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ORIGINAL ARTICLE

The effect of Emdogain® and platelet-derived growth factor on the osteoinductive potential of hydroxyapatite tricalcium phosphate

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Abstract The aim of this study was to determine whether hydroxyapatite β-tricalcium phosphate (HA-TCP) either alone or coated with Emdogain® (EMD) or recombinant human platelet-derived growth factor-BB (rhPDGF-BB) becomes osteoinductive in the murine thigh muscle model for osteoinduction. Twenty CD1 adult male mice had gelatin capsules implanted into the thigh muscle of both hind limbs. The capsules were either empty or contained one of the following: uncoated particulate HA-TCP, EMD-coated HA-TCP or rhPDGF-BB-coated HA-TCP. The implant sites were assessed histologically at 4 and 8 weeks. A semi-quantitative histological examination was performed to assess the inflammatory changes, reparative processes and osteoinduction within the graft site. At both 4 and 8 weeks, histological analysis failed to demonstrate any osteoinductive activity in any of the specimens from the experimental groups. A minimal chronic inflammatory response and foreign body reaction around the implanted materials was seen which reduced over time. The HA-TCP 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. 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 thigh muscle model of osteoinduction. Coating HA-TCP with EMD or rhPDGF-BB does not enhance its osteoinductive potential.

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Introduction

For many years, autogenous bone has been considered the gold standard for surgical augmentation of osseous defects. However, the amount of bone that can be harvested is limited and are associated with an increased risk of surgical complications and postoperative morbidity [1, 2]. Alternatives such as allografts, alloplasts or xenografts are available, although the latter two lack osteogenic cells or osteoinductive proteins and are, at best, only considered osteoconductive and limited in use to smaller osseous defects.

The biphasic calcium phosphate 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 [3, 4]. While alloplastic materials such as HA-TCP are generally considered to be osteoconductive and not osteoinductive, osteoinductivity of some HA-TCP preparations has been reported after intramuscular or subcutaneous implantation into experimental animals [5–11].

Clinically, the use of an alloplast with both osteoinductive and osteoconductive properties may result in greater and more rapid bone formation in osseous defects when compared to non-osteoinductive or weakly osteoinductive materials as demonstrated in a variety of animal models [8, 10, 12, 13]. Bone regeneration can be achieved through a tissue engineering strategy utilising an osteoconductive alloplast matrix to deliver osteogenic or osteoinductive

agents to the defect site. Porous calcium phosphate ceramics have been proposed as suitable carriers because their surfaces are chemically stable, they have a high adsorption capacity for proteins and provide a good substratum for the attachment of cells with osteogenic potential [14–17]. In addition, the adsorption of biologic agents onto CaP ceramics is rapid, demonstrating biphasic release kinetics both in vivo and in vitro with an initial rapid burst release followed by a sustained release over a longer period [18, 19]. Two commercial products available for use in periodontal regeneration which have significant biological or 'growth factor-like' properties are an enamel matrix protein derivative (EMD) marketed as Emdogain® (BIORA AB, Straumann, Malmö, Sweden) and a product containing recombinant human platelet-derived growth factor-BB (rhPDGF-BB) marketed as GEM 21S® (Osteohealth, Shirley, NY, USA). Both of these products have been combined with HA-TCP and evaluated for use in periodontal and bone regeneration [57–59].

To date, there have been no studies concerning the osteoinductive properties of HA-TCP when combined with EMD (Emdogain®) or rhPDGF-BB. On the basis that EMD influences mesenchymal cell differentiation and that rhPDGF-BB is a potent mitogenic stimulator of osteoblasts, we hypothesise that these biologic agents will have osteoinductive properties when combined with HA-TCP. Therefore, the aim of this study was to evaluate the osteoinductive capacity of HA-TCP alone or in combination with either an EMD or rhPDGF-BB in a murine thigh muscle model of ectopic bone formation.

Materials and methods

Animals

Twenty male CD1 mice of 6 to 8 weeks of age with a minimum weight of 30 g were used in this study (Animal Services Division, Institute of Medical and Veterinary Science, Adelaide, Australia). Ethics approval was granted by the Animal Ethics Committee of both the Institute of Medical and Veterinary Science (IMVS) and the University of Adelaide.

