the other groups, a reactive change was seen in myocytes adjacent to the implant site. The particle voids were lined by densely packed mesenchymal like cells with immature fibroblasts as well as macrophages and isolated multinucleated giant cells. This cell layer varied from one to 15 cells thick and in general appeared thicker than the HA-TCP + PDGF group but similar to the HA-TCP group. Immediately surrounding and continuous with this lining cell layer was a fibrous connective tissue layer containing mature spindle shaped fibroblasts and dense collagen fibres tissue (Figure 9A and 9B). This connective tissue encapsulated the particles and separated them from the surrounding muscle or adipose. The fibroblast response appeared to be greater than the other two groups with less inter-animal variation with most animals demonstrating a strong fibroblastic response. Vascular structures were seen within the fibrous connective tissue although this did not appear to be as intense as the HA-TCP + PDGF group. The cellular response within the tissues was similar to the other two groups with a minimal acute inflammatory response and the presence of minimal or no polymorphonuclear leucocytes. Low levels of chronic inflammatory cells and minimal multinucleated cells were detected within the fibrous connective tissue. In all samples, no osteoblastic activity or bone matrix synthesis was detected and there was no evidence of intramuscular bone or cartilage formation.



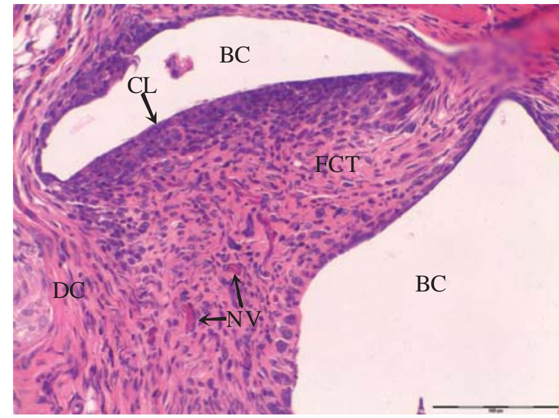


Figure 9A

Figure 9B

Figure 9A: Photomicrograph of H and E section from HA-TCP + EMD group at 4 weeks at 40x magnification (Bar = 500μm)

Figure 9B: Photomicrograph of H and E section from HA-TCP + EMD group at 4 weeks at 200x magnification (Bar = 100μm)

(B=Hind Limb Bone, BC=Bone Ceramic particle, CL=Cellular capsule layer, DC=Dense collagen fibres, FCT=Fibrous connective tissue, M=Muscle, NV=Neovascularisation,)

8 Week Groups

HA-TCP

Samples exhibited irregularly shaped voids representing the decalcified particles surrounded by a cellular layer. A black particulate matter was evident around the implanted particles in all samples although this appeared to be less than the 4 week samples. In the majority of samples the encapsulating cell layer was thinner than the 4 week specimens, ranging from one to five cells thick. Compared to the 4 week group, the morphology of this cell layer appeared to be more organised with less intercellular spaces with connective tissue fibres between these cells continuous with the fibrous connective tissue layer surrounding the particles. This outer connective tissue layer was comprised of mature fibroblasts with spindle shaped nuclei and collagen fibres **(Figure 10A and 10B)**. Similar to the findings from the other groups, the interparticulate connective tissue was generally denser than outlying tissue. Vasculature was evident although this was less than the 4

week sample as was the acute and chronic inflammatory response with only isolated polymorphonuclear leucocytes, macrophages and lymphocytes seen in some specimens. Similarly, multinucleated giant cells were rarely seen in all specimens. In several of the 8 week samples, adipose tissue was seen surrounding this outer connective tissue layer and similar to the 4 week samples, the presence of adipose tissue was associated with a reduced thickness of the cellular capsule, reduced vascularity and reduced density of the fibrous connective tissue. In all samples, no osteoblastic activity or bone matrix synthesis was detected and there was no evidence of intramuscular bone or cartilage formation.

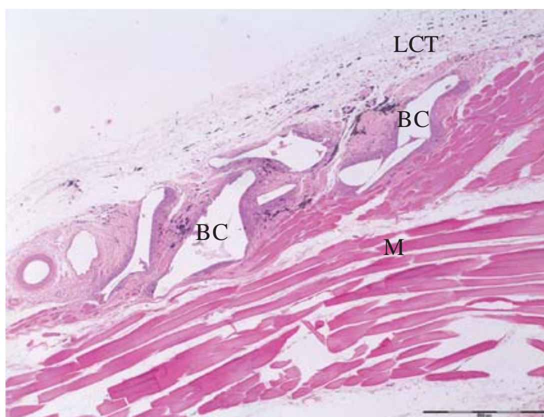


Figure 10A

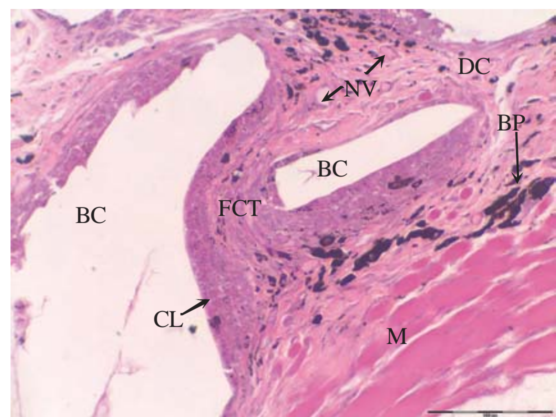


Figure 10B

Figures 10A: Photomicrograph of H and E section from HA-TCP group at 8 weeks at 40x magnification (Bar = 500μm)

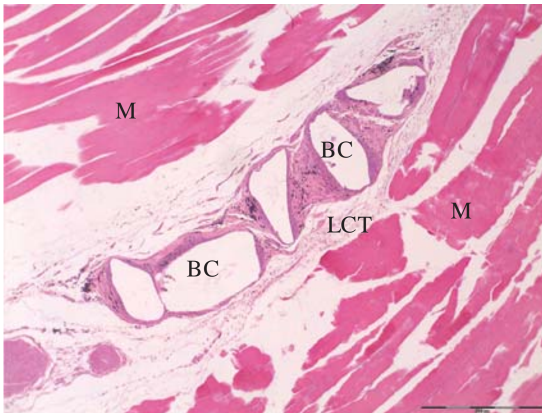
Figure 10B: Photomicrograph of H and E section from HA-TCP group at 8 weeks at 200x magnification (Bar = 100μm)

(BC=Bone Ceramic particle, BP=Black particulate material, CL=Cellular capsule layer, DC=Dense collagen fibres, FCT=Fibrous connective tissue, M=Muscle, NV=Neovascularisation)

