Effect of Platelet-Derived Growth Factor-BB on bone formation around dental implants: an experimental study in sheep

A report submitted to the University of Adelaide in partial fulfilment of the requirements of the Degree of Doctor of Clinical Dentistry (Periodontology)

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SUB CRUCE LUMEN

Contents

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Acknowledgements

Chapter 1. Literature review on outcomes of immediate implants and materials or techniques used for bone augmentation around immediate implants

1.1 Introduction

Strategies for implant therapy have previously involved waiting up to nine months post-extraction for implant placement. After placement, the implant was then left submerged and undisturbed for six to nine months for osseointegration to occur, followed by a subsequent surgical procedure prior to prosthetic reconstruction (Brånemark et al., 1977, Adell et al., 1981). This long treatment protocol was often unacceptable for patients and thus, strategies have been explored to reduce treatment time, such as the immediate placement of dental implants into fresh extraction sockets (Schulte et al., 1978, Lazzara, 1989, Becker et al., 1992, Becker et al., 1994c; Becker et al., 1997). Immediate implant placement is a popular treatment approach, particularly for the replacement of anterior teeth. It is defined as the placement of a dental implant into an extraction socket immediately after tooth extraction (Chen et al., 2009a). The main advantages of utilising this approach are to shorten treatment time and to reduce the number of surgical procedures (Lazzara, 1989, Becker et al., 1994c; Schropp and Isidor, 2008). Additionally, the failure rate has been reported to be less than 5%, which appears to be comparable to implants placed with a delayed approach, although most of the reported studies have had relatively short-term observational periods, with an average of one to three years (Fiorellini and Nevins, 2003, Esposito et al., 2006b, Quirynen et al., 2007; Chen and Buser, 2009b). However, although there are no long-term prospective clinical studies reporting on the aesthetic outcomes of immediate implants, short-term reports seem to suggest a high incidence of midfacial marginal soft tissue recession as well as a loss in the height of the interdental papillae (Chen and Buser, 2009b). Recent clinical data indicate that 8 to 20 % of immediate implants may have unsatisfactory aesthetic outcomes, with marginal tissue recession ≥1 mm (Lindeboom et al., 2006; Evans and Chen, 2008).

1.2 Dimensional alveolar ridge alteration following tooth extraction

The dimensional change of the alveolar ridge following tooth extraction was first described in humans in the 1960's. Study casts, subtraction radiography and direct ridge width measurements at re-entry surgery were used to document these changes (Pietrokovski and Massler, 1967, Johnson, 1969, Lekovic et al., 1998, Lekovic et al., 1997, Schropp et al.,

2003b;Iasella et al., 2003). Based on the results from these studies, most of the alterations of the alveolar ridge in both height and width dimensions occur during the first three months following tooth extraction, with bone resorption being more pronounced on the buccal than on the lingual side of the ridge (Pietrokovski and Massler, 1967, Johnson, 1969, Schropp et al., 2003a). The observation that bone resorption occurs to a greater extent on the buccal aspect may be explained by the quality of the buccal bone since buccal bone is predominantly composed of bundle bone (bone that lines the socket wall), which undergoes resorption over the first few weeks following tooth extraction (Araújo and Lindhe, 2005). Thus, the early resorption of the bundle bone may in part be responsible for the marked reduction in height and thickness of the buccal bone relative to the lingual aspect during the first few weeks after tooth loss (Araújo and Lindhe, 2005).

Using subtraction radiography, Schropp and co-workers reported a reduction in the bucco-lingual width dimension of 50% with two thirds of the change occurring by three months following tooth extraction at premolar and molar sites (Schropp et al., 2003a). A concomitant loss in vertical height of the buccal bone of approximately 0.8 mm was also observed at this time. Other clinical studies reported similar findings with an average loss between 0.7 to 2 mm in vertical height, and approximately 3 to 6 mm reduction in the horizontal dimension over a four to six month observational period following tooth loss (Lekovic et al., 1998, Lekovic et al., 1997, Iasella et al., 2003). Nonetheless, there are a variety of systemic and local factors that may influence the extent of reduction of the alveolar ridge following tooth extraction (Chen et al., 2004). Systemic factors may include the patient's general health and habits such as smoking, and local factors includes aspects such as the condition of the socket walls before and after tooth extraction, thickness of the buccal bone wall, tissue thickness, and the presence of local infection (Chen et al., 2004; Chen and Buser, 2009b).

1.3 What are the aesthetic outcomes of immediate implants?

Aesthetics in implant dentistry is a difficult area to evaluate as several different methods or indices exist. Aesthetics has been evaluated by patients using questionnaires (Kan et al., 2003; Chen et al., 2007), or by measuring a change in probing depths and attachment levels to determine the amount of recession of the mucosa and papillae. More recently, a number of scoring indices have been used to assess the aesthetic outcomes of implant therapy. These include the subjective esthetic score (SES) (Evans and Chen, 2008), white esthetic score

(WES) (Buser *et al* 2009; Belser *et al* 2009), pink esthetic score (PES) (Furhauser et al., 2005), or the papilla index score (Jemt, 1997). The PES uses seven variables to assess the soft tissues in relation to a reference tooth with scores of 0, 1, or 2 designated for each variable, with a maximum score of 14 meaning the best possible soft tissue aesthetic result (Furhauser et al., 2005). The SES uses a scoring system from 1 to 4, with 4 being the worst result in terms of a change in vertical height of the facial soft tissue margin as well as the facial soft tissue contour (Evans and Chen, 2008). The WES assigns a score from 0 to 2 for five parameters based on the implant restoration shape and dimensions (Buser *et al* 2009; Belser *et al* 2009). The papilla index score as described by Jemt (1997) is the most frequently used index to describe the form of the papilla. Scores of 1 to 3 are given based on the percentage of papilla fill, with a score of 3 meaning complete fill of the embrasure. Each method has its own advantages and disadvantages, with no consensus as to which method is superior, as well as who should perform the evaluation to provide the most reliable data. However, the method selected is important because a large variation in results can be reported when different approaches are employed. For instance, when two aesthetic indices were used to evaluate the results of a retrospective study of 85 patients receiving immediate implants (Chen et al., 2009), the SES system revealed 9.4% of sites with an unsatisfactory result (scores 3 and 4), while the PES showed 22% of patients with a suboptimal result (8 or 9). In addition, when patients who had received immediate implant treatment were questioned on whether they were satisfied with the result, 90% of patients said they were happy (Chen et al., 2007), whereas when the clinician performed the evaluation, it was reported that a good aesthetic outcome was achieved in only 66% of the cases. More recently, a combination of the PES/WES index has been demonstrated to be suitable for evaluating anterior single-tooth implant aesthetics (Buser *et al* 2009; Belser *et al* 2009), however, further trials are required to validate the clinical usefulness of the combination index (Belser *et al* 2009).

There are few long-term studies reporting on the aesthetic outcomes of anterior single tooth immediate implants. Most clinical studies have been short-term with a follow up of one to two years and with a small number of subjects (Chen and Buser, 2009b). A previous systematic review suggested that immediate implants may have good aesthetic outcomes based on the results of only two randomized controlled clinical trials with a small number of subjects involved (RCTs) (Esposito et al., 2006b). However, a recent review based on 21 studies reporting on the aesthetic outcomes of single tooth immediate implants placed in the anterior maxilla reported that recession of the midfacial buccal mucosa ≥1 mm and loss of papilla height developed in a high proportion of patients within the first year following

implant placement (Chen and Buser, 2009b). Furthermore, some studies have shown that a percentage of patients are at risk of developing significant recession >1 mm, which is visually detectable and may be considered a disappointing aesthetic outcome. A prospective case series which documented the aesthetic outcomes of 12 patients with 14 immediate implants placed reported about 21% of sites had midfacial soft tissue recession between 1 to 2 mm after one year (Juodzbalys and Wang, 2007). A retrospective case series of 42 patients with immediate implants followed for an average period of 1.5 years reported that although the mean recession in the midfacial mucosa was 0.9±0.78 mm, 19% of patients developed recession in the midfacial mucosa ≥1.5 mm (Evans and Chen, 2008).

1.4 Can immediate implants prevent alterations in the dimension of the alveolar ridge after tooth extraction?

In aesthetic implant dentistry, patients desire an imperceptible, natural-looking result. However, in the anterior zone, the loss of a tooth which results in alterations to bone and soft tissue dimensions makes it difficult or almost impossible to achieve such a result. However, for decades, clinicians have attempted to find a method to preserve the hard and soft tissues dimensions following tooth loss.

It was previously believed that immediate placement of implants could preserve the hard tissue architecture and dimensions of the extraction socket (Watzek et al., 1995). However, recent studies have shown that immediate implant placement failed to prevent bone modelling, and bone loss was observed to occur in both the vertical and horizontal dimensions following tooth extraction {Schropp, 2003; Botticelli et al., 2004a; Covani et al., 2004; Araújo et al., 2006). During a four month period following immediate implant placement in fresh extraction sockets in dogs, a marked reduction in width of both the buccal and lingual bone walls was observed (Botticelli et al., 2004b;Araújo et al., 2006). Resorption occurred to a greater extent on the buccal aspect with more than 50% reduction, while the lingual/palatal bone wall showed a loss of about 30% (Botticelli et al., 2004b; Araújo et al., 2006). Similar observations were described in a series of prospective clinical studies (Botticelli et al., 2004a; Covani et al., 2003; Covani et al., 2004; Covani et al., 2007). After six months of submerged healing, 10 patients receiving 15 immediate implants showed a mean loss of 3 mm or a 30% reduction in the horizontal width of the alveolar ridge (Covani et al., 2003; Covani et al., 2004). The mean loss in crestal height of the facial bone was reported in a subsequent study, being an average of 0.8 mm (Covani et al., 2007). However, no change was detected in 38%

of sites, while a loss of up to 1 mm developed in 50% of sites, and a loss up to 2 mm occurred in 15% of sites at 6 months of healing.

1.5 Can bone grafting around immediate implants compensate for crestal bone resorption following tooth loss?

Although based on a limited number of short-term studies, two comprehensive reviews have found that bone augmentation may be effective at achieving bone fill and maintaining soft tissue levels more coronally around immediate implants (Chen and Buser, 2009b;Esposito et al., 2006a). A small randomised controlled clinical trial reported that when bone graft material was used to augment around immediate implants, the mean buccal mucosal margin level was found to be 1 mm more coronal to the implant shoulder after six months compared to sites where no bone grafting material was used (Cornelini et al., 2004a). A recent prospective study performed bone grafting around immediate implants when a horizontal gap greater than 2 mm was present and observed that the midbuccal soft tissues underwent minimal recession at six months (van Kesteren et al., 2010). However, several studies have demonstrated that although bone augmentation can reduce the extent of resorption in the horizontal dimension (Chen et al., 2005; Covani et al., 2007; Botticelli et al., 2004a, Chen et al., 2007), it may not predictably reduce the loss in vertical height of the facial bone (Chen et al., 2007; Chen et al., 2005; Chen et al., 2009; Gher et al., 1994). A six month prospective controlled clinical trial demonstrated loss in vertical height of the facial bone (1 to 1.5 mm) in the maxillary anterior and premolar regions even though bone augmentation was performed around immediate implants, however, the sites that received augmentation showed less resorption in the thickness of the facial bone (14 to 24%) compared to sites that were not augmented which lost 50% in the thickness of the facial bone (Chen et al., 2007).

1.6 When should bone augmentation be performed around immediate implants?

When implants are placed immediately after tooth extraction, there is often a gap between the socket wall and the coronal neck of the implant due to an incongruity in the dimensions between the tooth socket and implant. As a result, several studies were performed to investigate the effect of various gap sizes around implants to determine when complete

bone healing could occur without the need for bone augmentative procedures (Schmitz & Hollinger 1986). This has been referred to as a "critical size" defect.