Dispensing 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 and 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 of particulate HA-TCP was placed into 30 gelatin 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 germicidal irradiation for a minimum of 24 h prior to implantation.

Preparation of biologic agents

Recombinant human platelet derived growth factor-BB (rhPDGF-BB) was purchased from PeproTech (Rocky Hill, NJ, USA) and 500 μg 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. This concentration is the same as a commercial preparation of rhPDGF-BB/β-TCP (Gem 21S®—Osteohealth, Shirley, NY, USA) used for periodontal regenerative therapy. The reconstituted sample was prepared immediately prior to the implantation procedure. A commercially available EMD (Emdogain®) preparation (30 mg/ml in a propyl glycol alginate carrier) was purchased from BIORA AB, Straumann (Malmö, Sweden, Lot number: F3752) and was opened at the time of implantation.

Combination of HA-TCP ceramic and biological agents

The biological agent (rhPDGF-BB or EMD) was mixed with HA-TCP within the gelatin capsule for 10 s prior to sealing of the capsule and intramuscular implantation.

Implantation procedure

Surgery was performed using inhalation anaesthesia induced with 2% *v/v* isoflurane with O_2 flow rates set at 2 l/min. Both hindlimbs were secured and the implantation sites were disinfected with alcohol swabs. An incision was made through the full thickness of the skin over the thigh, and an intramuscular pocket was created in the quadriceps muscle using blunt dissection. Sealed gelatin capsules containing the test and control materials were then inserted into the intramuscular pocket and the incision closed with wound autoclips. The implants were placed bilaterally, giving two implants per animal. The animals were given antibiotics for 7 days postoperatively (0.3 mg/ml enrofloxacin, Baytril25®, Bayer AG, Leverkusen, Germany). All animals were weighed and reviewed weekly until the completion of the experiment and were maintained on a normal water and food diet for the experimental period.

Experimental groups

Twenty mice were divided into four groups of five mice with implants placed into the left and right quadriceps

muscles. Table 1 summarises the allocation of graft material and growth factor for each site per group at each time period.

The control group had empty gelatin capsules implanted. For the EMD/HA-TCP implants, 28 μl of 30 mg/ml of EMD was added to capsules containing 10 mg HA-TCP and mixed with the dispensing pipette tip. For the PDGF/HA-TCP implants, 10 μl of 0.3 mg/ml rhPDGF-BB was added to capsules containing 10 mg HA-TCP and mixed with the dispensing pipette tip. These concentrations were based on the mixing ratios recommended by the manufacturers for Emdogain PLUS® (Emdogain®+Bone Ceramic®) and Gem 21S® (rhPDGF-BB+β-TCP) preparations.

Retrieval surgery

Retrieval of the implants was carried out at 4 and 8 weeks. At each of these time intervals, ten animals were euthanised using CO_2 inhalation. The left and right rear limbs were retrieved from each mouse and, after removal of skin and fur, placed into 10% buffered formalin for 3 days. Following fixation, the specimens were rinsed thoroughly in physiological buffered saline and then decalcified in 5% formic acid for 2 weeks with the solution being changed every second day. Completion of decalcification was confirmed by radiography and specimens were then processed for paraffin embedding and histological examination.

Histological evaluation

The specimens were sectioned (7 μm) along the transverse axis of the femur bone and then stained with haematoxylin and eosin, von Kossa and Perl's stain. Images were viewed 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 ×200 magnification were obtained (AnalySIS FIVE, Olympus Australia, Mount Waverly, VIC, Australia) and analysed with a separate computer image analysis program (Image J version 1.41o, National Institutes of Health, USA).

Three sections of the implanted area were examined for each animal. A semi-quantitative histological examination [20] was performed to assess the inflammatory changes, reparative processes and presence of bone formation (Table 2).