HA-TCP + PDGF

The 8 week specimens had a similar appearance to the 4 week specimens with a number of irregularly shaped voids representing the coated HA-TCP particles (**Figure 11A and 11B**). The black particulate matter was present with a similar distribution and density to the other 8 week groups

although this appeared to be less than the 4 week group. The particles were surrounded by a cellular layer of cells ranging from 1 to 10 cells thick, which had the appearance of immature fibroblasts with plump nuclei. A number of mature fibroblasts with narrow spindle shaped nuclei as well as collagen fibres were also seen within this cell layer. Compared to the 4 week samples, the thickness of the cell layer was similar but its morphology differed in that it appeared to be more fibrous with collagen fibres continuous with the surrounding fibrous connective tissue. Similar to other 4 and 8 week specimens, the interparticulate connective tissue was denser than that surrounding the graft area, which was a looser, less cellular connective tissue. Vasculature was seen within the fibrous connective tissue, generally associated with the dense interparticulate connective tissue. The degree of vascularity seen at 8 weeks was less than that demonstrated at 4 weeks but appeared to be greater than the other groups at this time period. The inflammatory and foreign body response was reduced when compared to the 4 week specimens with negligible acute inflammatory and minimal chronic inflammatory cells present as well as isolated multinucleated giant cells detected in only a few sites and specimens. Specimens from three animals demonstrated the presence of adipose tissue partially surrounding the particles although this was less than the 4 week specimens. In one specimen, large amounts of fibro-adipose tissue surrounded the graft particle and minimal vascularity as well as a thin cellular layer surrounding the implant was observed. In all samples, no osteoblastic activity or bone matrix synthesis was detected and there was no evidence of intramuscular bone or cartilage formation.



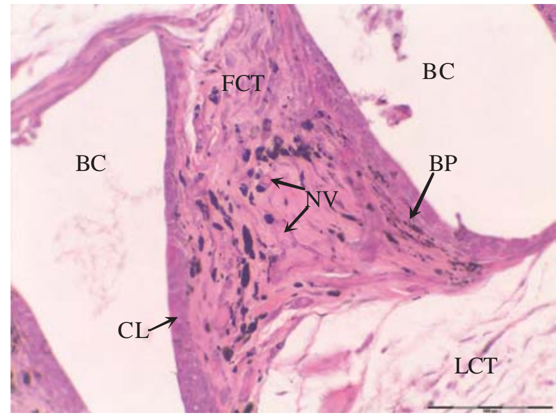


Figure 11A

Figure 11B

Figures 11A: Photomicrograph of H and E section from HA-TCP + PDGF group at 8 weeks at 40x magnification (Bar = 500μm)

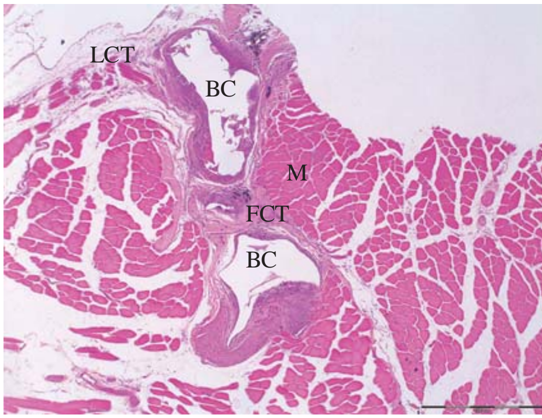
Figure 11B: Photomicrograph of H and E section from HA-TCP + PDGF group at 8 weeks at 200x magnification (Bar = 100μm)

(BC=Bone Ceramic particle, BP=Black particulate material, CL=Cellular capsule layer, FCT=Fibrous connective tissue, M=Muscle, NV=Neovascularisation)

HA-TCP + EMD

The 8 week appearance of these specimens at low magnification was similar to the 4 week group with several voids surrounded by a cellular layer, an outer fibrous connective tissue network and adjacent skeletal muscle or adipose tissue. In all samples, the black particulate matter present in the earlier samples was evident with a similar distribution and density to the other 8 week groups although this appeared to be less than the 4 week group. A darker staining cellular layer of one to 10 cells thick with the appearance of mesenchymal like cells or active fibroblasts were present surrounding the particles although this was thinner and more fibrous compared to the 4 week samples. Fibrous connective tissue consisting of mature fibroblasts with elongated nuclei and collagen fibres were seen continuous with and surrounding this cellular layer. This appeared to be less dense than the 4 week group (**Figure 12A and 12B**) and the interparticulate fibrous connective tissue was denser than the surrounding fibrous connective tissue. Four out of five animals

demonstrated particles that were enclosed by increased amounts of adipose tissue with limited fibrous connective tissue encapsulating the graft particles (Figure 12C) that were greater than the 4 week samples and other 8 week groups. Similar to other groups, samples with increased amounts of adipose tissue were associated with reduced vascularity and a thinner cell layer encapsulating the particles. The degree of vascularity in this group was predominantly associated with dense fibrous connective tissue and was reduced when compared to the 4 week samples as well as the 8 week HA-TCP + PDGF samples. The inflammatory response was reduced when compared to the 4 week specimens and similar to the other 8 week groups with negligible acute inflammation and minimal chronic inflammation present. A few macrophages and giant cells were identified but these were not common findings. In addition, in all samples, no osteoblastic activity or bone matrix synthesis was detected and there was no evidence of intramuscular bone or cartilage formation.



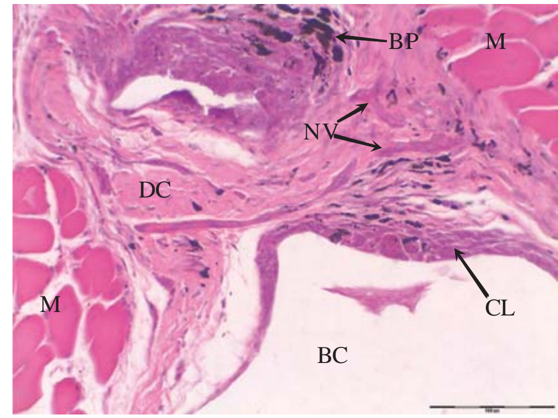


Figure 12A

Figure 12B

Figures 12A: Photomicrograph of H and E section from HA-TCP + EMD group at 8 weeks at 40x magnification (Bar = 500μm)

Figure 12B: Photomicrograph of H and E section from HA-TCP + EMD group at 8 weeks at 200x magnification (Bar = 100μm)

(BC=Bone Ceramic particle, BP=Black particulate material, CL=Cellular capsule layer, DC=Dense collagen fibres, FCT=Fibrous connective tissue, M=Muscle, NV=Neovascularisation)

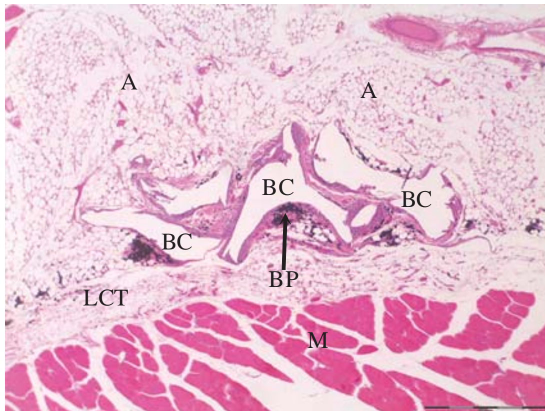


Figure 12C: Photomicrograph of H and E section from HA-TCP + EMD group at 8 weeks at 40x magnification demonstrating adipose tissue surrounding graft particles (Bar = 500μm)

(A=Adipose tissue, BC=Bone Ceramic particle, BP=Black particulate material, LCT=Loose connective tissue, M=Muscle)

Other Histological Stains

Perl's Stain

Several slides from each specimen were stained with Perl's stain to determine whether the black particles identified in the H and E slides were haemosiderin.