Most studies seem to agree that marginal defects (with all bony walls intact) less than 2 mm wide may resolve spontaneously without any need for bone augmentation (Hämmerle et al., 2002, Botticelli et al., 2003b, Chen et al., 2004, Jung et al., 2007, Paolantonio et al., 2001, Covani et al., 2003). However, in gaps wider than 2 mm, bone augmentation has been recommended (Chen et al., 2007; Polyzois et al., 2007; Cornelini et al., 2004a, Chen and Buser, 2009b). No difference in bone healing around immediate implants with marginal defects ≤2 mm wide was observed compared to implants placed in healed sites with no defects in 48 patients (Paolantonio et al., 2001). Re-entry surgery performed after six months revealed complete bone fill in all of the defects, which was confirmed histologically with direct bone to implant contact at previously exposed implant surfaces. Similarly, a prospective case study of 10 patients observed complete healing of the residual defects around immediate implants when the marginal gap present was ≤2 mm wide (Covani et al., 2003). In contrast, when marginal defects surrounding immediate implants were greater than 2 mm, most of the defects failed to resolve with complete bone fill (Botticelli et al., 2004a). A prospective clinical study on 18 patients with 21 immediate implants showed complete resolution of defects in only 22% of sites with initial marginal gaps between 2 to 3 mm wide, compared to 78% of sites with marginal defects less than 2 mm wide (Botticelli et al., 2004a).

Defect depth has also been shown to have an influence on bone healing in peri- implant defects (Schropp et al., 2003a, Yoon et al., 2008). In a surgically created defect model where coronal bony defects were prepared in the mandible of dogs at two depths of 2.5 mm and 5 mm, sites with the 5 mm deep defect exhibited almost 30% less bone to implant integration compared to the 2.5 mm deep defects after eight and 12 weeks of healing (Yoon et al., 2008). Furthermore, after 12 weeks of healing, complete bone healing was not observed in the 5 mm deep defects while the 2.5 mm deep defects exhibited complete healing. Similar results were observed in a prospective clinical study, where about 30% of sites with a defect depth up to 4 mm failed to heal completely after three months (Schropp et al., 2003a).

1.7 What grafting materials or techniques are available for bone augmentation and are they effective at promoting new bone formation around immediate implants?

Many bone grafting materials, in combination with or without membranes, have been used successfully to achieve bone fill in defects around immediate implants (Schwartz-Arad and Chaushu, 1997, Becker, 2003; McAllister and Haghighat, 2007; Berglundh and Lindhe, 1997, Chen et al., 2005; Chen et al., 2007; Chen and Buser, 2009b). These include autogenous bone grafts, allografts, xenografts and alloplasts. The biologic basis for using these materials for bone grafting involves three processes: osteogenesis, osteoconduction, and osteoinduction (Lang et al., 1998). Osteogenesis occurs when vital bone forming cells such as osteoblasts and precursor osteoblasts are transplanted to a defect site where they may form new bone; osteoconduction occurs when a material provides space to serve as a scaffold for the ingrowth of precursor osteoblasts into the defect; and osteoinduction involves converting pluripotent, mesenchymal-derived cells into bone forming cells with the subsequent formation of bone (Lang et al., 1998).

The perfect bone grafting material has yet to be identified, as each type appear to have its own advantages and disadvantages for use in different clinical situations such as maxillary sinus augmentation, horizontal and vertical ridge augmentation, ridge preservation and around immediate implants (Darby et al., 2009; Jensen and Terheyden, 2009; Chiapasco and Zaniboni, 2009). In addition, the material chosen may be due to operator preference, substitution rate and what is commercially available (Darby *et al* 2009). Each type of bone graft will be considered, as well as the clinical evidence to support its use in bone augmentation, particularly in marginal defects surrounding immediate implants.

1.7.1 Guided bone regeneration (GBR)

The GBR technique is based on the principles of guided tissue regeneration and was proposed in order to create space around the bony defect to allow bone forming cells to populate and form bone without interference from other tissue cells (Dahlin et al., 1988, Dahlin et al., 1989, Becker and Becker, 1990, Dahlin et al., 1991) .

The use of GBR in treating osseous defects around implants has been established since the early 1990s, as a clinically successful and well-documented procedure performed either

alone or in combination with bone grafts (Becker and Becker, 1990, Dahlin et al., 1991; Simion et al., 1994). A small clinical trial involving 40 patients with denuded implant surfaces reported 95% bone fill in defects grafted with autograft/membrane after six months of healing compared to 60% fill in sites receiving autograft only (Schlegel et al., 1998). However, almost 33% of the sites with the membrane became exposed and required premature removal (Schlegel et al., 1998).

Several prospective clinical studies have observed almost complete defect fill when a non-resorbable (e-PTFE) membrane was used in small defects around immediate implants and when no membrane exposure occurred (Becker et al., 1994c; Lang et al., 1994). Less resorption of the buccal plate has also been reported when e-PTFE membranes were used around immediate implants, compared to sites grafted with autografts only after six months, in a clinical trial involving 62 patients receiving immediate implants in the anterior maxilla and premolar sites (Chen et al., 2005). However, the main disadvantage of non-resorbable membrane types is the predisposition for wound dehiscence and exposure of the membrane leading to the need for early membrane removal and thus resulting in poorer treatment outcomes in terms of bone fill (Zitzmann et al., 1997, Becker et al., 1994c). As a result, certain types of resorbable membranes (e.g. BioGide) that have less risk of premature membrane exposure were developed in order to provide a good alternative to non-resorbable membranes and are also easier to manage when they become exposed. However, because these membranes are soft, they have a tendency to collapse into the defect unless they are supported by the addition of bone grafting materials or bone substitutes which maintain the space for bone regeneration. The use of membrane-supporting materials has been recommended whenever resorbable membranes are used and good clinical outcomes have been achieved with such an approach (Zitzmann et al., 1997, Cornelini et al., 2004a, Chen et al., 2007). One clinical study involving 18 implants with dehiscence/fenestration type defects demonstrated that when a bone graft was combined with a GBR procedure, both resorbable and non-resorbable membranes were successful at achieving an average of 94% bone fill after seven months of healing (Simion et al., 1997).

1.7.2 Autogenous bone grafts

Autogenous bone grafts are bone grafts taken from the same person and are regarded as the "gold standard" for bone augmentation, as they remain the only bone graft to contain osteoinductive proteins, osteoblasts and osteoprogenitor cells, which are effective in

stimulating new bone formation (Buser et al., 1998, Jensen et al., 2007; Hallman and Thor, 2008). Additionally, they are biocompatible and the graft particles are considered osteoconductive as they provide a three-dimensional scaffold into which new bone may grow. In fresh autogenous bone grafts, several growth factors have been detected, including members of the transforming growth factor-β superfamily (bone morphogenetic proteins), angiogenic factors (vascular endothelial growth factor, fibroblast growth factor), platelet-derived growth factor, and insulin growth factor I, which have chemotactic and mitogenic effects on cells (Schmidmaier et al., 2006). Therefore, based on these properties, defects grafted with autogenous bone show the fastest rate of new bone formation compared to other types of bone graft or substitutes (Buser et al., 1998). It was demonstrated that at four weeks of healing, autografts showed the greatest percentage of newly formed bone with 87% of the surface of the graft particles already covered with bone compared to alternative bone graft materials when used to fill large contained defects in the mandible of pigs (Buser et al., 1998). Similar findings were reported in a clinical study when autografts were compared to an allograft material (DFDBA) for augmenting extraction sockets (Becker et al., 1994a). Biopsies after several months of healing revealed a greater amount and maturity of new bone formed within a shorter healing time at sites augmented with the autogenous grafts (Becker et al., 1994a). However, the main disadvantages of using autografts are donor site morbidity, limited volume from intraoral sites and an increase in surgical time to harvest the grafts, particularly if extra-oral grafts are required (Moy et al., 1993).

Autogenous grafts can be harvested from intraoral or extraoral sites. The site chosen depends on the amount of bone required for augmentation. When large quantities of bone are required, extraoral sites such as the iliac crest, tibia or skull are common areas, however, when sites requiring augmentation are small, intraoral grafts are preferred as it causes significantly less discomfort and morbidity to the patient. A popular approach to collect sufficient quantity of autogenous bone intraorally is with the use of bone collectors (bone traps). These comprise of filters placed in a suction device to collect bone particles/debris during implant osteotomy preparation without causing additional discomfort to the patient (Blay et al., 2003). A small clinical study demonstrated that bone collected with a bone collector during drilling of osteotomy sites for implant placement was capable of achieving bone fill in small defects such as fenestrations and dehiscences around implants (Blay et al., 2003). However, the use of particulate autogenous bone collected from intraoral sites show faster resorption due to the smaller graft particles (Springer et al., 2004a, Springer et al., 2004b). In addition, several studies and reviews have indicated that bacterial contamination of

the bone collected with a large number of microorganisms always occurred even when strict protocols such as pre-operative chlorhexidine rinse, antibiotic prophylaxis, and the use of a dedicated suction reserved only for aspiration of bone and coagulum at the surgical site were followed (Blay et al., 2003; Etcheson et al., 2007; Esposito et al., 2006a, Tezulas and Dilek, 2008; Graziani et al., 2007). Thus, the value in using these graft particles has been questioned due to the potential for the implant surface to be contaminated from the graft particles which may compromise implant treatment outcomes (Esposito et al., 2006a, Tezulas and Dilek, 2008; Graziani et al., 2007). Nonetheless, good clinical outcomes have been reported with the use of autogenous bone particulate at peri-implant defects (Becker et al., 1994b;Blay et al., 2003; Schlegel et al., 1998, Simion et al., 1997, Chen et al., 2005). A clinical trial involving 54 implants in 30 patients reported almost complete defect fill of defects surrounding implants during second-stage surgery when autogenous bone chips harvested intraorally were used to fill the defects after implant placement (Becker et al., 1994b). In addition, other studies have reported an average bone fill of 95% when implants with dehiscence/fenestration type defects were filled with autograft and a non-resorbable membrane after six months of healing (Schlegel et al., 1998, Simion et al., 1997).

Furthermore, the technique to collect and process particulate bone grafts has been shown to affect cell vitality, and thus the osteogenic potential of the graft. An *in vitro* study demonstrated that bone milling can reduce the vitality of osteoblasts in cancellous bone, but milling cortical bone resulted in the particles being more osteoconductive (Springer et al., 2004b). Nonetheless, the application of different techniques to preserve cell vitality in particulate bone grafts may be irrelevant considering similar results in the amount of new bone formation were reported (Coradazzi et al., 2007).

An alternative bone graft or substitute with a slower resorption rate to autogenous bone has been suggested to be more useful to compensate for the socket modelling changes following tooth loss when augmentation is required around immediate implants (Chen et al., 2007). A prospective clinical study involving 62 patients reported significant resorption of the labial plate at six months even though autogenous grafts with or without a membrane were used to graft around immediate implants (Chen et al., 2005). However, in a subsequent prospective study performed by the same authors, the use of an anorganic bovine bone material to graft around immediate implant sites showed a reduction in the horizontal dimension of 25% compared to 50% loss in width dimension at implant sites when no grafting material were used (Chen et al., 2007).

1.7.3 Allografts

Allografts are bone grafts harvested from cadavers and processed by methods such as freezing or demineralising and freezing to remove all viable cells, which reduces the risks for immune rejection. Thus, they have been termed demineralised freeze-dried bone allograft (DFDBA), and freeze-dried bone allograft (FDBA). These materials are supplied in particulate or block form. The advantages of these grafts compared to autografts are availability in large quantities, and elimination of the need for a donor site. However, disadvantages include unpredictable or poor bone formation with some preparations of commercially available DFDBA, which may be due to the age of the person or the site where the graft was acquired from (Shigeyama et al., 1995; Becker et al., 1995), and the risk for disease transmission of human immunodeficiency virus (HIV). Although the risk is minimal, concern still exists for some patients regarding their absolute non-infectivity since several reports using different methods to freeze and freeze-dry contaminated bone specimens failed to eliminate the virus (Buck et al., 1990, Marthy and Richter, 1998) with suggestions that the best protection from the virus remains with strict donor selection criteria (Buck et al., 1990, Marthy and Richter, 1998).