Inflammation was recorded as either acute (if a predominantly polymorphonuclear (PMN) leucocyte cell infiltrate was detected around the implanted material) or chronic (if the cell infiltrate consisted predominantly of plasma cells, monocytes/macrophages or lymphocytes). 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 degree of fibrosis around implanted materials and the density of the fibrous network were also included in the histological assessment. For the assessment of the vascular response around the implanted particles, the total vasculature area was measured and recorded as a percentage of the total area examined. The distribution of adipose tissue was determined by the extent of adipose tissue and adipocytes detected around the implanted particles and recorded. The total area of adipose tissue was measured and recorded as a percentage of the total area examined. The thickness of the cell layer encapsulating the particle was measured and recorded. Osteoinduction was reported if matrix resembling the osteoid matrix of woven bone or presence of osteocytes was detected adjacent to implanted materials. The mean and standard deviation for each semi-quantitative histological category were calculated for each experimental group and at each time period.

Statistical analyses

The collected data were analysed using the non-parametric Kruskal–Wallis test to determine any statistical differences between the three experimental groups and the Mann–Whitney test applied for comparisons between any two groups. Statistical analyses were performed using the statistical and graphing package (GraphPad Prism 5.0, GraphPad Software Inc, La Jolla, USA). Values of $P < 0.05$ were considered statistically significant.

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

Group no.	No. of mice	Experimental period (weeks)	Left thigh	Right thigh
1	5	4	Control (gelatin capsule)	HA-TCP+EMD
2	5	8	Control (gelatin capsule)	HA-TCP+EMD
3	5	4	HA-TCP	HA-TCP+rhPDGF-BB
4	5	8	HA-TCP	HA-TCP+rhPDGF-BB

Table 2 Histomorphometric assessment criteria

Acute inflammation
- 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
- 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
- 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—density
- 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—area
- 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—area
- 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
- 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
- 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

Results

Histological evaluation

A qualitative and histomorphometric evaluation was conducted on all specimens. Since the control group showed no histological evidence or reaction to the implanted empty gelatin capsules, this group was not included in any subsequent histological evaluation.

Qualitative analysis of 4-week specimens

The histological appearance of the implant sites for the different treatments after 4 weeks is shown in Fig. 1. These sites were characterised by the presence of irregularly shaped voids representing the decalcified HA-TCP particles. These voids were encapsulated by a dense cellular layer of one to 15 cells resembling metabolically active fibroblasts with plump nuclei and granular cytoplasm. Macrophages and isolated multinucleated giant cells were also seen within this cell layer. Surrounding the cellular layer was a fibrous connective tissue layer consisting of mature spindle-shaped fibroblasts and considerable neovascularisation. The density of the fibrous connective tissue between particles appeared to be greater than that surrounding the total graft area adjacent to muscle. Within the fibrous connective tissue, only a minimal acute inflammatory response was noted. A low level chronic inflammatory response was seen with low numbers of macrophages and lymphocytes identified throughout the surrounding connective tissue while minimal multinucleated cells were seen. In all samples, no osteoblastic activity or bone matrix synthesis was detected and there was no evidence of intramuscular bone or cartilage formation.