4 Week Groups

No staining indicating the presence of haemosiderin was identified. The black material had a similar appearance and distribution around the implanted particles to specimens stained with other staining protocols **(Figure 13A-13C)**.

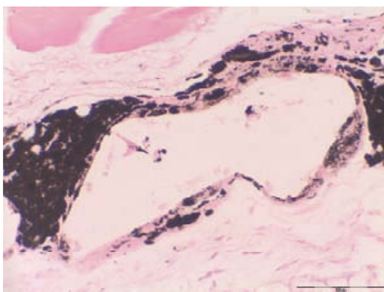


Figure 13A

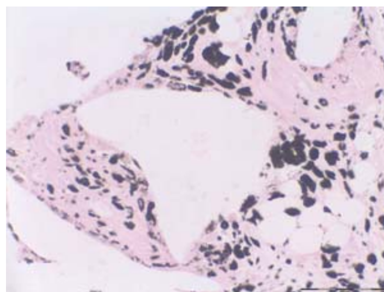


Figure 13B

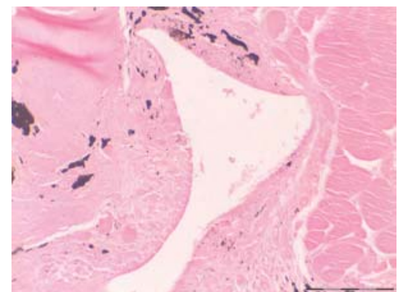


Figure 13C

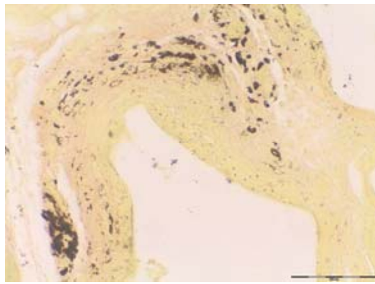
Figures 13A-13C: Photomicrograph of 4 week sections stained with Perl's stain from HA-TCP (Figure 13A), HA-TCP + PDGF (Figure 13B) and HA-TCP + EMD (Figure 13C) groups at 200x magnification (Bar = 100μm)

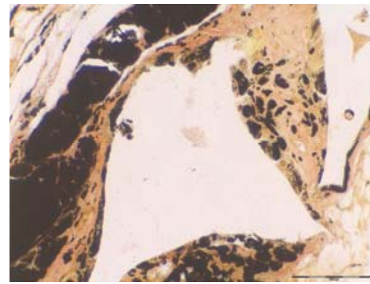
Von Kossa's stain

Several slides from each specimen were stained with Von Kossa's stain to determine whether the black particles identified in the Haematoxylin and Eosin slides were calcium particles.

4 Week Groups

No staining indicating calcification was seen in the specimens with the black material distributed in a similar manner and intensity as seen in other specimens with different staining protocols **(Figure 14A-14C)**.





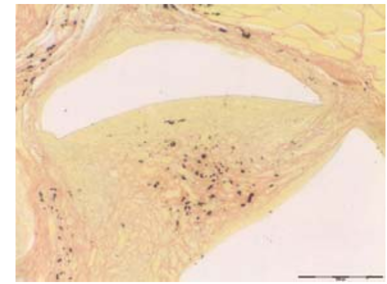


Figure 14A Figure 14B Figure 14C

Figures 14A-14C: Photomicrograph of 4 week sections stained with von Kossa's stain from HA-TCP (Figure 14A), HA-TCP + PDGF (Figure 14B) and HA-TCP + EMD (Figure 14C) groups at 200x magnification (Bar = 100μm)

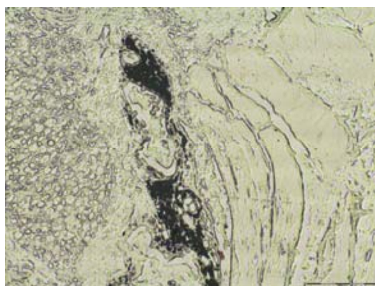
Unstained sections

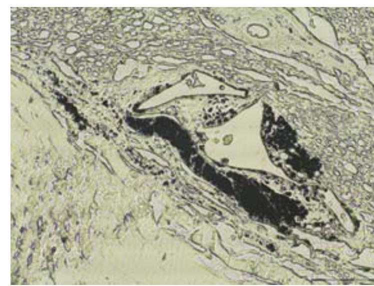
Unstained sections from each specimen were assessed to determine whether the black material identified in the Haematoxylin and Eosin slides were a result of the staining process.

4 Week Groups

HA-TCP, HA-TCP + PDGF, HA-TCP + EMD

The black material was present with a similar appearance and intensity as seen in the stained sections **(Figure 15A-15C)**.





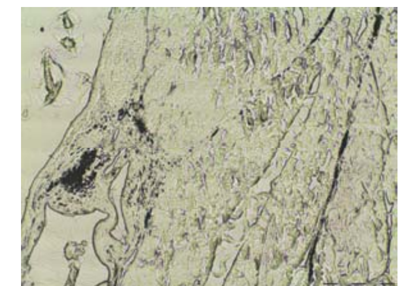


Figure 15A Figure 15B Figure 15C

Figures 15A-15C: Photomicrograph of 4 week unstained sections from HA-TCP (Figure 15A), HA-TCP + PDGF (Figure 15B) and HA-TCP + EMD (Figure 15C) groups at 100x magnification (Bar = 200μm)

2.4.5 Histomorphometric analysis

4 Week Samples (Table 2)

The inflammatory response to implantation was similar amongst the three groups with low levels of acute inflammatory cells seen in all groups. No difference existed in the chronic inflammatory response between the HA-TCP and the HA-TCP + EMD groups. However the HA-TCP + PDGF group demonstrated a smaller chronic inflammatory response when compared to the other two groups. This was statistically significant only when compared to HA-TCP + EMD. A minimal foreign body reaction was observed at 4 weeks with no difference between the three groups. The fibrous connective tissue response varied amongst the three groups with the greatest distribution of fibrous connective tissue around the graft particle seen in the HA-TCP+ EMD group which was significantly greater when compared to the HA-TCP + PDGF group. Similarly, a significantly greater fibrous connective tissue response was seen in the HA-TCP or EMD groups compared to the PDGF group. No significant differences were reported in the vascular distribution between the three groups; however a greater total vascular area was demonstrated in the HA-TCP + PDGF group when compared to the other groups although this did not reach statistical significance. Adipose tissue was a more common finding in the HA-TCP + PDGF group when compared to the other two groups and was distributed to a wider degree around the implanted graft particle when compared to HA-TCP and HA-TCP + EMD specimens although this was only significant when compared to HA-TCP + EMD. The total area of adipose tissue measured followed a similar pattern with the greatest amount of adipose tissue measured in the HA-TCP + PDGF group and the least in the HA-TCP + EMD group with the difference between these two groups statistically significant. The greatest thickness of the cellular capsule encapsulating the ceramic particle was seen in the HA-TCP group followed by the HA-TCP + EMD group. In contrast, the HA-TCP + PDGF group demonstrated the thinnest cell layer; however this was only significant when compared to the HA-TCP group. No evidence of osteoinduction was detected in any of the three groups.