Allografts appear to be effective for use in sinus augmentation (Valentini and Abensur, 1997, Whittaker et al., 1989, Cammack 2nd et al., 2005), localised ridge augmentation (Cochran and Douglas, 1993; Cammack 2nd et al., 2005) and ridge preservation procedures (Iasella et al., 2003; van Kesteren et al., 2010). However, the bone quality of sites augmented with allografts may be inferior and the resorption rate of the graft particles may be slower than with autogenous grafts as it contains no viable cells (Becker et al., 1994a, Iasella et al., 2003). When compared to autogenous bone graft for socket preservation, DFDBA showed minimal new bone formation on the graft particles, and minimal resorption of the particles after three to 13 months in seven patients (Becker et al., 1994a). A six month clinical study involving 24 patients demonstrated that the use of FDBA for ridge augmentation maintained some of the width of the alveolar ridge and vertical height dimensions, but histological examination of the grafted sites revealed an inferior bone composition of 28% new bone and 37% residual FDBA fragments, compared to 54% new bone at non-augmented sites (Iasella et al., 2003). However, another study analysed biopsies from 93 patients who received maxillary sinus or ridge augmentation grafts of demineralized freeze-dried bone allograft (DFDBA) or freeze-dried bone allograft (FDBA), and reported on average 42% new bone for either grafts at six to 36 months of healing (Cammack 2nd et al., 2005). When used around immediate

implants in a clinical study involving 36 patients, DFDBA showed greater bone fill and higher frequency of sites with complete resolution of osseous defects at six months post-surgery compared to immediate implant sites left empty (Gher et al., 1994). A more recent prospective clinical trial reported minimal recession in the midbuccal soft tissue position when DFDBA was used to fill horizontal defects greater than 2 mm around immediate implants at six months (van Kesteren et al., 2010).

1.7.4 Xenografts

Xenografts are bone grafts derived from a different species from the recipient. Deproteinised bovine bone (DBB) is the most researched xenograft material and is widely used for bone augmentation because it has a structure similar to human bone. Deproteinised bone means that all proteins in it have been extracted to avoid immune rejection. However, as this procedure eliminates the osteoinductive ability, DBB acts solely as an osteoconductive scaffold (Berglundh and Lindhe, 1997, Jensen et al., 2009; Abushahba et al., 2008; Polyzois et al., 2007). A commercially available DBB product, Bio-Oss® (Geistlich AG, Wolhusen, Switzerland) has been extensively studied for a wide range of applications including sinus floor augmentation, socket and ridge preservation, dehiscence or fenestration defects around implants (Hämmerle et al., 1998, Hämmerle and Lang, 2001; Zitzmann et al., 1997, Chen et al., 2007; Chen et al., 2009).

Concern has been raised with the use of DBB due to the risks of disease transmission from cattle to humans with the occurrence of bovine spongiform encephalopathy (BSE) and Creutzfeldt Jakob Disease (CJD). However, the risks of transmitting diseases through the use of such materials from a commercial product such as Bio-Oss® (Geistlich AG, Wolhusen, Switzerland) appear to be low due to the application of stringent protocols in sourcing and processing of the bovine bone (Sogal and Tofe, 1999, Wenz et al., 2001).

DBB is considered a relatively non-resorbable grafting material (Hallman et al., 2001; Hallman et al., 2002; Schlegel and Donath, 1998, Yildirim et al., 2000; Yildirim et al., 2001). From a biological perspective, resorption requires adhesion molecules (arginine–glycine–asparagine sequences) for osteoclasts to attach to plasma and extracellular matrix proteins (Pierschbacher and Ruoslahti, 1984), but since DBB has been deproteinised and is free of proteins, resorption of the graft particles appears to be difficult. Studies examining biopsies harvested from humans after 3 years (Hallman et al., 2001) and 9 years (Traini et al., 2007)

reported DBB particles to be in close contact with giant cells, but without exhibiting signs of resorption.

The use of DBB may be associated with a risk for fibrous encapsulation of the graft particles. It has been reported that implants inserted into jaw defects in dogs that were augmented with DBB showed limited osseointegration after several months of healing (Carmagnola et al., 2000). Fibrous encapsulation of the graft particles was observed resulting in limited direct contact between the graft particles with new bone. In contrast, it has been reported that bovine graft particles are surrounded by new bone with a bone density similar to non-grafted sites after seven months of healing in jaw defects created in dogs (Berglundh and Lindhe, 1997) and more recent animal experiments have reported similar observations (Abushahba et al., 2008; Polyzois et al., 2007).

The rate of bone healing at sites augmented with DBB appears to be slower than with the use of autografts, but at a similar rate when compared to bone substitutes. Less mature bone, less graft to bone contact and less newly formed bone was observed at four weeks when DBB was used to compare against particulate autografts to fill large defects in the mandible of minipigs (Jensen et al., 2009). However, in comparison to bone substitutes such as biphasic calcium phosphates with a high hydroxyapatite content, similar amounts of new bone formation has been observed although the DBB graft particles displayed higher fractions in contact with newly formed bone.

Although autogenous bone is considered the ideal grafting material as it results in the fastest rate of healing in bony defects, DBB has been considered a suitable alternative, particularly for use around immediate implants (Esposito et al., 2008). The rationale for the use of DBB relates to its non-resorbable property, as autografts appear to resorb too rapidly to compensate for the changes in socket dimensions following tooth loss. The use of DBB appears to be clinically relevant in the anterior zone, particularly when aesthetics are a concern. Results from prospective clinical studies have suggested that the changes in socket dimensions as a result of post-extraction socket modelling may be modified in the presence of a graft material with a slow resorption rate such as DBB (Cornelini et al., 2004b;Chen et al., 2007). When DBB was used to graft around immediate implant sites, the extent of horizontal resorption was reduced to 25% of the original buccal dimension (Chen et al., 2007). In comparison, sites that received autogenous bone or no grafting material observed a 50% reduction in the width of the buccal bone (Botticelli et al., 2004a, Chen et al., 2005; Chen et

al., 2007). A recent Cochrane Systematic Review, although based on a limited number of studies, concluded that immediate implant sites treated with DBB with a membrane may result in a better position of the soft tissue margin compared to barrier membranes alone (Esposito et al., 2008).

1.7.5 Alloplasts

Most alloplastic materials consist of hydroxyapatite (HA), tricalcium phosphate (TCP), or biphasic calcium phosphate (a mixture of HA/TCP). Calcium phosphates are popular materials for filling bone defects since the composition closely resembles the inorganic phase of bone (Buser et al., 1998). These synthetic substitutes have been used for over 25 years in orthopaedic and dental applications as they are biocompatible and have no risk of disease transmission. Alloplasts are considered osteoconductive and not osteoinductive materials, as they work simply by providing a physical scaffold for bone ingrowth (Gatti et al., 1990, Buser et al., 1998, Jensen et al., 2006; Jensen et al., 2007; Jensen et al., 2009).

Calcium phosphate in the form of tricalcium phosphate (TCP) can be sintered into a uniform material, resulting in α or β-tricalcium phosphate, which is a purified, multicrystalline, and porous form of calcium phosphate, similar to natural bone mineral (Szabó et al., 2001). These materials dissolve in surrounding tissues due to a higher pH, with α-TCP being more soluble and having a higher and faster resorption rate than β-TCP. The resorption of β-TCP has also shown to be fairly rapid with almost complete resorption by eight weeks (Jensen et al., 2007), which may make this material useful in augmenting small localised bony defects by allowing for complete substitution of the particles with new bone (Buser et al., 1998, Jensen et al., 2007).

The exact mechanism of how β-TCP allows bone formation is still debated, but it appears to be based on a chemical reaction with the surrounding tissues which results in the disintegration of the material. Upon dissolution of β-TCP, calcium and phosphate ions are released into the surrounding area promoting osteogenesis (Gatti et al., 1990, Kamitakahara et al., 2008; Jensen et al., 2007; Araujo et al., 2010). A cell-mediated reaction involving macrophages or osteoclasts that actively phagocytise the dissolved particles then follows (Jensen et al., 2007).

The pore size is an important characteristic of β-TCP which determines whether these materials form bone. Pore sizes greater than 100 μm have shown to enhance the formation of new capillaries and bone as it allows for ingrowth and attachment of these cells, while a smaller pore size may not permit cell or capillary invasion and thus, may not allow bone formation to occur (Jensen et al., 2007). Several studies in large animal models have demonstrated that similar amounts of new bone formation could be achieved with β-TCP with a pore size between 200 to 400 μm, in comparison to autografts with almost 40% new bone fill achieved after four weeks of healing in contained defects (Jensen et al., 2006; Jensen et al., 2007).

More recently, biphasic alloplastic materials have become commercially available for use in bone regeneration of osseous defects (Jensen et al., 2007; Jensen et al., 2009). One such product available for clinical use is Bone Ceramic® (Institut Straumann, Basel, Switzerland). The material is produced by sintering HA and TCP to a chemically united material, which is porous and has a high affinity to proteins. The approach of combining a non-resorbable material with a highly resorbable material may be useful in clinical situations where limited degradation is preferred to preserve bone volume, while still allow for some bone healing to occur such as around immediate implant sites. However, no studies have investigated the use of the material for such sites. Further, the outcomes of varying the ratio of HA/TCP to modulate the resorption rates and osteoconductive property of the material have been investigated in a minipig model (Jensen et al., 2007; Jensen et al., 2009). It appears increasing the β-TCP content of a biphasic material corresponds to an increase in the rate of bone formation, while increasing the HA content results in a similar rate of bone formation as well as the non-resorbable property observed with a bovine bone graft (Bio-Oss) (Jensen et al., 2007; Jensen et al., 2009).

1.7.5.1 Combination of β-TCP with growth factors

There has been a growing interest in the use of β-TCP as a scaffold for growth factors, since cells can be attached to the β-TCP particles (Lee et al., 2000). A product called MD05 (Scil Technology GmbH, Denmark) which consists of two components; a ß-TCP scaffold combined with a growth factor, a recombinant version of human growth differentiation factor-5 (rhGDF-5), has commenced Phase IIa clinical trials as a regenerative bone substitute material for dental implant applications as well as for the treatment of intrabony defects affecting teeth. Superior results in terms of bone regeneration have been reported in animal

models (Poehling et al., 2006b;Weng et al., 2009) but results from clinical trials have yet to be published. Another commercially available product which combines β-TCP with a platelet-derived growth factor-BB (PDGF-BB) is GEM 21S™ (Luipold Pharmaceuticals, NY, USA). The product received FDA approval in 2005 following results from a large multicentre clinical trial reporting that the product was safe and effective for use in the treatment of periodontal intrabony defects (Nevins et al., 2005). However, the use of GEM 21S™ in peri-implant defects has not been investigated.

1.8 Molecular agents and growth factors with potential to enhance bone regeneration

For decades, researchers have been seeking an alternative to autogenous bone with similar or even better osteoinductive and osteoconductive properties for bone augmentation. A promising approach is the use of growth factors or morphogens since it is well known that these agents are present at low concentrations in bone matrix and plasma and regulate tissue repair through stimulatory effects on angiogenesis and cell proliferation, differentiation, and matrix synthesis (Hallman and Thor, 2008). Growth factors are primarily mitogenic (induce cell proliferation) and chemotactic (recruit cells); whereas morphogens act mainly by osseoinduction, which cause stem cells to differentiate into bone forming cells (Urist, 1965). Agents which have been investigated to stimulate and enhance bone regeneration include bone morphogenetic proteins (BMPs), platelet-rich plasma (PRP), enamel matrix proteins, peptide P-15 (P-15), recombinant human growth differentiation factor 5 (rhGDF-5), basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-β), insulin growth factor-I (IGF-I) and platelet-derived growth factor (PDGF).