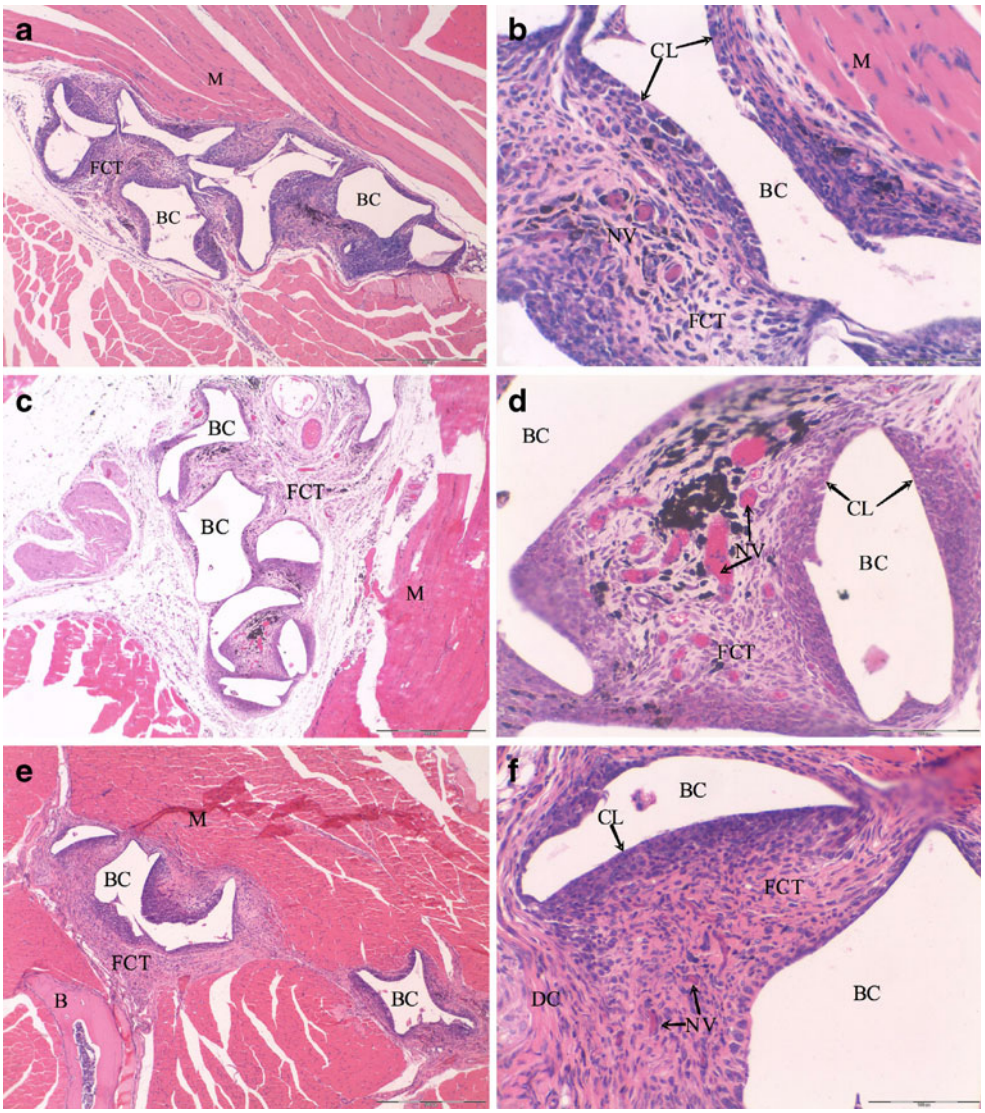

Fig. 1 Photomicrograph of H&E-stained sections of implants retrieved at 4 weeks. **a** HA-TCP group at 4 weeks at ×40 magnification (*bar*=500 μm). **b** HA-TCP group at 4 weeks at ×200 magnification (*bar*=100 μm). **c** HA-TCP+PDGF group at 4 weeks at ×40. **d** HA-TCP+PDGF group at 4 weeks at ×200. **e** HA-TCP+EMD group at 4 weeks at ×40. **f** HA-TCP+EMD group at 4 weeks at ×200. *BC* bone ceramic particle, *CL* cellular capsule layer, *DC* dense collagen, *FCT* fibrous connective tissue, *M* muscle, *NV* neovascularisation

Qualitative analysis of 8-week specimens

The 8-week specimens had a similar appearance to the 4-week specimens with a number of irregularly shaped voids representing the HA-TCP particles (Fig. 2). The cell layer encapsulating these voids 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. The surrounding connective tissue layer was comprised of mature fibroblasts with spindle-shaped nuclei and collagen fibres and was generally denser than the outlying tissue surrounding the total implant site. Vascularisation was evident although this was less than the 4-week samples 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 all samples, no osteoblastic activity or bone matrix synthesis was detected and there was no evidence of intramuscular bone or cartilage formation.

Histomorphometric analysis—4 weeks

The inflammatory response to implantation was similar amongst the three groups with low levels of acute inflammatory cells seen in all groups (Table 3). No differences 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 ($P<0.05$). A minimal foreign body reaction was observed at 4 weeks with no