8 Week values (Table 2)

At 8 weeks, a minimal acute and chronic inflammatory response as well as a foreign body response was demonstrated with no significant differences demonstrated between the three groups. The greatest distribution of fibrous connective tissue was seen in the HA-TCP group after 8 weeks and the least around HA-TCP + PDGF particles. Although no statistically significant differences existed between the HA-TCP + EMD and HA-TCP + PDGF groups, both these groups demonstrated statistically reduced distribution of fibrous connective tissue around the graft particle than the HA-TCP group after 8 weeks. Although a difference in the distribution of fibrous connective tissue was demonstrated, there were no differences between the three groups in the density of the fibrous connective tissue at this time period. A greater distribution of vasculature in the HA-TCP + PDGF group was demonstrated when compared to the other two groups and this was statistically significant when compared to the HA-TCP + EMD group. Similarly, the total vascular area measured was greatest in the HA-TCP + PDGF group and least in the HA-TCP + EMD group with a significant difference demonstrated between the two. Although the distribution and total vasculature area around the graft particle was greater in the HA-TCP + PDGF group when compared to the uncoated HA-TCP group this was not statistically significant. A difference was demonstrated in the extent of adipose tissue distribution as well as the total area of adipose tissue measured. Significantly greater distribution of adipose tissue was seen in the HA-TCP + EMD group when compared to the other two groups which exhibited similar levels of adipose tissue distribution. The total area of adipose tissue measured was also significantly greater in the HA-TCP + EMD group compared to the other two groups and although the PDGF coated group had a greater area of adipose tissue than the uncoated particles, this was not statistically significant. No differences in the thickness of the cellular layer lining the particle were seen between the three groups. In addition, there was no evidence of osteoinduction in any of the three groups.

4 week versus 8 week results (Table 2)

HA-TCP

A significant reduction in the inflammatory and foreign body response was seen from 4 to 8 weeks. A similar distribution of fibrous connective tissue around the implanted particle was seen at the two time periods and although the density of this tissue was reduced at 8 weeks, this was not statistically significant. Similarly, a non-statistically significant reduction in the distribution and total vasculature area as well as adipose tissue surrounding the graft particle was demonstrated at 8 weeks. At this time period, there was also a statistically significant reduction in the thickness of the lining cell layer encapsulating the graft particle.

HA-TCP + PDGF

Similar to the HA-TCP group, a significant reduction was demonstrated in the inflammatory and foreign body response between 4 and 8 weeks. No differences existed in the distribution of the fibrosis connective tissue and although a small increase in the density was demonstrated, this was not statistically significant. Similarly, a small non significant increase in the vasculature distribution was seen but this coincided with a non significant reduction in the total vasculature area measured. In the 8 week samples, there was a significant reduction in the degree of adipose tissue distribution as well as the total amount of adipose tissue measured when compared to the 4 week samples. No difference was seen in the thickness of the lining cell layer encapsulating the graft particle between the two time periods.

HA-TCP + EMD

Similar to the other two groups, a significant reduction in the chronic inflammatory and foreign body reaction scores were demonstrated between 4 and 8 weeks while no significant difference existed for the acute inflammatory score between the two time periods. A significant reduction existed in the distribution of fibrous connective tissue around the graft particle at 8 weeks and

although this was associated with a reduction in the density of these tissues this did not reach statistical significance. A general reduction in the vascular distribution as well as the total vascular area measured existed between 4 and 8 weeks, however this was not statistically significant. A change in the distribution of adipose tissue as well as the total adipose tissue area existed between 4 and 8 weeks with a significant increase in both these parameters measured. In addition, there was a significant reduction in the lining cell thickness between the two time periods.

Table 2: Histomorphometric results of HA-TCP, HA-TCP + PDGF and HA-TCP + EMD at 4 and 8 weeks

AVERAGE VALUES Mean ± SD Median (25th percentile, 75th percentile)	4 WEEK			8 WEEK		
	HA-TCP	HA-TCP + PDGF	HA-TCP + EMD	HA-TCP	HA-TCP + PDGF	HA-TCP + EMD
Number of samples	12	15	15	15	13	15
Acute Inflammation	0.6 ± 0.5 1.0 (0, 1.0) ¶	0.7 ± 0.5 1.0 (0, 1.0) ¶	0.3 ± 0.5 0 (0, 1.0)	0.1 ± 0.3 0 (0, 0) ¶	0.1 ± 0.3 0 (0, 0) ¶	0.1 ± 0.4 0 (0, 0)
Chronic Inflammation ♦	1.8 ± 0.4 2.0 (2.0, 2.0) ¶	1.4 ± 0.5 1.0 (1.0, 2.0) * ¶	1.9 ± 0.5 2.0 (2.0, 2.0) * ¶	0.3 ± 0.5 0 (0, 1.0) ¶	0.8 ± 0.4 1.0 (0.5, 1.0) ¶	0.6 ±0.5 1.0 (0, 1.0) ¶
Resorption/Foreign Body Reaction	0.8 ± 0.4 1.0 (1.0, 1.0) ¶	0.8 ± 0.4 1.0 (1.0, 1.0) ¶	0.7 ± 0.5 1.0 (0, 1.0) ¶	0.1 ± 0.4 0 (0, 0) ¶	0.2 ± 0.4 0 (0, 0.5) ¶	0.1 ± 0.1 0 (0, 0) ¶
Fibrosis Distribution ♦ ♣	3.5 ± 0.5 3.5 (3.0, 4.0)	3.1 ± 1.1 3.0 (2.0, 4.0) *	3.9 ± 0.4 4.0 (4.0, 4.0) * ¶	3.7 ± 0.5 4.0 (3.0, 4.0) ‡ ⊗	3.1 ± 0.9 3.0 (3.0, 4.0) ⊗	3.3 ± 0.5 3.0 (3.0, 4.0) ‡ ¶
Fibrosis Density ♦	3.0 ± 0.6 3.0 (3.0 3.0) †	2.2 ± 0.7 2.0 (2.0, 3.0) * †	3.5 ± 0.7 4.0 (3.0, 4.0) *	2.5 ± 1.0 3.0 (2.0, 3.0)	2.7 ± 0.8 3.0 (2.0, 3.0)	2.9 ± 0.8 3.0 (2.0, 3.0)
Vascularity Distribution ♣	2.1 ± 0.7 2.0 (2.0, 2.8)	1.9 ± 1.1 2.0 (1.0, 3.0)	1.9 ± 0.8 2.0 (1.0, 3.0)	1.7 ± 1.1 2.0 (1.0, 3.0)	2.5 ± 0.8 3.0 (2.0, 3.0) ∫	1.6 ± 0.8 2.0 (1.0, 2.0) ∫
Vascularity (% of total area)	1.4 ± 0.8 1.5 (0.7, 2.1)	2.2 ± 2.1 1.2 (0.5, 4.5)	1.1 ± 1.1 1.0 (0.4, 1.5)	0.9 ± 0.7 1.0 (0.4, 1.7)	1.4 ± 0.9 1.2 (0.7, 2.5) ∫	0.7 ± 0.7 0.6 (0.2, 1.0) ∫
Vascularity Score	1.9 ± 0.9 2.0 (1.0, 3.0)	2.4 ± 1.3 2.0 (1.0, 4.0)	1.5 ± 0.8 1.0 (1.0, 2.0)	1.3 ± 0.8 1.0 (1.0, 2.0)	1.9 ± 0.9 2.0 (1.0, 3.0) ∫	1.1 ± 0.6 1.0 (1.0, 1.0) ∫
Adipose Distribution ♦ ♣	0.7 ± 0.8 0.5 (0, 1.0)	1.5 ± 1.4 1.0 (0, 3.0) * ¶	0.3 ± 0.9 0 (0, 3.0) * ¶	0.4 ± 0.8 0 (0, 1.0) ‡	0.5 ± 0.9 0 (0, 1.0) ∫¶	2.0 ± 1.3 2.0 (1.0, 3.0) ‡ ∫¶
Adipose (% of total area) ♦ ♣	4.2 ± 8.0 0.3 (0, 6.0)	22.4 ± 24.2 10.2 (0, 53.4) * ¶	1.8 ± 5.7 0 (0, 0) * ¶	2.2 ± 7.0 0 (0, 1.4) ‡	4.8 ± 13.3 0 (0, 3.5) ∫¶	17.8 ± 13.9 21.2 (0.4, 31.5) ‡ ∫¶
Adipose Score ♦ ♣	0.8 ± 1.0 0.5 (0, 1.0)	2.0 ± 1.8 2.0 (0, 4.0) * ¶	0.3 ± 0.8 0 (0, 0) * ¶	0.4 ± 0.8 0 (0, 1.0) ‡	0.5 ± 1.1 0 (0, 1.00) ∫¶	2.4 ± 1.6 3.0 (1.0, 4.0) ‡ ∫¶
Capsule Thickness ♦	1.9 ± 0.7 2.0 (1.3, 2.0) † ¶	1.3 ± 0.5 1.0 (1.0, 2.0) †	1.8 ± 0.7 2.00 (1.00, 2.00) ¶	1.3 ± 0.7 1.0 (1.0 1.0) ¶	1.5 ± 0.5 2.0 (1.0, 2.0)	1.3 ± 0.6 1.0 (1.0, 2.0) ¶
Osteoinduction	0	0	0	0	0	0