1.8.1 Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins (BMPs) offer an intriguing approach to bone regeneration as they are osteoinductive, which means that these proteins can cause chemotaxis, proliferation, and differentiation of osteoprogenitor cells into osteoblasts (Wozney et al., 1988). BMPs were first discovered in 1965 in the form of protein extracts taken from demineralized bone matrix (Urist, 1965). Since then, more than 30 BMPs have been characterized using recombinant biotechnology. However, recombinant human BMP-2 (rhBMP-2) (Jung et al., 2008) and BMP-7 (osteogenic protein-1, OP-1) (Rutherford et al.,

1992) have shown the most promise for bone regeneration in experimental and clinical studies, although the former has received more attention in craniofacial use.

For almost a decade, rhBMP-7 has been in clinical use in Europe and the United States for a variety of orthopaedic indications, including non-union fractures in several different bones, as an alternative to autogenous bone grafts with promising results (Garrison et al., 2007; White et al., 2007). Additionally, several preclinical studies using large animal models have demonstrated the osteoinductive potential of rhBMP-7 in a number of bone regenerative applications including peri-implant defects (Cook et al., 1995b), vertical ridge augmentation (Leknes et al., 2008b;Susin et al., 2010), extraction sockets (Cook et al., 1995a), and maxillary sinus augmentation (Margolin et al., 1998, McAllister et al., 1998). An early pilot study using BMP-7 around immediately placed implants in monkeys found that bone formation was accelerated within three weeks with newly formed bone in close apposition to the titanium implants (Rutherford et al., 1992). A recent study of supra-alveolar critical-size peri-implant defects in the mandible of dogs used implants coated with two dose levels of rhBMP-7 at 1.5 and 3.0 mg/ml (Susin et al., 2010). Significant vertical bone regeneration as well as osseointegration was observed after eight weeks of healing with similar results achieved at both dose levels although the higher dose was associated with some local side effects (Susin et al., 2010). However, when the results were compared to a parallel study using rhBMP-2 with the same dose levels of 1.5 and 3.0 mg/ml (Leknes et al., 2008a, Wikesjö et al., 2008), similar results were achieved in terms of vertical height gain, but the rhBMP-2 treatment showed significantly greater area of new bone formation. Nevertheless, rhBMP-7 is able to induce localised alveolar bone formation as untreated critical-size defects do not regenerate spontaneously (Wikesjö et al., 2006).

Several animal and human investigations have shown promising results in bone healing with the application of rhBMP-2 for augmentation in the maxilla or mandible (Howell et al., 1997, Boyne et al., 1997, Cochran et al., 1999, Cochran et al., 2000; Jung et al., 2003; Jones et al., 2006; Park et al., 2007; Wikesjö et al., 2009). These studies have shown that rhBMP-2 can induce greater and more rapid bone formation in a wide variety of defect models including ridge augmentation procedures (Cochran et al., 2000; Howell et al., 1997, Fiorellini et al., 2005), peri-implant defects (Cochran et al., 1997, Sigurdsson et al., 1997, Cochran et al., 1999, Jones et al., 2006; Park et al., 2007; Jung et al., 2003), and in sinus floor elevation procedures (Boyne et al., 1997, Triplett et al., 2009). Defects treated with rhBMP-2 showed the greatest amount of new bone as well as more mature bone at 4 and 12 weeks of healing in

surgically created peri-implant defects in the mandible of dogs (Cochran et al., 1999, Jones et al., 2006). Furthermore, the approach of combining rhBMP-2 with an anorganic bovine bone graft and a collagen membrane has been suggested as an alternative to the use of autogenous block grafts for extended alveolar bone defects (Jung et al., 2003). A prospective clinical trial showed more mature new bone, an increase in graft to bone contact, and more predictable outcomes with GBR with the addition of rhBMP-2 after six months of healing. However, the effects of rhBMP-2 may be limited to the early phase of bone repair, as some studies reported that although the amount of new bone formed at an earlier time point was significantly greater than the controls, after a longer healing period no significant differences were observed (Cochran et al., 1999, Jones et al., 2006; Jung et al., 2003).

Even with such promising results, there are still no commercial products containing rhBMP-2 available for clinical use as a bone-stimulating factor for bone regeneration in implant dentistry. The difficulties of developing a BMP product may relate to the high costs involved with the production of BMPs. Other difficulties include establishing the therapeutic dose of rhBMP-2 for human intraoral use that maximises the therapeutic effect yet limits any systemic effects, and identifying the ideal carrier type which can deliver the proteins at the optimal dose and for sufficient duration. Positive results from clinical trials using rhBMP-2 employed a high dose level between 0.18 to 2.89 mg due to the fast degradation of the proteins *in vivo* (Jung et al., 2003; Howell et al., 1997), which is several magnitudes higher than the concentration of naturally occurring BMPs within human bone matrix (1µg/g bone) (Urist et al., 1983). However, more recently, the use of rhBMP-2 was explored via a gene delivery approach as an alternative to deliver these proteins at a significantly lower dose (micrograms) in order to reduce the costs and the potential for negative systemic side effects (Park et al., 2007). The study created large peri-implant defects in the calvarial of minipigs and found greater bone fill in defects and greater bone to implant contact (BIC) than controls after one and four weeks of healing (Park et al., 2007). Nonetheless, until these issues are addressed, the clinical application of these proteins for bone regeneration in implant dentistry appears somewhat limited.

1.8.2 Platelet-rich plasma (PRP)

The concept of adding platelet-rich plasma (PRP) to bone grafts was introduced in the mid 1990s in order to deliver a high concentration of autogenous growth factors to enhance bone regeneration for maxillofacial and dental applications (Tayapongsak et al., 1994;

Whitman et al., 1997, Marx et al., 1998). PRP is thought to be an inexpensive source of growth factors with three to four times the concentration of platelets compared to that of blood plasma, and therefore was thought to have higher levels of platelet-derived growth factors (Sánchez et al., 2003; Tonelli et al., 2005; Hallman and Thor, 2008). Platelets are essential for haemostasis, inflammation and wound healing. During the early stages of wound healing, platelet-released growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) initiate a cascade of events that result in wound healing. PRP is prepared by using whole blood taken from the patient and placing the blood in a centrifuge to separate the red blood cells from the platelets.

When used clinically to treat large mandibular defects, PRP combined with autogenous bone resulted in more trabecular bone formation observed at six months with more than twice the rate of bone maturation, and about 20% greater bone density compared to sites treated with the use of autogenous grafts alone (Marx et al., 1998). However, it is difficult to establish whether the positive result was due to the autograft alone rather than the addition of PRP, since when PRP was combined with other graft materials, no improvement in bone formation was found (Sánchez et al., 2003) (McAllister and Haghighat, 2007). More recently, several clinical trials involving PRP for sinus augmentation have been performed, and the results published have reported a large variation in the clinical outcomes (Consolo et al., 2007; Schaaf et al., 2008). A well-designed clinical trial involving 16 patients with bilateral symmetrical maxillary sinus atrophy received either a combination of PRP/autogenous bone on one side or autogenous bone alone contralaterally (Consolo et al., 2007). Histological results after four months revealed that in sites treated with PRP, a greater volume of bone as well as a denser radiographic appearance of the new bone was observed. However, the benefit gained from addition of PRP appeared restricted to the early stages of healing, with almost no difference observed at intervals longer than six months. By contrast, a larger prospective clinical trial involving 53 patients demonstrated no additional value at four months post-surgery with the addition of PRP to autogenous bone at promoting greater volumes of new bone for sinus floor augmentation (Schaaf et al., 2008). Thus, the usefulness of PRP in enhancing bone regeneration has been questioned in several reviews in regards whether the extra cost and time spent on the PRP procedure could be justified since the evidence for any clinical benefit of PRP appears to be somewhat limited and controversial (Sánchez et al., 2003; Hallman and Thor, 2008). Interestingly, a recent *in vitro* study suggested that the lack of clinical benefits reported in previous studies with PRP use may be due to the concentration of PRP used, as the effect of PRP on osteoblasts may be concentration specific (Creeper et al.,

2009). Using cultures of human osteoblasts, a concentration of 100% PRP was shown to compromise the vitality of cells, whereas 50% PRP or platelet-poor plasma demonstrated the best outcomes in terms of cell migration, proliferation, and differentiation over a five day period. Nonetheless, to date, there are no controlled clinical trials supporting the use of PRP to improve bone regeneration around immediate implants.

1.8.3 Enamel Matrix Derivative (EMD)

A growth factor-like agent has been developed for periodontal regeneration of intrabony defects enamel matrix derivative (EMD) which is commercially available in most countries (Institut Straumann, Basel, Switzerland). The product was developed by isolating enamel matrix proteins from developing porcine teeth. These proteins are then extracted and purified and the freeze-dried protein extract is solubilised in a propylene glycol alginate carrier solution (Hammarstrom et al., 1997, Sculean et al., 1999). *In vitro* studies have shown positive effects of EMD on proliferation of PDL cells, gingival fibroblasts, and cementoblasts (Gestrelius, 1997; Wennström and Lindhe, 2002), and clinically EMD has been shown to promote wound healing in periodontal intrabony defects (Giannobile W, 2003; Bosshardt, 2008). However, the use of enamel matrix derivative (EMD) for bone regeneration in peri-implant bone defects has shown limited benefit. A small clinical study applied EMD in combination with a resorbable membrane around immediate implants in 32 patients (Cangini and Cornelini, 2005). However, healing was reported to be better at sites without EMD after one year.

1.8.4 Peptide P-15

Peptide P-15 (P-15) is a synthetic clone of a 15 amino acid sequence of type I collagen, which competes for cell-surface sites for attachment of collagen. P-15 has been shown to behave in a way similar to collagen that is responsible for cell migration, differentiation, and proliferation (Bhatnagar et al., 1999, Qian and Bhatnagar, 1996). A commercial tissue-engineered bone replacement graft product, PepGen P-15 (Dentsply Friadent, Mannheim, Germany) is a combination of a xenograft (deproteinized bovine bone) with a synthetic peptide (P-15). PepGen P-15 has been evaluated and shown to be effective in treating periodontal intrabony defects (Yukna et al., 1998, Yukna et al., 2000), shown to be a useful material for sinus augmentation procedures (Krauser et al., 2000a; Degidi et al., 2004; Scarano et al., 2006), and shows potential to accelerate bone fill in an extraction socket (Hahn

et al., 2003). However, most clinical studies evaluating PepGen P-15 have assessed its use for sinus grafting (Krauser et al., 2000b;Degidi et al., 2004; Scarano et al., 2006) and are poorly designed involving only a small number of patients. In one case report, PepGen P-15 was shown to accelerate bone formation when used for sinus augmentation, which achieved a similar amount of bone fill at four months of healing as control sites at eight months of healing (Krauser et al., 2000a). In contrast, other studies using PepGen P-15 for sinus augmentation revealed no clinical benefit in the amount of new bone formed when PepGen P-15 was used compared to controls (Degidi et al., 2004; Scarano et al., 2006). However, it has also been suggested that to observe a significant benefit with the use of P-15, biopsies of sites augmented with P-15 should be performed at earlier time points since the effect of P-15 peptide was believed to stimulate cell attachment and accelerate bone formation, which is more obvious during the early phase of bone repair (Degidi et al., 2004).

The use PepGen P-15 around immediate implants has also been explored (Tehemar et al., 2003). However, the results are preliminary with data limited to one study in a dog model (Tehemar et al., 2003). When PepGen P-15 was used alone or in combination with a non-resorbable ePTFE membrane, a higher level of bone to implant contact was observed after three months of healing compared to control implants that received no treatment or the ePTFE membrane alone.

1.8.5 Recombinant human growth differentiation factor-5 (rhGDF-5)

Growth differentiation factor-5 (GDF-5) is a member of the BMP superfamily. It is a naturally occurring protein required for proper skeletal and joint development (Francis-West et al., 1999). A recombinant version of human GDF-5 (rhGDF-5) has been developed which has been shown to have osteoinductive potential in preclinical animal studies (Spiro et al., 2000; Yoshimoto et al., 2006). When rhGDF-5 was implanted in subcutaneous or intramuscular sites, local areas of ectopic bone formation were observed (Spiro et al., 2000; Yoshimoto et al., 2006).