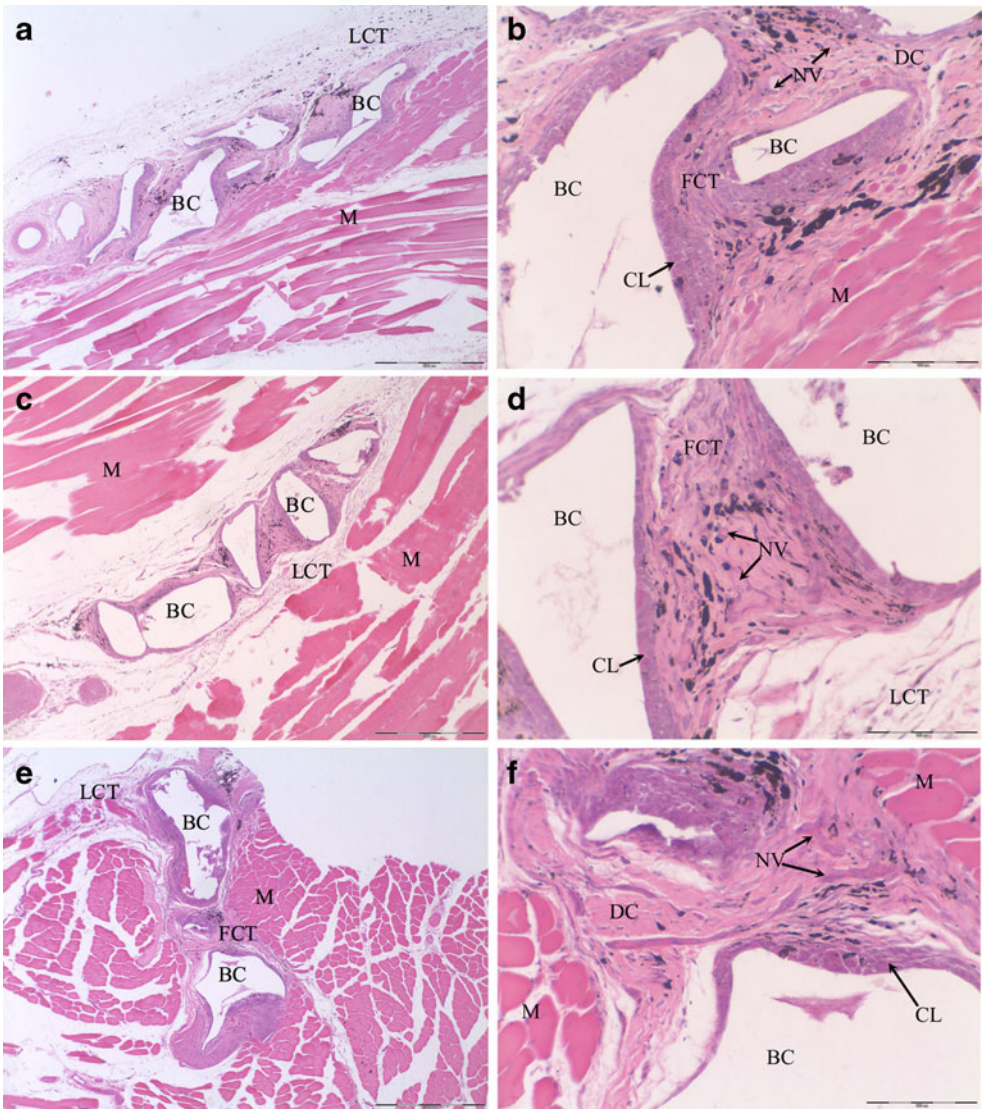

Fig. 2 Photomicrograph of H&E-stained sections of implants retrieved at 8 weeks. **a** HA-TCP group at 8 weeks at ×40 magnification (*bar*=500 μm). **b** HA-TCP group at 8 weeks at ×200 magnification (*bar*=100 μm). **c** HA-TCP+PDGF group at 8 weeks at ×40. **d** HA-TCP+PDGF group at 8 weeks at ×200. **e** HA-TCP+EMD group at 8 weeks at ×40. **f** HA-TCP+EMD group at 8 weeks at ×200. *BC* bone ceramic particle, *CL* cellular capsule layer, *DC* dense collagen, *FCT* fibrous connective tissue, *LCT* loose connective tissue, *M* muscle, *NV* neovascularisation

Table 3 Histomorphometric results of HA-TCP, HA-TCP+PDGF and HA-TCP+EMD at 4 and 8 weeks