Key

♦ Kruskal-Wallis Test significant at ($P<0.05$) (4 Weeks)

♣ Kruskal-Wallis Test significant at ($P<0.05$) (8 Weeks)

Mann Whitney Test significant at ($P<0.05$) for 4 week groups

* Significant difference between HA-TCP + PDGF versus HA-TCP + EMD groups

† Significant difference between HA-TCP versus HA-TCP + PDGF groups

Mann Whitney Test significant at ($P<0.05$) for 8 week groups

∫ Significant difference between HA-TCP + PDGF versus HA-TCP + EMD groups

⊗ Significant difference between HA-TCP versus HA-TCP + PDGF groups

‡ Significant difference between HA-TCP versus HA-TCP + EMD groups

Mann Whitney Test significant at ($P<0.05$)

¶ Significant difference between group at 4 and 8 weeks

2.5 Discussion

The aim of this study was to investigate the osteoinductive potential of a commercially available particulate HA-TCP ceramic alone or combined with the growth factors rhPDGF-BB or EMD.

2.5.1 Experimental design

The ectopic bone model allows assessment of the osteoinductive potential of a material as it allows detection of new bone formation in a non-osseous site (Urist, 1965, Winter and Simpson, 1969) with the murine intramuscular implantation model widely utilised (Becker *et al*., 1995, Garraway *et al*., 1998, Yoneda *et al*., 2003, Pekkarinen *et al*., 2005, Ranly *et al*., 2005, Machado *et al*., 2006). A negative control group consisting of mice that had an empty gelatine capsule implanted into the hindlimb was included in the current study. Although a positive control group was not included, the osteoinductive potential of a composite graft of HA-TCP and BMP in the murine hindleg muscle model has previously been demonstrated (Pekkarinen *et al*., 2005).

Previous studies investigating the osteoinductive potential of HA-TCP have reported ectopic bone formation in dogs and pigs approximately six weeks after intramuscular implantation (Yang *et al*., 1996, Yang *et al*., 1997) while subcutaneous implantation in mice and intramuscular implantation in rats has demonstrated bone formation by 90 days post implantation (Yuan *et al*., 2006b). Similarly, the soft tissue implantation of composite grafts of HA-TCP combined with BMP or MSCs into rodents have demonstrated osteoinduction after 8 weeks (Oda *et al*., 1997, Alam *et al*., 2001, Arinzeh *et al*., 2005) suggesting that the implantation period utilised in this study was suitable for the detection of ectopic bone formation.

The outcomes of experiments utilising animal models may be influenced by interanimal variability (Zbinden *et al*., 2007). Variability may exist due to inherent genetic or physiological differences between animals within the investigated groups or may be created due to differences in the macro and microenvironments of the animals prior to, or during the experimental phase as a

result of the interaction between animals or between the animal and it's environment (Howard, 2002).

A limitation of the current study is the small number of mice within each experimental group, with the possibility that the results reported may be due to variation between individual mice rather than true differences or similarities between experimental groups. The inclusion of a greater number of mice in each experimental group would reduce this effect. However, the experimental protocol utilised in the current study was designed to minimise the effect of interanimal variability. Mice were of the same gender and of similar weight prior to the experimental procedure and were selected randomly for inclusion into each experimental group. In addition, all animals were maintained for a conditioning period after arrival and were individually housed in similar conditions and on similar diets.

2.5.2 Radiographic findings

Due to the radiopaque nature of Bone Ceramic®, the presence of radiopacity within the thigh muscle does not confirm the presence of new bone formation. Greatest dispersion was seen in the uncoated particles at both time periods while less dispersion was seen in the composite graft groups. The addition of the liquid rhPDGF-BB to the HA-TCP produced a slurry at the bottom of the capsule, reducing dispersion of the graft material after implantation. In contrast, due to the viscous nature of the PGA carrier of EMD, graft material may have adhered to the side of the capsule after mixing accounting for some of the graft dispersion observed radiographically.

2.5.3 Histological findings

The absence of major adverse effects at the implant site in the three experimental groups during the experimental period suggests that these materials are safe for use.

The voids seen histologically represent the decalcified Bone Ceramic® particles, with a similar appearance as seen in other histological studies (Jensen *et al.*, 2007, Schwarz *et al.*, 2007, Sculean

et al., 2008b, Friedmann *et al*., 2009) and has been described as having a fragmentary, potsherd-like shape (Klein *et al*., 2009).

Specimens from all three experimental groups failed to demonstrate the presence of osteoinduction with a lack of osteoid formation on the surface of the implanted graft particles.

2.5.4 Inflammatory response

An acute inflammatory response arises due to surgical trauma to vascularised connective tissues during graft placement (van der Meulen and Koerten, 1994) and depending on the extent of injury, resolution is rapid with biocompatible materials (Anderson *et al*., 2008). At both time periods, the implants in all three groups were well tolerated and surrounded by a fibrous connective tissue with minimal cellular inflammatory response with a reduction in acute inflammatory cells between 4 and 8 weeks.