RhGDF-5 has received growing interest for clinical use in dental and maxillofacial applications due to the positive results achieved in bone regeneration from animal trials (Poehling et al., 2006a; Weng et al., 2009; Polimeni et al., 2010). A product called MD05 (Scil Technology GmbH, Bayern, Germany) commenced Phase IIa clinical trials as a regenerative bone substitute material for dental implant applications. The material has two

components: a synthetic ß-tricalcium phosphate (ß-TCP), and rhGDF-5. MD05 has demonstrated superior bone regeneration after six weeks of healing when used in critical-size calvarial defects in a rat model (Poehling et al., 2006b). In this study, the amount of new bone formed was about five times greater with MD05 than with the other bone substitutes tested, and the defects were completely healed by six weeks. Further, the amount of fibrous tissue in the defects was also significantly lower in the MD05 group. In addition, a recent pilot animal study demonstrated a potential benefit of rhGDF-5 in combination with β-TCP for the treatment of bony defects around implants (Weng et al., 2009). In that study, a critical size defect model in the mandible of dogs was used to examine the effect of rh-GDF-5. Results after two months of healing showed that defects filled with rh-GDF-5 combined with β-TCP showed a greater amount of new bone formation around the implants compared to defects filled with the control materials. However, results from clinical trials using MD05 in bone defects surrounding implants have not yet been reported.

1.8.6 Fibroblast growth factor (FGF)

Since the early 1990s, the bone regenerative potential of basic fibroblast growth factor (bFGF) has been investigated in several animal models (Aspenberg et al., 1991; Yamada et al., 1997a; Hosokawa et al., 2000; Akagawa et al., 2009). The growth factor bFGF is produced by a variety of cells including macrophages, mesenchymal cells, chondrocytes and osteoblasts (Hallman and Thor, 2008). The mechanism of action is thought to involve the recruitment of mesenchymal stem cells, osteoblasts, and endothelial cells, as well as stimulating these cells to differentiate or multiply (Gospodarowicz, 1990, Kimura et al., 1995; Power et al., 2002).

A commercial product of bFGF (Fiblast Spray; Kaken Pharmaceutical Co Ltd, Tokyo, Japan) has been developed for topical use in the treatment of non-healing leg ulcers. Since it was launched in the United States (US) in 2001 it has generated more than USD$35 million in worldwide revenue annually. Moreover, bFGF is the only growth factor that has received approval for clinical use in Japan (Kurokawa et al., 2003), which indicates why most of the research on FGF has come from Japan. In recent reports, the human recombinant form of FGF-2 (rhFGF-2) has been recommended for clinical use to accelerate bone repair in osteoarthritis patients undergoing tibial osteotomy (Kawaguchi et al., 2007). A clinical study examined rhFGF-2 at three dose levels (200, 400 and 800 µg) in a gelatin-hydrogel complex in 59 patients and found the 800 µg dose of rhFGF-2 increased the frequency of patients with

radiographic bone union, decreased the average healing time, and resulted in less post-operative pain (Kawaguchi et al., 2007). In addition, the trial revealed no clinical adverse outcomes associated with the three dosages of rhFGF-2 examined over the four months duration of the study.

The use of FGF-2 in dental applications has also been documented. Periodontal tissue regeneration using FGF-2 has reached phase II clinical trials in Japan with results suggesting it is safe and effective for use in stimulating periodontal regeneration (Kitamura et al., 2008). A randomised controlled clinical trial involving 74 patients with 2- or 3-wall intraosseous defects treated with varying concentrations of FGF-2 (0.03, 0.1 or 0.3%) in a hydroxypropylcellulose vehicle showed an increase in radiographic alveolar bone height with almost twice the gain in defects treated with 0.3% FGF-2 compared to control sites (1.85 mm versus 0.95 mm) after nine months. No adverse events were identified related to the application of the agent. However, FGF remains in the experimental stage for bone augmentation applications in the maxilla/mandibular regions, as the optimal concentration for bone regeneration has not been confirmed, although a few preclinical animal studies have recently shown potential for its use to augment bony defects around implants (Hayashi et al., 2007; Akagawa et al., 2009). When used with a gelatin-hydrogel complex, varying concentrations of bFGF (1, 10 and 100 µg) were shown to be useful in regenerating bone around fenestrated implants (Hayashi et al., 2007). A similar finding was also observed in a subsequent study, which demonstrated that a 10 µg dose of bFGF combined with a slow degradation-type gelatin-hydrogel complex was effective at accelerating bone regeneration around fenestrated implants after four weeks in a dog model, while a 1 µg dose was not (Akagawa et al., 2009).

1.8.7 Transforming growth factor-beta (TGF-β)

Transforming growth factor-beta (TGF-β) is an endogenous osteoinductive protein produced by osteoblasts, which is involved in inducing the proliferation and differentiation of osteogenic cells (Linkhart et al., 1996) as well as inhibiting osteoclasts precursors (Bonewald and Mundy, 1990). The highest concentration of TGF-β is found in platelets (Assoian et al., 1983), but in terms of quantity, it is most abundant in bone (Seyedin et al., 1985).

For bone regenerative applications, the human recombinant form of TGF-β (rhTGF-β) has shown to induce bone fill in skull defects of rabbits (Beck et al., 1993), enhance fracture

healing in rabbit tibiae (Lind et al., 1993), and has achieved significant bone regeneration in alveolar ridge defects in canines (Ruskin et al., 2000). However, in contrast to BMPs, ectopic bone formation has not been observed with the use of TGF-β (Lieberman et al., 2002).

The development of TGF-β for bone regeneration remains in the experimental stage since the dose level and a suitable carrier for TGF-β has yet to be determined, although studies have revealed that the use of a biodegradable gelatin-hydrogel, or a collagen sponge appear to be promising carriers to release TGF-β in a biologically active form (Yamamoto et al., 2000; Hong et al., 2000; Ueda et al., 2002; Srouji et al., 2005). Following treatment with 0.1 µg TGF-β in a hydrogel complex or collagen sponge, significant new bone formation was observed in rabbit skull defects at six weeks (Hong et al., 2000; Ueda et al., 2002). Thus far, there are no reports of the use of rhTGF-β in peri-implant defects.

1.8.8 Insulin-like growth factor (IGF)

Insulin-like growth factors (IGFs) exert important actions on cells during skeletal growth and in bone formation and are considered essential regulators of bone remodelling (Canalis et al., 1993). It has been proposed that IGFs are stored in bone matrix and thus, when resorption occurs in the bone remodelling cycle, IGFs are released and function to increase osteoprogenitor cells by stimulating cell proliferation to increase the number of osteoblasts to replace the bone that was removed by resorption (Mohan and Baylink, 1991; Dequeker et al., 1993). Thus, the use of IGFs may be useful for regenerating bone. There are two isoforms of insulin-like growth factors (IGFs), IGF-I and II, with both having similar biological activities although IGF-I is the more potent form involved in bone formation, whereas IGF-II has a greater role in the later stages of endochondral bone formation. IGF-I and II are the most abundant growth factors stored in bone matrix (Mohan and Baylink, 1991). Cells that produce IGFs include osteoblasts, chondrocytes and endothelial cells (Solheim, 1998).

No studies have applied IGF-1 solely to a bone defect without a combination of other growth factors to promote bone regeneration at peri-implant defects. Previous studies have employed a combination of PDGF-BB with IGF-1 which has shown promising results for faster bone regeneration and osseointegration (Becker et al., 1992; Nociti Jr et al., 2000; Lynch et al., 1991b). However, the reason for a combination approach may be due to the need to combine IGF-I with a growth factor that is able to inhibit bone resorption because although

IGFs have the ability to stimulate osteoblast proliferation and differentiation, they have also been shown to stimulate osteoclast formation and thus, bone resorption (Linkhart et al., 1996).

1.8.9 Platelet-derived growth factor (PDGF)

Among all the growth factors, platelet-derived growth factor (PDGF) has received the most attention as a growth factor agent for clinical use in periodontal regeneration, and shows good potential to enhance bone regeneration. PDGF is a powerful chemotactic agent for inflammatory cells, mesenchymal and bone forming cells during the initial stages of fracture healing. PDGF is synthesized by platelets, monocytes, macrophages, endothelial cells and osteoblasts (Andrew et al., 1995). It is released by blood platelets at the site of injury, which stimulates a cascade of events that leads to fracture healing, since it has both a direct mitogenic effect on osteoblasts and osteoclasts, as well as an indirect effect by stimulating inflammatory cells such as macrophages to secrete other growth factors which assists in bone repair (Andrew et al., 1995; Fujii et al., 1999, Lieberman et al., 2002; Graham et al., 2009). Initially, PDGF was thought to be composed of only two polypeptide chains, A and B, but through the use of genomic and biochemical methods, chains C and D have been identified. These chains join together to form five different dimeric PDGFs, and of the five, four are homodimers (AA, BB, CC, DD) and one is a heterodimer (AB). Chains A and C are expressed in epithelial, muscle, and neuron cells; Chain B is mainly expressed in platelets, osteoblasts and endothelial cells; and Chain D is expressed by fibroblasts and smooth muscle cells. The most biologically potent of the PDGF isoforms is PDGF-BB, as it appears to bind to osteoblasts with the greatest affinity (Zhang et al., 1991; Centrella et al., 1991).

There have been over 100 studies published on the effects of PDGF on wound healing, periodontal regeneration, and on periodontal ligament (PDL) cells and bone cells since it was first reported in the late 1980s that periodontal regeneration could be achieved with the application of PDGF (Lynch et al., 1989). PDGF appears to have mitogenic and chemotactic effects on PDL cells and alveolar bone cells, and it has been shown to promote regeneration of the entire periodontal complex with new bone, periodontal ligament, and cementum observed in both *in vitro* and *in vivo* studies (Oates et al., 1993; Nevins et al., 2003; Camelo et al., 2003; Howell et al., 1997, Wang et al., 1994; Lynch et al., 1989, Lynch et al., 1991b;Giannobile et al., 1996, Cho et al., 1995)Rutherford *et al* 1992; (Matsuda et al., 1992). However, the use of PDGF for bone regeneration in peri-implant defects remains limited with reports published only in preclinical animal models.

1.8.9.1 Effect of PDGF on bone healing in peri-implant bone defects

The approach of using PDGF-BB to accelerate and promote bone repair in peri-implant defects appears promising, since PDGF-BB is a key regulatory molecule in bone repair and regeneration (Graham et al., 2009). However, the evidence for the use of PDGF-BB to treat peri-implant defects is weak, with experiments to date consisting of pilot studies in various animal models and having several flaws in the study design, including the use of small peri-implant defects which makes obvious differences difficult to demonstrate, as well as the use of low concentrations of PDGF-BB with a carrier that does not provide sufficient mechanical support to maintain the space for optimal bone regeneration. Although such limitations exist, most of the studies performed on PDGF reported a positive effect on bone formation (Lynch et al., 1991a; Becker et al., 1991; Nociti Jr et al., 2000). There have been reports of contradictory findings to suggest that PDGF inhibits bone formation (Ranly et al., 2005; Roussy et al., 2007), however those studies tested PDGF-BB in an environment unsuitable for the growth factor to demonstrate its potential for bone regeneration, considering PDGF has a mitogenic and chemotactic effect on osteoblasts, and not an osteoinductive effect on mesenchymal cells as observed with BMPs. Thus, when gel capsules containing PDGF-BB were inserted into the muscles of mice, it was not surprising that negative results were reported. In addition, if the defect size chosen is too small, the difference in new bone formation may not be apparent with the use of the growth factor. A study using a 1.25 mm wide circumferential defect around implants reported improved bone fill after three and eight weeks post-implant placement with almost 30% more bone fill in defects treated with PDGF-BB/IGF-I at three weeks compared to control defects in dogs (Nociti Jr et al., 2000). However, a repeat of the same study, but with a smaller peri-implant defect observed only minor differences in bone fill between groups (Stefani et al., 2000). The authors suggested that the defect size chosen may be responsible for the negative result, since the implants were placed almost or in contact with bone, which may have limited the potential for any significant difference in bone regeneration to be observed (Stefani et al., 2000).