Average values (mean ± SD)	4 weeks			8 weeks		
	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 e	0.7±0.5 e	0.3±0.5	0.1±0.3 e	0.1±0.3 e	0.1±0.4
Chronic inflammation*	1.8±0.4 e	1.4±0.5 a, e	1.9±0.5 a, e	0.3±0.5 e	0.8±0.4 e	0.6±0.5 e
Resorption/foreign body reaction	0.8±0.4 e	0.8±0.4 e	0.7±0.5 e	0.1±0.4 e	0.2±0.4 e	0.1±0.1 e
Fibrosis density*	3.0±0.6 b	2.2±0.7 a, b	3.5±0.7 a	2.5±1.0	2.7±0.8	2.9±0.8
Vascularity (% of total area)	1.4±0.8	2.2±2.1	1.1±1.1	0.9±0.7	1.4±0.9 c	0.7±0.7 c
Adipose (% of total area)**	4.2±8.0	22.4±24.2 a, e	1.8±5.7 a, e	2.2±7.0 d	4.8±13.3 c, e	17.8±13.9 c, d, e
Capsule thickness*	1.9±0.7 b, e	1.3±0.5 b	1.8±0.7 e	1.3±0.7 e	1.5±0.5	1.3±0.6 e
Osteoinduction	0	0	0	0	0	0

Mann–Whitney test significant at $P<0.05$ for 4-week groups: (a) significant difference between HA-TCP+PDGF vs HA-TCP+EMD groups, (b) significant difference between HA-TCP vs HA-TCP+PDGF groups; Mann–Whitney test significant at $P<0.05$ for 8-week groups: (c) significant difference between HA-TCP+PDGF vs HA-TCP+EMD groups, (d) significant difference between HA-TCP vs HA-TCP+EMD groups; Mann–Whitney test significant at $P<0.05$: (e) significant difference between groups at 4 and 8 weeks

*$P<0.05$, Kruskal–Wallis test (4 weeks); **$P<0.05$, Kruskal–Wallis test (8 weeks)

difference between the three groups. The fibrous connective tissue response varied amongst the three groups with the greatest reaction demonstrated around the graft particles coated with EMD and was significantly greater than the HA-TCP+PDGF group ($P<0.001$). Although no differences were noted in the vascular distribution between the three groups, there was a tendency toward a greater total vascular area in the HA-TCP+PDGF group when compared to the other groups; however, this was not of statistical significance. Adipose tissue was a more common finding in the HA-TCP+PDGF group when compared to the other two groups. 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 ($P<0.05$). No evidence of osteoinduction was detected in any of the three groups at the 4-week time point.

Histomorphometric analysis of 8-week specimens

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 ($P=0.9465$ (acute inflammation), $P=0.1353$ (chronic inflammation)) (Table 3). The greatest fibrous connective tissue reaction was seen in the group with HA-TCP alone and the least in the HA-TCP+PDGF group. Although there appeared to be a visual difference in the fibrous tissue reaction between the three groups, this was not substantiated when the groups were compared by statistical analyses. A greater total vascularised area was demonstrated in the HA-TCP+PDGF group and the least seen in the HA-TCP+EMD group, and this difference was statistically significant ($P<0.05$). The total area of adipose tissue measured around the ceramic particles was significantly greater in the HA-TCP+EMD group compared to the other two groups ($P<0.01$) (HA-TCP+EMD vs HA-TCP), ($P<0.01$) (HA-TCP+EMD vs HATCP+PDGF). 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 at the 8-week time point.

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 rhPDGF-BB or EMD in a murine ectopic bone formation model. Previous studies investigating the osteoinductive potential of various HA-TCP preparations have reported ectopic bone formation after intramuscular and subcutaneous implantation in mice, dogs and pigs by 6–12 weeks [5, 8, 9, 21, 22]. Similarly, the soft tissue implantation of composite grafts of HA-TCP combined with bone morphogenetic proteins (BMPs) or mesenchymal stem cells into rodents has demonstrated osteoinduction after 3–8 weeks [23–26], suggesting that the implantation period utilised in this study was suitable for the detection of ectopic bone formation.

In the present study, specimens from all three experimental groups failed to demonstrate the presence of osteoinduction, and this is in agreement with a number of other studies which reported a lack of osteoinduction

following implantation of HA-TCP into experimental animals [23, 27, 28].