Following the acute inflammatory response, a chronic inflammatory response at the implant site is seen with the predominance of mononuclear cells such as lymphocytes and monocytes. All experimental groups demonstrated low levels of lymphocytes and plasma cells with macrophages being the predominant chronic inflammatory cell type. A reduction in the chronic inflammatory cell infiltrate was seen between 4 and 8 weeks which is consistent with a normal healing response. The HA-TCP + PDGF group demonstrated the smallest inflammatory response after 4 weeks although this was only significant when compared to the HA-TCP + EMD group. As wound healing is divided into two phases with a inflammatory phase followed by the reparative phase (Linares, 1996) it is possible that the presence of PDGF may have enhanced the progression of the healing process with earlier resolution of the chronic inflammatory reaction.

It has been suggested that EMD may have an anti-inflammatory effect (Myhre *et al*., 2006, Sato *et al*., 2008). This was not supported in the current study with the greatest distribution of chronic inflammatory cells present in the EMD group at four weeks. However, *in vitro* studies have reported increases in levels of inflammatory mediators such as cyclooxygenase-2 (COX-2) after

treatment with EMD (Takayanagi *et al.*, 2006) while *in vivo*, an increase in inflammatory cells has been reported after the subcutaneous implantation of EMD containing collagen membranes (Yuan *et al.*, 2003).

A minimal foreign body reaction was demonstrated in all groups with only isolated multinucleated foreign body giant cells identified which decreased over time suggesting biocompatibility as well as the low resorbability of the Bone Ceramic® material. These cells may form from fusion of macrophages around the graft material (Behling and Spector, 1986) and may be involved in resorption and phagocytosis of CaP ceramics (Wada *et al.*, 1989, Basle *et al.*, 1993, Heymann *et al.*, 2001, Wenisch *et al.*, 2003).

2.5.5 Encapsulation/Fibrosis

After implantation, the HA-TCP particles were enclosed within a fibroblast rich connective tissue with a cellular layer lining the graft particles, which became less cellular and more fibrous over time. A thinner capsule layer was seen generally in specimens that demonstrated greater adipose tissue deposition. The formation of a fibrous capsule around a biocompatible implant occurs after resolution of the inflammatory response with the migration and proliferation of macrophages, endothelial cells and fibroblasts (Anderson and Miller, 1984, Anderson *et al.*, 2008) resulting in connective tissue deposition which becomes increasingly acellular and avascular over time (Ziats *et al.*, 1988).

Surrounding this capsular layer was a fibrous connective tissue similar to those previously reported (Schwarz *et al.*, 2007) with mature fibroblasts exhibiting a traditional spindle shaped appearance. The increased density and distribution of the fibrous connective tissue observed around the HA-TCP particles in the EMD group at 4 weeks may be explained by an increase in the levels of local TGF-β1 as a result of EMD application. A TGF-β or a TGF-β like molecule has been identified as a bioactive factor in EMD (Kawase *et al.*, 2002, Hama *et al.*, 2008) with this growth factor a potent

growth factor regulating numerous cellular functions including extracellular matrix production (Barrientos *et al*., 2008).

2.5.6 Effect of particle size and resorbability on soft tissue reaction

The 400-700 μm particle size of the Bone Ceramic® may account for the tissue response demonstrated in the current study which is consistent with others reporting a minimal inflammatory reaction and fibrous encapsulation of the particles (Cui *et al*., 1995, Alam *et al*., 2001, Develioglu *et al*., 2005, Fellah *et al*., 2007, Fellah *et al*., 2008). Although CaP ceramics are bioinert, the implantation of small particles (≤20 μm) into non-osseous sites are commonly associated with localised foreign body and inflammatory reactions characterised by the presence of macrophages and multinucleated giant cells (van der Meulen and Koerten, 1994, Dupraz *et al*., 1998, Ooms *et al*., 2003). In rats, implantation of particles with diameters less than 100 μm were associated with the greatest inflammatory reactions up to three weeks post implantation while minimal inflammatory reactions were associated with larger particles of 200-400 μm (Malard *et al*., 1999). Similarly, three weeks after intramuscular implantation of HA-TCP granules into rats, thicker fibrous tissue capsule formation were observed around particles with a diameter of 80-200 μm compared to those <20 μm associated with the greatest inflammatory reaction (Fellah *et al*., 2008). The predominant cell types associated with the smaller particles were multinuclear giant cells and lymphocytes within the interstitial tissue between the HA-TCP particles as well as macrophages in contact with the ceramic particle.

The dissolution properties of the CaP ceramic and duration of implantation may also play a role in the tissue reaction. Dissolution is influenced by the type of CaP ceramic with the extent of dissolution greatest for TCP and least for HA (LeGeros, 1993) as well as by differences in ceramic preparation that modify specific surface area such as sintering temperature (LeGeros, 1993, Laquerriere *et al*., 2003). The high sintering temperature of the Bone Ceramic® at 1100-1500°C results may result in low microporosity and low specific surface area resulting in a low dissolution

rate. A low inflammatory response and minimal resorption of Bone Ceramic® particles have been reported *in vivo* after time periods ranging from nine to ten weeks (Fleckenstein *et al.*, 2006, Schwarz *et al.*, 2007) up to nine months (Jensen *et al.*, 2007, Sculean *et al.*, 2008b).

Conversely, ceramics with greater dissolution properties may enhance the inflammatory response with the presence of macrophages and multinucleated giant cells stimulated by the continued release of degradation products from the graft material. *In vitro*, greater production and release of proinflammatory cytokines such as Il-1β were reported when macrophages were cultured with highly soluble 100% TCP granules compared to less soluble HA-TCP granules (Curran *et al.*, 2005). *In vivo*, nonresorbable, highly crystalline HA granules stimulated fibrous encapsulation when implanted subcutaneously into rats while a resorbable, poorly crystalline CaP apatite generated inflammatory and foreign body reactions (Eid *et al.*, 2001). Similarly, the presence of multinuclear giant cells around a highly soluble HA-TCP ceramic with a high TCP component has been reported after subcutaneous implantation into rats (Oda *et al.*, 1997).

The presence of inflammatory and foreign body cells may also be associated with the length of implantation period. Although macrophages were undetected around a HA ceramic implant one and three months after subcutaneous implantation in rats, the appearance of these cells six months after implantation may suggest material biodegradation resulting in a localised inflammatory reaction (Cui *et al.*, 1995). Similarly, no inflammatory response was reported 30 days after implantation of porous HA-TCP granules with a 65:35 HA:TCP ratio and diameter of 900-1200um (Cerasorb®) into rat cranial defects (Develioglu *et al.*, 2005). After three months, occasional multinucleated giant cells were detected around this material, while a pronounced multinucleated giant cell response was detected at six and 18 months, (Develioglu *et al.*, 2006, Develioglu *et al.*, 2007).

2.5.7 Vascularisation

Neovascularisation and angiogenesis results from a complex series of events involving endothelial cell organisation into immature vessels and association with outer mural cells such as

pericytes and vascular smooth muscle cells (vSMCs) with subsequent matrix deposition and vessel maturation (Yancopoulos *et al*., 2000).