Earlier studies in animal models have focussed particularly on PDGF and rhIGF-I combination for bone regeneration in defects around implants (Lynch et al., 1991a; Stefani et al., 2000; Becker et al., 1991). The first pilot study reported positive results during early bone healing with the use of a PDGF and rhIGF-I combination around rough titanium implants in dogs (Lynch et al., 1991a). The study employed a 4 µg dose of each growth factor with a

methylcellulose gel acting as a carrier and control. Implants with 1 mm diameter holes in the apical area were coated with the growth factor gel and after one week, the percentage of new bone in the apical holes was significantly greater at sites treated with the growth factor, with the difference in percentage of bone fill being almost 10% higher than in the controls. After three weeks of healing, the percentage of bone fill remained about 30% higher than at the control sites (Lynch et al., 1991a). Positive results were also reported in subsequent animal studies (Becker et al., 1992; Nociti Jr et al., 2000). The effect of a 5 µg dose of PDGF/IGF-I in dogs revealed greater bone formation around immediate implants with a buccal dehiscence defect (exposing the coronal six threads of the implants) in comparison to GBR (ePTFE), and GBR with a bone graft (DFDBA) (Becker et al., 1992). After four months of healing, sites treated with PDGF/IGF-I showed 18% higher BIC compared to sites receiving the other treatments. Re-entry surgery performed at four months showed the extent of bone healing with almost complete coverage of the six exposed threads being comparable in defects treated with GBR alone and with the growth factor treated sites. However, when the thickness of the regenerated buccal bone was analysed, defects treated with PDGF/IGF-I demonstrated almost double the thickness of bone at the coronal three threads compared to the GBR only defects.

1.8.9.2 Potential of using PDGF-BB with different carrier types on bone regeneration

Combining growth factors with various scaffolds to achieve more predictable outcomes for bone regeneration is a developing area of research. Allografts, alloplasts and xenografts are generally considered osteoconductive materials, and therefore an approach to combine these materials with growth factors seems appealing (Jiang et al., 1999, Stephan et al., 2000; Lee et al., 2000; Schwarz et al., 2009b)

Several studies have reported positive outcomes with the combination of PDGF-BB and a xenograft (DBB) to enhance and support bone formation (Simion et al., 2006; Schwarz et al., 2009a; Nevins et al., 2009). The approach of using bovine bone as a suitable carrier is based on the non-resorbable property of the graft particles once applied *in vivo* (Schwarz et al., 2009a). A proof-of-principle study using PDGF-BB in combination with a deproteinised bovine block (DBB) showed the potential to regenerate significant amounts of new bone in the vertical dimension after four months of healing in mandibular ridge defects of dogs (Simion et al., 2006). Another study examined the use of DBB soak-loaded with rhPDGF-BB (0.3 mg/ml) and observed greater new bone formation after three weeks in lateral ridge defects of dogs (Schwarz et al., 2009a). However, the study was funded by the manufacturers

of the bovine graft material, which may question the validity of the results. Nevertheless, results from *in vitro* studies have shown good support with the use of DBB as a carrier for PDGF-BB with enhanced proliferation of cultured rat osteoblastic cells when PDGF-BB was applied compared to osteoblasts treated with DBB only (Jiang et al., 1999, Stephan et al., 2000). Furthermore, histological results of a recent clinical study on seven patients revealed robust new bone formation and intimate contact between the new bone and bovine graft particles when PDGF-BB (0.3 mg/ml) combined with BioOss collagen (90% DBB and 10% porcine collagen) was applied to extraction sockets in order to preserve the ridge dimensions after four to six months of healing (Nevins et al., 2009), however the study was another that was sponsored by the company that manufactured the materials.

Synthetic bone substitutes have also been proposed as suitable carriers for PDGF (Lee et al., 2000; Schwarz et al., 2009b). A study reported large amounts of new bone formation in rat calvarial defects was achieved after two and four weeks of healing when an extremely small dose of PDGF-BB (0.2µg) was combined with a chitosan/TCP sponge as a carrier (Lee et al., 2000). The carrier was proposed to be able to regulate the release of PDGF to maintain an effective therapeutic concentration of PDGF for bone formation. The use of biphasic calcium phosphate (BCP) comprising of 60% HA and 40% β-TCP, as a carrier for PDGF-BB showed promise for bone regeneration in lateral ridge defects in the mandible of dogs (Schwarz et al., 2009b). A slightly greater amount of new bone formation, as well as a greater proportion of mature bone was reported after three weeks of healing.

Moreover, due to the high degradation rate of PDGF-BB by proteinases once applied *in vivo*, it has been suggested that a gene delivery approach may be more suitable for larger defects where a sustained PDGF release could attract osteogenic cells for a prolonged time to achieve greater amounts of new bone formation (Chang et al., 2010). Gene therapy to deliver PDGF-BB was recently shown to be a safe and effective approach at accelerating bone repair and result in successful implant osseointegration in the rat model (Chang et al., 2010).

1.9 Conclusions

The use of immediate implants is a popular treatment approach to shorten the treatment time for implant therapy, as survival rates are high and appear to be equivalent to implants placed in healed extractions sites. However, it creates a new challenge for clinicians to achieve good predictable outcomes in terms of aesthetics, as a high proportion of patients are

at risk for developing some recession that may be visible over the first year following immediate implant placement (Chen and Buser, 2009b). The use of bone grafts or agents with or without membranes has been recommended to help promote bone fill in marginal defects surrounding immediate implants as well as compensate for the changes in ridge dimensions following tooth loss. However, to date, there is still no single material regarded as being superior for augmentation around immediate implants due to the limited number of well-designed clinical trials with sufficient patients and follow-up (Chen et al., 2004; Esposito et al., 2006a; Esposito et al., 2008; Chen et al., 2009).

Although autogenous bone has been considered the ideal bone grafting material, the problem of bacterial contamination when intraoral bone grafts are harvested, and the lack of a proven method to completely decontaminate the graft particles while maintaining cell viability, means that the risk for infective complications will always be present whenever these grafts are used around implants. Further, the resorption rate of the graft particles may occur too quickly and thus may not provide sufficient new bone to compensate for the external dimensional changes of the socket following tooth loss (Chen et al., 2005). As a result, various grafting materials and agents have been suggested as alternatives to autogenous bone. In particular, the use of bovine bone (DBB) in conjunction with GBR has shown some promise for use around immediate implants (Cornelini et al., 2004a; Esposito et al., 2006a; Chen et al., 2007; Esposito et al., 2008; Chen and Buser, 2009b).

The use of growth or differentiation factors is an emerging approach with significant potential to improve bone regeneration, as preclinical and clinical studies have demonstrated superior outcomes in terms of the amount and rate of new bone formation when these agents were compared to traditional bone grafting materials. Although much work remains to be done before some of the agents become a clinical reality, several agents have shown promise. One such agent which has been clinically tested and shown to be safe and effective for human intra-oral use in the treatment of periodontal osseous defects is platelet-derived growth factor-BB in combination with an alloplastic material, β-TCP. However, to date, there have been no studies evaluating this combination for bone regeneration in defects surrounding implants.

In conclusion, the future of bone regeneration appears exciting, particularly if a product could be developed encompassing growth or differentiation factors with or without a combination of bone grafts or substitutes for use in procedures such as around immediate implants to achieve optimal aesthetic outcomes routinely for all patients. Furthermore, a

product which is simple to use, cost-effective, has a low risk for post-operative complications, and can result in shortened treatment time by accelerating bone healing would be regarded by clinicians as the gold standard material for bone regeneration.

1.10 Hypothesis

Our hypothesis is that the application of rhPDGF-BB with β-TCP should result in faster and more bone regeneration in critical size bony defects around dental implants.

1.11 Aim of the study

The aim of this investigation was to examine the effect of a combination of purified recombinant human platelet-derived growth factor (rhPDGF-BB) with a synthetic beta-tricalcium phosphate (β-TCP) on bone healing around dental implants with critical size circumferential defects.

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Chapter 2. Effect of PDGF-BB on bone formation around dental implants: an experimental study in sheep

Tina Choo, P. Mark Bartold, Victor Marino

2.1 Abstract

Immediate placement of implants into fresh extraction sockets has the potential of shortening the total treatment time for patients. However, when implants are placed at the time of tooth extraction, there is often a gap between the walls of the extraction socket and the implant surface. This gap is usually widest in the coronal aspect of the socket. Hence, management of this bony defect around dental implants may play an important role in improving implant success in terms of aesthetic outcomes. The use of growth or differentiation factors is an emerging approach with significant potential to improve bone regeneration. One such agent which has been clinically tested and shown to be safe and effective for human intra-oral use in the treatment of periodontal osseous defects is platelet-derived growth factor-BB in combination with an alloplastic material, β-TCP. However, to date, there have been no studies evaluating this combination for bone regeneration in defects surrounding implants.

Objectives: The aim of this investigation was to examine the effect of a combination of purified recombinant human platelet-derived growth factor (rhPDGF-BB) mixed with a synthetic beta-tricalcium phosphate (β-TCP) on bone healing around dental implants with critical size circumferential defects.

Material and methods: Three critical-size circumferential defects (10 mm wide x 3 mm deep) were prepared in the ilium of six sheep. Three dental implants were placed into the centre of each defect and the 3.25 mm circumferential gap was filled with either: (a) blood clot alone; (b) β-TCP; (c) rhPDGF-BB (0.3 mg/ml) with β-TCP. The sheep were sacrificed at 2 and 4 weeks and histologic and histomorphometric analysis was performed to determine the percentage of new mineralised bone formation and residual β-TCP graft particles in the defects.

Results: Defects filled with rhPDGF-BB/β-TCP showed the highest rate of bone formation after 2 and 4 weeks with limited degradation of the β-TCP particles over 4 weeks. Defects filled with β-TCP showed the least bone fill after 2 and 4 weeks, and faster

degradation of the β-TCP particles over 4 weeks compared with defects filled with rhPDGF-BB/β-TCP. Percentage of new mineralised bone was comparable in defects with blood clot alone and β-TCP after 4 weeks of healing, but there was a collapse in the defect area in defects with blood clot alone. In comparison, the space was maintained when β-TCP was used in defects at 4 weeks.

Conclusions: The combination of rhPDGF-BB with β-TCP enhanced bone regeneration in contained peri-implant bone defects during the early stages of bone healing.

2.2 Introduction

Immediate implant placement is a popular treatment approach for the replacement of anterior teeth. Advantages of utilising such an approach include shortening treatment time and reducing the number of surgical procedures for the patient (Lazzara 1989, Becker *et al* 1994c; Schropp and Isidor 2008). However, although the failure rate has been reported to be comparable to implants placed with a delayed approach (Esposito et al., 2006b, Quirynen et al., 2007; Chen and Buser, 2009b), immediate implants create a new challenge for clinicians to achieve a natural-looking aesthetic result, particularly in anterior teeth in the aesthetic zone, where tooth loss may result in significant alterations to the hard and soft tissue dimensions. Short-term reports have suggested a high incidence of midfacial soft tissue recession with a loss in papillae height affecting a significant proportion of patients within the first year following immediate implant placement (Lindeboom et al., 2006; Evans and Chen, 2008; Chen and Buser, 2009b).

In addition to the modelling changes following tooth extraction, when implants are placed immediately into an extraction site, there is often a gap between the socket wall and the coronal neck of the implant due to incongruity in the dimensions between the tooth socket and implant. As a result, several investigations were performed to evaluate the effect of various gap sizes around implants to determine the "critical size" of a defect, which refers to a defect that does not spontaneously regenerate without adjunctive measures (Schmitz & Hollinger 1986). Most studies seem to agree that marginal defects around implants (with all bony walls intact) less than 2 mm wide appears to resolve spontaneously without any need for bone augmentation (Hämmerle et al., 2002, Botticelli et al., 2003b, Covani *et al* 2003; Chen et al., 2004; Jung et al., 2007). However, in gaps wider than 2 mm, bone augmentation has been

recommended to promote complete bone fill (Cornelini et al., 2004; Chen et al., 2007; Polyzois et al., 2007; Chen and Buser, 2009b).