Early studies have suggested that the presence of PDGF may enhance the osteoinductive activity of demineralised bone matrix by accelerating the growth of the newly formed hard tissue through its mitogenic and chemotactic effects [29]. Recent studies have suggested that PDGF-BB inhibits osteoinduction by demineralised bone matrix [30]; however, the osteoinductive potential of PDGF-BB alone was not assessed. A further study by the same group [71] failed to demonstrate osteoinductive properties of PDGF-BB containing platelet-rich plasma. The findings of the current study support this, with rhPDGF-BB exhibiting no osteoinductive properties.

Several in vitro studies have reported the presence of numerous biologic molecules in EMD or enamel extracts including BMP or BMP-like molecules [31–34]. 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 [33]. Furthermore, EMD has been suggested to stimulate the release of osteoinductive growth factors such as BMP-2 and BMP-4 from wound macrophages [35]. However, the lack of osteoinductive activity of EMD in the current study supports previous findings where EMD combined with various graft materials implanted into non-osseous sites failed to demonstrate osteoinduction [36–39].

The lack of osteoinductive activity of the test materials in the current study may be attributed to the physicochemical properties of the Bone Ceramic® or its ability to adsorb growth factors. Osteoinduction associated with implanted bioscaffolds 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 [10, 12, 40–43]. The HA-TCP used in this study (Straumann Bone Ceramic®) has a high interconnected macroporosity with a macropore size of 100–500 μm and a median pore diameter of 200 μm [44] which is in the range reported to be ideal for promotion of angiogenesis and osteoblast growth [11, 45–47]. However, the high sintering temperature of this material at 1,100–1,500°C may result in low surface microporosity and specific surface area [48, 49] explaining the absence of bone formation in the current study.

The rate of adsorption and release kinetics of the EMD and rhPDGF-BB from the HA-TCP used in this study, while not specifically studied, could have affected the osteoinductive potential. Ideally, adsorption and release of biological agents should mimic concentrations seen during normal wound healing [19, 50]. Most studies investigating composite grafts of biologic agents and CaP ceramics have allowed uptake of the agent for up to 72 h [18, 24, 51, 52]. In addition, greater protein adsorption occurs in ceramics with a higher Ca/P ratio and greater specific surface area [24, 53]. In the current study, the low microporosity of the HA-TCP ceramic and the combination of the biological agent with the HA-TCP particles immediately prior to implantation may have resulted in insufficient adsorption of the growth factor to the HA-TCP material with an insufficient concentration for osteoinduction [23, 24].

Angiogenesis is critical for normal bone healing [60], and the addition of rhPDGF-BB to HA-TCP resulted in a greater vascular response by the end of the experimental period. PDGF-BB displays angiogenic activity in vivo [61], and the combination of 0.3 mg/ml rhPDGF-BB to HA-TCP or a bovine bone mineral xenograft material during guided bone regeneration procedures in dogs demonstrated enhanced angiogenesis when compared to graft material alone [59, 62].

PDGF-BB may enhance angiogenesis by directly effecting pericytes, vSMCs, and endothelial cells [63–66] or indirectly by promoting the production of angiogenic growth factors such as VEGF [67, 68] or FGF2 [69, 70]. It is possible that the angiogenic effects demonstrated in the PDGF group are mediated directly by increased local concentrations of PDGF-BB or indirectly via endogenous VEGF and FGF2. Clinically, this could lead to increased wound neovascularisation and a more effective healing response.

In all specimens including the control group some ‘black material’ was noted including the control group suggesting that the source of this material did not originate from the HA-TCP particles or biological agents used. Tissues stained with Perl’s and von Kossa’s stain did not identify this black material as haemosiderin or calcium (results not shown). In addition, this material was noted in unstained specimens 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 gelatin 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 [54] 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 [55, 56].

In conclusion, 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 the HA-TCP

studied nor the addition of EMD or rhPDGF-BB provided the necessary three-dimensional scaffold or inductive factors required for differentiation of recruited mesenchymal progenitor cells into an osteoblastic lineage. The soft tissue response to these materials demonstrates 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.

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Conflict of interest The authors declare that they have no conflict of interest with the organisation that sponsored this study.

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