The addition of EMD to HA-TCP did not enhance the angiogenic response. Previous *in vivo* reports have suggested a proangiogenic effect of EMD with significantly greater new blood vessel formation around an EMD loaded collagen membrane after subcutaneous implantation in mice (Yuan *et al*., 2003). Similarly, greater production of fibrovascular granulation tissue after application of EMD to skin wounds in rabbits was seen when compared to the PGA carrier alone (Mirastschijski *et al*., 2004).

Although an overall effect *in vivo* has been demonstrated, recent *in vitro* reports have suggested a stimulatory effect of EMD on the proliferation of human microvascular endothelial cells (HMVECs) (Johnson *et al*., 2009). In contrast, others have suggested that EMD has a mixed or no effect on cell proliferation of human umbilical vein endothelial cells (HUVECs) (Yuan *et al*., 2003, Schlueter *et al*., 2007) although a positive chemotactic effect on these cells were demonstrated at lower EMD doses (Bertl *et al*., 2009).

Although not evident in the current study, EMD may also have an indirect effect on angiogenesis by release of the proangiogenic growth factor, vascular endothelial growth factor (VEGF) (Ferrara and Davis-Smyth, 1997, Nissen *et al*., 1998) from resident cells (Mirastschijski *et al*., 2004).

In contrast, the addition of rhPDGF-BB to the HA-TCP particles resulted in greater vascularity after four weeks, which was significant after 8 weeks. All members of the PDGF family, including PDGF-BB display potent angiogenic activity *in vivo* (Oikawa *et al*., 1994, Cao *et al*., 2002) with PDGF-BB able to induce angiogenesis in the mouse cornea (Cao *et al*., 2002). More recently, the combination of Bone Ceramic® and 0.3mg/ml rhPDGF-BB as a bone graft material in dogs demonstrated greater transglutaminase II antigen reactivity, an enzyme directly involved in the process of angiogenesis (Haroon *et al*., 1999) when compared to sites without the addition of rhPDGF-BB (Schwarz *et al*., 2009),.

PDGF-BB may mediate its proangiogenic effects through its mitogenic and chemotactic effects on pericytes and vSMCs (Westermark and Heldin, 1993, Lindahl *et al*., 1997) or effects on endothelial cells (Risau *et al*., 1992, Castellon *et al*., 2002). PDGF-BB may also exert an angiogenic effect indirectly by enhancing the secretion of other growth factors such as VEGF. *In vitro*, rhPDGF-BB stimulated osteoblast VEGF mRNA transcription in a time and dose dependent manner (Bouletreau *et al*., 2002) while *in vivo*, the application of rhPDGF-BB to localised periodontal osseous defects resulted in an increased concentration of VEGF detected in wound fluid (Cooke *et al*., 2006). Others have suggested a cooperative effect between PDGF-BB and FGF2 (Millette *et al*., 2005) with upregulation of FGF receptor expression by PDGF-BB in human vSMCs (Nissen *et al*., 2007). In the current study, it is possible that the angiogenic effect demonstrated in the PDGF group may be mediated by the direct effect of increased levels of PDGF-BB as well as indirectly via endogenous VEGF and FGF2.

2.5.8 Adipose tissue

Adipose tissue was seen surrounding the HA-TCP particles although this was limited to a few specimens in the HA-TCP group while large amounts of adipose tissue were seen in the PDGF group at 4 weeks and in the EMD group at 8 weeks. The presence of adipose tissue around CaP ceramics has previously been reported after the implantation of macroporous calcium phosphate cement into goats (Bodde *et al*., 2007).

The reason for the large amounts of adipose tissue deposition in some specimens is unknown but may be a result of inadvertent placement or migration of the graft material from the intramuscular pocket subcutaneously. Others have suggested that the presence of adipose tissue around an implant may indicate implant compatibility (Kaminski *et al*., 1977) with the development of adipose tissue only around non-reactive materials after intramuscular implantation into rabbits.

On the other hand, the presence of adipose tissue may have arisen as a result of surgical trauma (Signorini and Campiglio, 1998, Copcu and Sivrioglu, 2003) although formation is more

commonly associated with blunt trauma. Another possible reason for adipose tissue development could be related to the process of "adipogenic healing" (Xaymardan *et al.*, 2002). The authors reported on a sequence of wound healing in which highly vascular granulation tissue matured into adipose tissue instead of fibrous scar tissue. The appearance of adipocytes was demonstrated three weeks after intramuscular implantation of space creating nylon mesh tubes or porous polyvinyl and gelatine sponge material into mice. It was suggested that adipogenic healing occurred as a result of invasion, proliferation and differentiation of reparative granulation tissue into the maintained space by adipocyte precursor cells (Xaymardan *et al.*, 2002).

It is also possible that increased amount of adipose tissue in the PDGF group at 4 weeks may be related to its proangiogenic as well as cell proliferative and differentiation effects on preadipocytes (Butterwith and Goddard, 1991, Bachmeier and Loffler, 1995, Staiger and Loffler, 1998, Widberg *et al.*, 2009). Recent *in vivo* findings suggest that PDGF-BB may enhance adipose tissue formation with the application of 300 ng/ml of PDGF-BB to a murine tissue engineering chamber model significantly increasing adipogenesis after 6 weeks. Increased adipogenesis was associated with increased angiogenesis and infiltration of mesenchymal progenitor cells (Rophael *et al.*, 2007).

The reason for the increased deposition of adipose tissue in the EMD group at 8 weeks remains unknown although there have been reports of the negative effects of EMD on adipogenic differentiation with a marked reduction in lipoprotein lipase gene in pluripotent mesenchymal cells (Ohyama *et al.*, 2002).

2.5.9 Black material

The black material present within the specimens remains unidentified. This material was identified in all groups including the control group suggesting that the source of this material did not originate from the HA-TCP particles. Tissues stained with Perl's and von Kossa's stain did not identify this black material as haemosiderin (Iancu, 1992) or calcium (Symonds, 1990). In addition,

several unstained specimens were analysed, with the presence of this material suggesting that the origin of this material was not a result of the staining process.

The source of this material may be from a contaminant within the gelatine capsule or as a result of the fixation process. The formation of formalin pigment, also known as acid formaldehyde haematin occurs due to the action of formaldehyde on haemoglobin at acid pH (Pizzolato, 1976) resulting in a brown, intracellular and extracellular granular deposit. Although this is commonly associated with tissues that have been fixed in simple formalin fixatives such as 10% formalin or 10% formal saline, the formation of formalin pigment may have occurred with the use of neutral buffered formalin if the buffer was exhausted in the presence of an acidic blood and tissue pH following CO_2 inhalation euthanasia (Rothe, 1983, Angus *et al.*, 2008).

2.5.10 Comparisons to other studies

HA-TCP

HA-TCP associated osteoinduction has been demonstrated after soft tissue implantation in a variety of large and small animal models including rodents (Yuan *et al.*, 1998a, Kurashina *et al.*, 2002, Habibovic *et al.*, 2005b, Le Nihouannen *et al.*, 2005, Yuan *et al.*, 2006b, Habibovic *et al.*, 2008). However, other studies have also reported similar results to the current study with a lack of osteoinduction after implantation of HA-TCP (Oda *et al.*, 1997, Eid *et al.*, 2001, Fellah *et al.*, 2008).