For many years, numerous investigations were performed to identify the perfect material or technique for bone regeneration around immediate implant sites. However, to date, there is still no single material regarded as being superior (Chen et al., 2004; Esposito et al., 2006a; Esposito et al., 2008; Chen et al., 2009). Although autogenous bone grafts have long been regarded as the gold standard for bone augmentation, the main disadvantages for their use around immediate implants relate to limited volume, donor site morbidity, and the potential for infection as a result of graft contamination when intraoral bone grafts are harvested (Blay et al., 2003; Esposito et al., 2006a; Etcheson et al., 2007; Graziani et al., 2007; Tezulas and Dilek, 2008). As a result, various other grafting materials and agents have been developed as alternatives to autogenous bone.

Calcium phosphates are popular alloplast materials used to fill bone defects, since their composition closely resembles the inorganic phase of bone. These materials are considered osteoconductive, as they work simply by providing a physical scaffold for bone ingrowth (Gatti et al., 1990). Further, calcium phosphate in the form of tricalcium phosphate (TCP) can be sintered into a uniform material, resulting in α or β-TCP, which is a purified, multicrystalline, and porous form of calcium phosphate, similar to natural bone mineral (Szabó et al., 2001). The resorption of β-TCP is fairly rapid, with almost complete resorption by eight weeks (Jensen et al., 2007), and thus it may be useful to resolve bony defects as it allows for complete substitution of the particles with new bone (Jensen et al., 2007).

The use of growth or differentiation factors is an emerging approach with significant potential to improve bone regeneration, as preclinical and clinical studies have demonstrated superior outcomes in terms of the amount and rate of new bone formation when these agents were compared to traditional bone grafting materials. These factors are present at low concentrations in bone matrix and plasma, and are essential mediators of tissue repair through their stimulatory effects on angiogenesis, cell proliferation, cell differentiation and matrix synthesis.

Among all the growth factors, platelet-derived growth factor (PDGF) has received the most attention. It is synthesized by platelets, monocytes, macrophages, endothelial cells and osteoblasts (Andrew et al., 1995) and is composed of a combination of four polypeptide

chains (A, B, C, D) that join together to form five different dimeric PDGFs. Of the five, PDGF-BB is considered biologically the most potent of the PDGF isoforms as it appears to bind to osteoblasts with the greatest affinity (Zhang et al., 1991; Centrella et al., 1991).

PDGF-BB shows two effects on cells in order to enhance bone regeneration: it has a direct mitogenic effect on osteoblasts and osteoclasts, and an indirect effect by stimulating inflammatory cells such as macrophages to secrete other growth factors to assist in wound repair (Andrew et al., 1995; Lieberman et al., 2002; Graham et al., 2009). In recent times, a commercial product consisting of PDGF-BB in combination with β-TCP (GEM 21S[®]) has shown to be safe and effective for clinical use in periodontal regeneration (Nevins et al., 2003; Nevins et al., 2005). However, to date there have been no studies investigating the effect of GEM 21S[®] on bone regeneration in defects surrounding dental implants. Therefore, the aim of the current investigation was to examine the effect of a combination of rhPDGF-BB with β-TCP on bone formation in critical size peri-implant defects.

2.3 Materials and Methods

2.3.1 Animal model and study design

A total of six sheep, aged 3 to 5 years of age were included in the study. All sheep were deemed healthy, not gestating females and weighed on average 68 kg. This study was conducted in strict accordance with the guidelines of the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985), the Australian Code of Practice for the care and use of animals for scientific purposes of the National Health and Medical Research Council and The Institute of Medical and Veterinary Sciences Animal Ethics Committee (Project Approval No 126/08).

2.3.2 Surgical procedure

Anaesthesia was induced in the sheep with thiopentone (10-15 mg/kg) and maintained with 2.5% isoflurane in 4L of O_2 through tracheal intubation. The animals were placed in a lateral recumbent position for access to the pelvis. The surgical sites were disinfected with a sterile swab of povidone-iodine and a skin incision was made from the mid- point of the iliac crest running 15 cm perpendicular from this point. The fascia was cut and the muscles and tendons were separated by blunt dissection to expose an area of about 3.5 cm in diameter in the wing of the ilium. Standard drilling procedures according to the manual of the

NobelReplace® system (Nobel Biocare, Sweden) were used to prepare the osteotomy sites under copious irrigation with chilled sterile saline. Once the implant osteotomy sites were prepared, standardised circumferential bone defects were made (10 mm diameter, 3 mm depth) with a counterbore drill fitted with a custom-made 3 mm deep stop collar (Figure 1), resulting in a 3.25 mm wide gap between the implant surface and the surrounding bone wall to a depth of 3 mm (Figure 2). The size of the defects was in accordance with the definition of a critical-size defect (Schmitz & Hollinger 1986). The sites were then rinsed with saline before implant placement. A dental implant (3.5 x 10 mm) Ti-Unite® NobelReplace® Straight Groovy (Nobel Biocare, Sweden) was inserted into each osteotomy site and positioned so that the margin coincided with the level of the bone crest (Figure 3). Each sheep received three implants with a total of 18 implants inserted into six sheep. NobelReplace® titanium healing caps were placed on all the implants.

The three defects in each sheep were randomly assigned to be filled with one material prior to surgery by an independent investigator not involved with the surgery. The materials used to fill the defects were:

1. Blood clot alone (control group);

2. β-TCP (GEM 21S®, Osteohealth, Luitpold Pharmaceutical Inc Shirley, NY, USA): 0.5 cc of β-TCP particles (size 0.25-1.0 mm) mixed with 0.5 ml saline;

3. Growth Factor Enhanced Matrix (GEM 21S®) (Osteohealth, Luitpold Pharmaceutical Inc Shirley, NY, USA) containing recombinant human platelet derived growth factor-BB: 0.5 ml solution of rhPDGF-BB (0.3 mg/ml) in a syringe with 0.5 cc of β-TCP particles (size 0.25-1.0 mm).
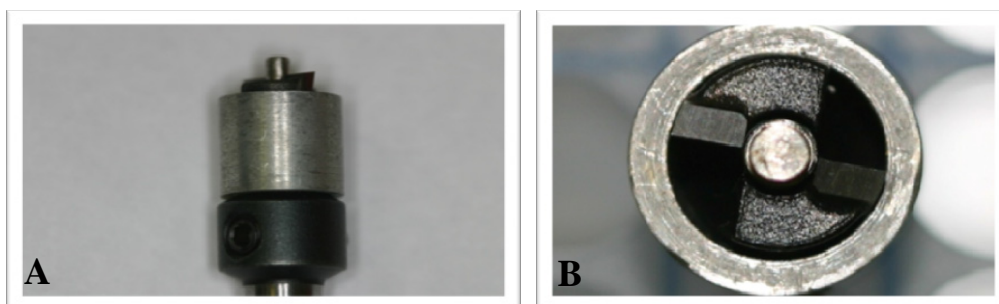

Figure 1. Customised defect drill with stop collar (10 mm diameter, 3 mm depth)
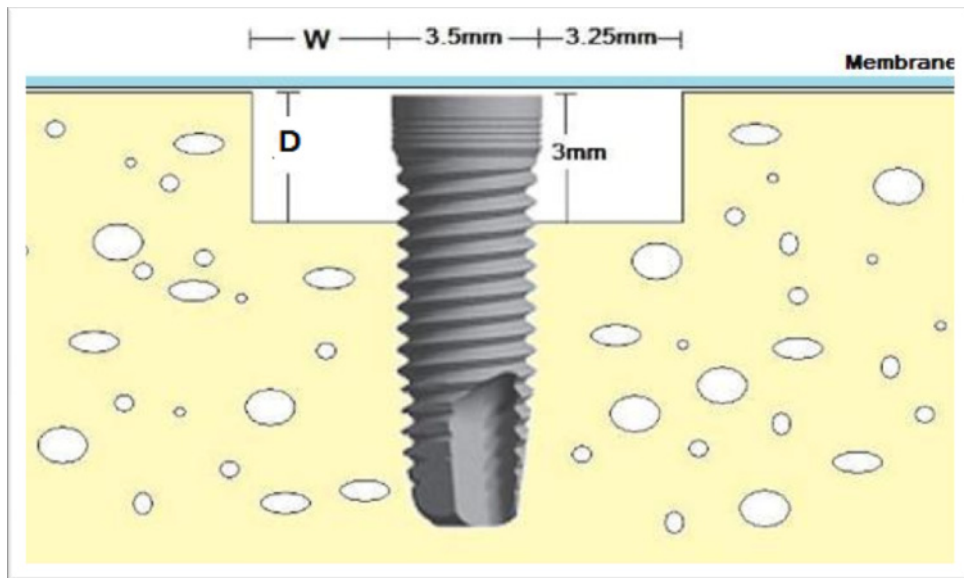
Figure 2. Critical-size coronal circumferential bone defect 3.25 mm wide, 3 mm deep
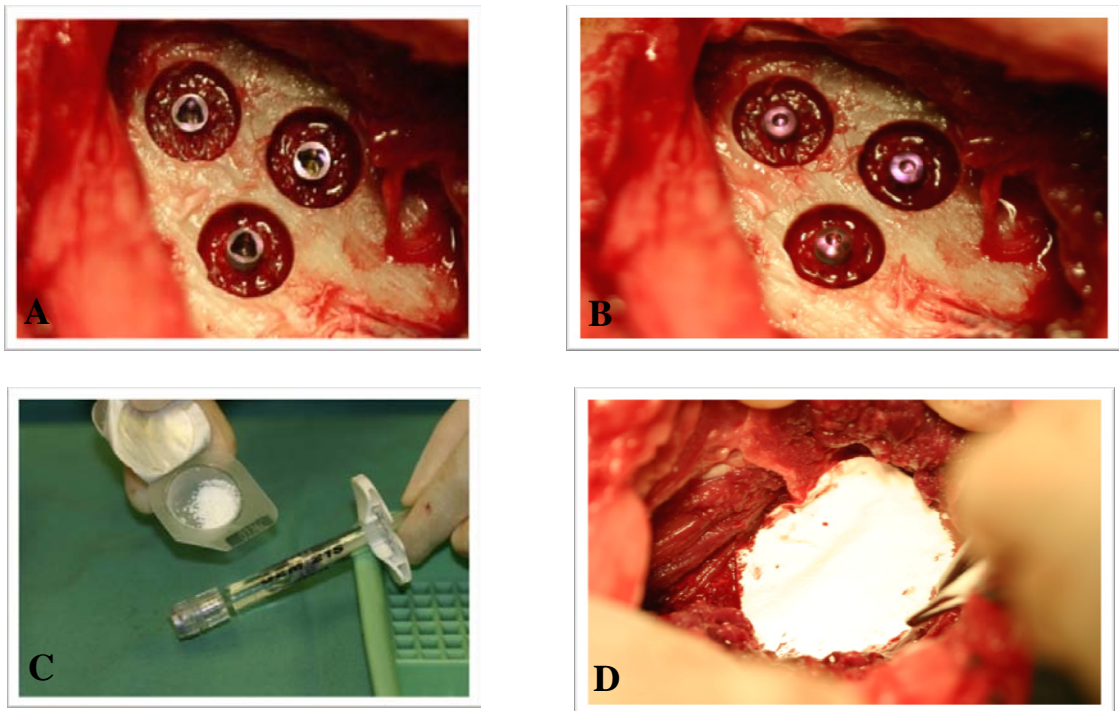

Figure 3. Arrangement of the surgically created peri-implant defects and implant placement in the sheep ilium (a) and with healing caps in place (b). GEM 21S: β-TCP was mixed with rhPDGF-BB (0.5 ml pre-filled syringe with 0.3 mg/ml PDGF-BB) 15 minutes before being implanted into the surgically created defects (c). BioGide membrane trimmed and in positioned to cover the defects (d).