PDGF

This is the first study to report on the osteoinductive potential of rhPDGF-BB and demonstrates a lack of osteoinduction around HA-TCP particles coated with this growth factor. Early studies have suggested that adjunctive PDGF may enhance the osteoinductive activity of demineralised bone matrix (Howes *et al.*, 1988) although recent studies have suggested an inhibitory effect of PDGF-BB on demineralised bone matrix osteoinduction (Ranly *et al.*, 2005). However, the osteoinductive potential of rhPDGF-BB alone was not investigated in this study. A further study by

the same group (Ranly *et al.*, 2007) investigated the role of platelet rich plasma (PRP) containing PDGF on the osteoinductive ability of demineralised bone matrix with the intramuscular implantation of 25 µl of PRP alone into mice failed to demonstrate any osteoinductive activity. The findings of the current study seem to support the findings in the current study that rhPDGF-BB even when combined with a carrier does not provide the osteoinductive stimulus necessary for ectopic bone formation.

EMD

Several *in vitro* studies have reported the presence of BMP or BMP-like molecules in EMD or enamel extracts including BMP-2, BMP-4 and BMP-6 (Iwata *et al.*, 2002, Suzuki *et al.*, 2005, Narukawa *et al.*, 2007). When a pluripotent mouse fibroblastic cell line was cultured with EMD, increased mRNA levels of osteogenic and chondrogenic related transcription factors were detected which were possibly mediated by a BMP-6 like molecule present in EMD (Narukawa *et al.*, 2007). Furthermore, EMD has been suggested to stimulate release of osteoinductive growth factors such as BMP-2 and BMP-4 from wound macrophages (Fujishiro *et al.*, 2008). However, the lack of osteoinductive activity of EMD in the current study supports those previously reported when EMD combined with a collagen carrier (Yoneda *et al.*, 2003) or graft material were implanted into non-osseous sites (Boyan *et al.*, 2000, Donos *et al.*, 2006, Plachokova *et al.*, 2008).

The reasons for a lack of osteoinductive activity in the current study may be attributed to the physicochemical properties of the Bone Ceramic® as well as the methodology used. Material induced osteoinduction appears to be dependent on the presence of certain structural elements such as macroporosity and a microporous surface to create a suitable microenvironment for cell differentiation and new bone formation (Yuan *et al.*, 1998a, Habibovic *et al.*, 2005b, Habibovic *et al.*, 2006a, Habibovic *et al.*, 2006b, Habibovic *et al.*, 2008).

A lack of ectopic bone formation in the current study may be attributed to the physicochemical properties of the Bone Ceramic®. This material has a high interconnected macroporosity with a macropore size of 100-500 μm and a median pore diameter of 200 μm (Klein *et al.*, 2009) which is in the range reported to be ideal for promotion of angiogenesis and osteoblast growth (Hulbert *et al.*, 1970, Klawitter *et al.*, 1976, Tsuruga *et al.*, 1997, Chang *et al.*, 2000). However, the high sintering temperature of Bone Ceramic® at 1100-1500°C may result in low surface microporosity and specific surface area (Kitsugi *et al.*, 1987, LeGeros, 1993) explaining the absence of bone formation in the current study.

The use of a murine model may also account for variability in osteoinduction. Although the combination of CaP ceramics and osteogenic cells or BMP have been demonstrated in the murine model (Oda *et al.*, 1997, Alam *et al.*, 2001, Arinzeh *et al.*, 2005), the degree of CaP ceramic induced osteoinduction can vary depending on the animal model as well as between individual animals (Yang *et al.*, 1996, Habibovic *et al.*, 2005b, Habibovic *et al.*, 2006a). Osteoinduction has been demonstrated regularly in large animal models such as dogs, baboons, sheep and goats (Yamasaki and Sakai, 1992, Ripamonti, 1996, Le Nihouannen *et al.*, 2005, Habibovic *et al.*, 2008) while less consistently in small animal models, including the murine model (Ohgushi *et al.*, 1989, Goshima *et al.*, 1991, Ohgushi *et al.*, 1993, Klein *et al.*, 1994, Yang *et al.*, 1996, Kurashina *et al.*, 2002, Yuan *et al.*, 2006b). This may be related to differences in levels of osteoinductive activity or levels of endogenous proteins such as BMP between species or between individual animals (Ripamonti, 1996, Marusic *et al.*, 1999, Habibovic *et al.*, 2005b) which could affect the level of mesenchymal cell differentiation into an osteogenic lineage.

Variability in the osteoinductive response may also be attributed to the size of the implanted grafts (Habibovic *et al.*, 2006a). Recent studies on osteoinduction have utilised large blocks or cylinders of CaP ceramics compared to particulate graft materials. This may provide a more mechanically stable surface necessary for bone growth (Szmukler-Moncler *et al.*, 1998) as well as provide a greater surface area for cell adhesion and proliferation for bone formation. The use of

smaller or particulate graft materials may explain the lack of osteoinduction seen in the current study due to the reduced surface area or presence of graft micromotion (Yuan *et al.*, 1998a, Yuan *et al.*, 2001, Habibovic *et al.*, 2006a).

The rate of adsorption and release kinetics of the growth factors, EMD and rhPDGF-BB from HA-TCP may have affected the osteoinductive potential. Ideally, adsorption and release of growth factors should allow a sufficient concentration of a biologically active growth factor that mimics concentrations seen during normal wound healing (Winn *et al.*, 1999, Whitaker *et al.*, 2001). Most studies investigating composite grafts of growth factors and CaP ceramics have allowed uptake of the growth factor uptake for up to 72 hours under specific conditions (Laffargue *et al.*, 2000, Alam *et al.*, 2001, Ziegler *et al.*, 2002, Bateman *et al.*, 2005). In addition, greater protein adsorption occurs in ceramics with a higher Ca/P ratio and greater specific surface area (Alam *et al.*, 2001, Zhu *et al.*, 2008). In the current study, the low microporosity of the HA-TCP ceramic and the combination of growth factor with the HA-TCP particles immediately prior to implantation as used clinically may have resulted in insufficient adsorption of the growth factor to the HA-TCP material with an insufficient concentration for bone induction (Oda *et al.*, 1997, Alam *et al.*, 2001).

2.5.11 Conclusions and future directions

The present investigation failed to demonstrate any osteoinductive properties of a commercially available HA-TCP ceramic (Straumann Bone Ceramic®) when implanted alone or combined with rhPDGF-BB or EMD. These studies suggest that neither Straumann Bone Ceramic® nor the addition of EMD or rhPDGF-BB provides the three dimensional scaffold or inductive factors required for differentiation of mesenchymal progenitor cells into an osteoblastic lineage. The soft tissue response to these materials demonstrate that these materials are biocompatible with no adverse reactions reported and by the end of the experimental period the HA-TCP particles were encapsulated by an organised fibrous connective tissue.

As the combination of these growth factors and CaP ceramics are marketed for clinical use for bone regeneration they may have osteopromotive effects. Further research on the osteopromotive effects of EMD and rhPDGF-BB combined with HA-TCP should be undertaken to determine whether these materials enhance the rate and degree of bone formation in critical sized osseous defects.

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