A resorbable membrane (Bio-Gide®, Geistlich AG, Wolhusen, Switzerland) was trimmed to the appropriate shape and adapted so that the membrane completely covered all implants and defects and extended beyond the defect margins by approximately 2 mm (Figure 3d). All implants were left to heal submerged and the muscle layers were closed using a resorbable suture (Polyglactin; Coated Vicryl® 0, Ethicon, Johnson & Johnson Medical Ltd, USA). Fascia, subcutis and skin were sutured closed using resorbable sutures (POLYSORB™ 2-0; Braided LACTOMER)

2.3.3 Postoperative management

All animals received Rilexine IM (150 mg/10 kg) 30 minutes before surgery for prophylaxis antibiotic cover and Clavulox (3 ml) for three days postoperatively. Post surgery pain and inflammation was managed with a non-steroidal anti-inflammatory and analgesic (Rimadyl 50 mg/25 kg IM b.i.d.). For the first four days, the sheep were kept single and healing was monitored twice daily.

2.3.4 Retrieval surgery and histological preparation

The sheep were sacrificed with IV injection of 20 ml pentobarbitone (325 mg/ml). Three sheep with each of the different filling materials were sacrificed at 2 weeks, and the remaining three at 4 weeks. The pelvis bone was removed and sectioned down to contain the surgical implant/defect sites. The presence and location of the implants was assessed radiographically. The collected specimen was then fixed in 10% neutral-buffered formalin solution for 7 days. Each implant site was sectioned into cubes (1.5^3 cm) with a low speed diamond saw and the specimens were prepared for non-decalcified bone histology (Donath and Breuner, 1982). The process involved dehydration of the bone in increasing concentrations of ethanol solutions (70, 85, 95, and 100%) at room temperature, followed by embedding in methylmethacrylate. Sections were cut in the middle of the implant parallel to the longitudinal axis of the implant in sections approximately 1 mm in thickness using a low speed diamond saw (blade thickness 381 μm). For each implant the two most complete central sections were selected and glued with Loctite 358 UV cure (glass adhesion glue) to a clear 1 mm thick glass slide and ground to a final thickness of approximately 80 to 100 μm by microgrinding and polishing. The sections were surface stained in 0.1% toluidine blue solution for 7 hours. Excess stain was removed by rinsing the sections with distilled water.

2.3.5 Histological examination

The sections were coded and randomised prior to analysis. Two sections from each implant were analysed and the histomorphometric analysis was conducted on one side of each section. All measurements were repeated at different time intervals to ensure consistency of measurements. General histological examination and histometric measurements were performed with a Leica microscope (LEICA DM6000 LB, Leica Microsystems, Germany) with an attached camera. An automated image analysis software (Leica QWin V3, Leica Microsystems, Germany) was used to determine the following measurements in the defect area:

1. Amount of new (mineralised) bone in mm²;
2. Amount of residual β-TCP in mm².

The periphery of the defect area was traced with a cursor and the area was recorded. The area of new mineralised bone and residual β-TCP particles was determined in the same manner. The amount was then converted and expressed as a percentage of the total defect area.

2.3.6 Statistical analysis

In order to compare the percentage of new bone according to filling type and time period, a linear mixed effects model was fitted to the data. In the model, the type of filling material, time period, and the interaction between materials and time period were entered as predictor variables. Animal identification was entered as a random effect to account for potential dependence in measurements from the same animal. Where the model predicted a significant difference ($P<0.05$), further post-hoc testing was applied to the groups being compared. All calculations were performed using SAS Version 9.2 (SAS Institute Inc Cary, NC, USA). The GraphPad Prism program (Prism 5; GraphPad Software Inc., CA, USA) was used to create graphic images.

2.4 Results

2.4.1 Surgery and postoperative period

All surgery was uneventful and the animals recovered well, eating and walking normally within 24 hours following surgery. All implant sites healed uneventfully with no signs of complications.

2.4.2 Histological Analysis: qualitative

One implant was lost in the 4-week control group during retrieval; therefore it could not be included in the analysis.

2.4.2.1 Two-week healing period (Figure 4a-c)

Figure 4 shows the histological results after 2 weeks of healing in the control group (a), β-TCP group (b), and GEM 21S group (c). Figures 4a and 4c show new woven bone growth following the same pattern originating from existing trabecular bone at the bottom and lateral wall of defect. The new mineralised bone detected has a light brown stained appearance. At this stage of healing, most of the defects were filled with fibrous connective tissue. New mineralised bone was rarely seen in defects filled with β-TCP particles (Figure 4b). The β-TCP particles were identified as large, black masses occupying most of the defect area of Figure 4b and 4c. The particles appeared to be well contained in the membrane protected defects and were surrounded by thin fibrous tissue in Figure 4b and 4c.

2.4.2.2 Four-week healing period (Figure 4d-f)

Figure 4d-f shows the results after four weeks of healing in the control group (d), β-TCP group (e), and GEM 21S group (f). All groups demonstrated a similar pattern of new bone growth. In the control group (Figure 4d) most sections showed bone formation to be limited and confined to the margins of the defect with no sections showing any bone formation at the top of the defect. Further, all sections showed partial collapse of the defect area.
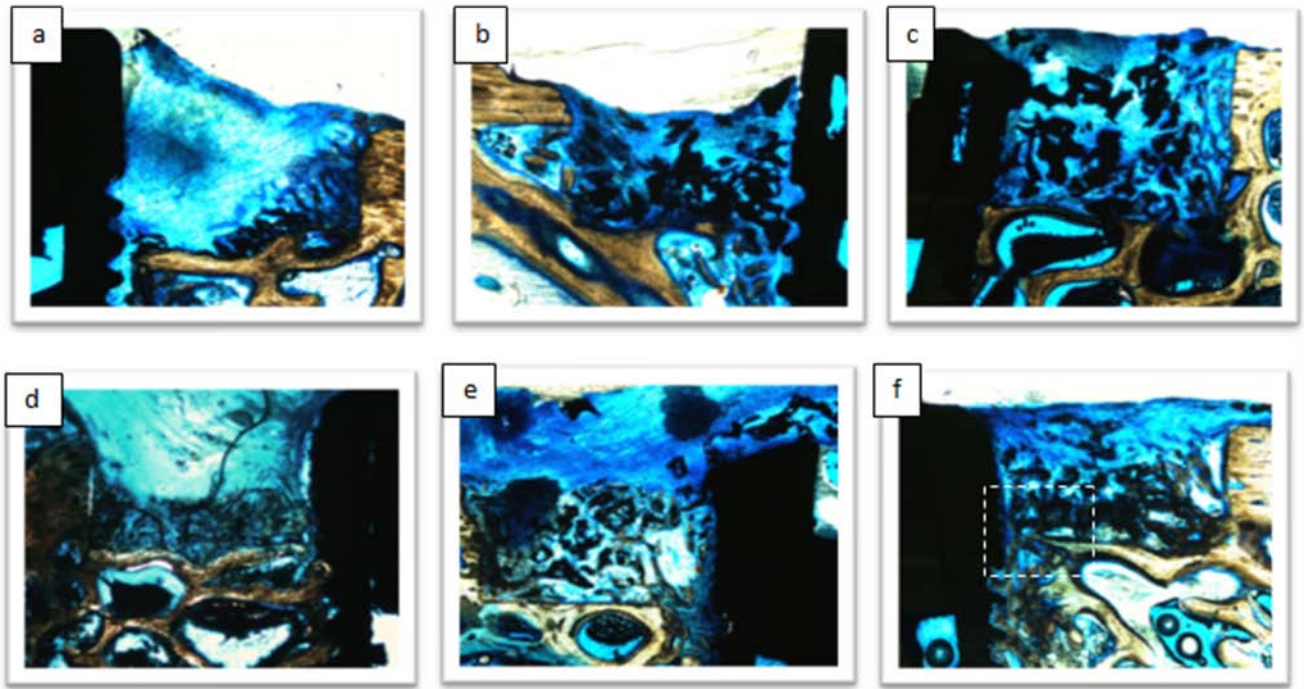
Figure 4. 2 weeks of healing in the control group (a), β-TCP group (b), and GEM 21S group (c). 4 weeks of healing in the control group (d), β-TCP group (e), and GEM 21S group (f). Undecalcified ground section, toluidine blue surface stain; original magnification x2.5.

In defects filled with β-TCP granules only (Figure 4e), new bone extended from the margins of the defect and into the middle of the defect. All sections showed that the defect area was maintained. Some sections showed new mineralised bone extending to the top of the defect. New bone was most often found around β-TCP particles and in between, forming branches of woven bone, however, the amount of new bone was inconsistent, with some sections showing only thin and sparse amount of new bone around the graft particles. Fibrous soft tissue was frequently observed adjacent to the implant surface and around residual β-TCP particles. Smaller and fewer remnants of β-TCP granules were observed than at two weeks of healing. In comparison, all sections treated with PDGF-BB (Figure 4f) consistently showed a larger amount of new bone growth from both the bottom and lateral walls of the defect, as well as around and in between the graft particles bridging the new branches of woven bone together. New bone was in contact with the implant surface in the majority of the sections (Figure 5). The new bone appeared thicker and more mature compared to the other groups, but not as mature as the existing bone. Almost complete defect fill was noted in all the sections. The β-TCP particles appeared smaller and were regularly surrounded by newly formed woven bone (Figure 6). Figures 6a and 6b show changes in size of β-TCP particles

from 2 to 4-weeks of healing with new bone enclosing the particles in PDGF-BB filled defects.
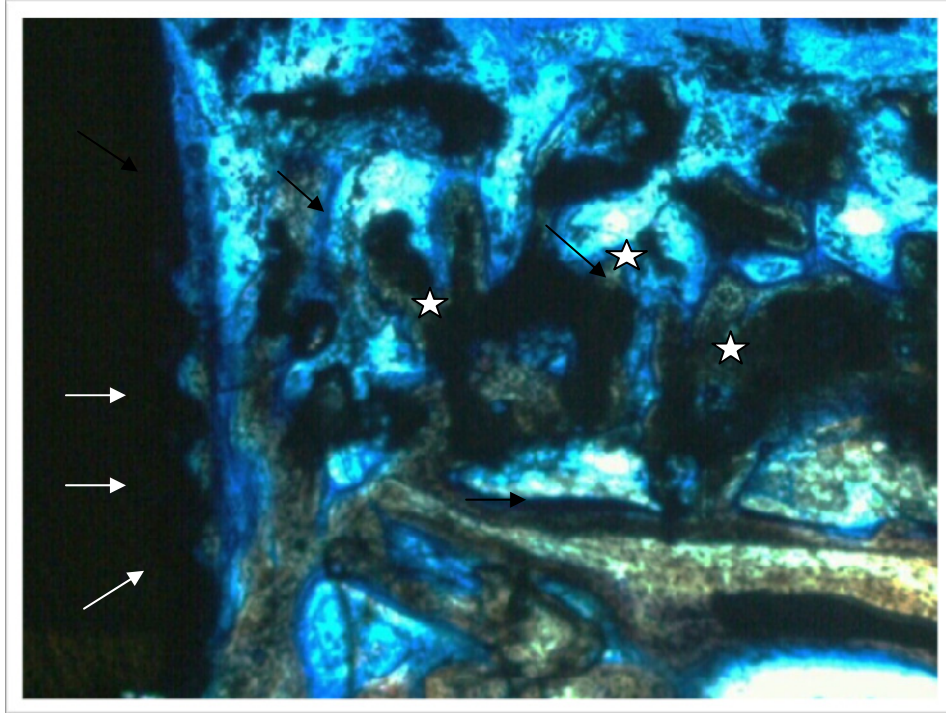

Figure 5. A magnified view of box inset in Figure 4f. Abundant new bone growth (arrows) sprouting from the base of the defect and extending directly onto threads of the implant. Residual β-TCP particles (*) is being replaced by new bone. Undecalcified ground section, toluidine blue surface stain; original magnification x5.
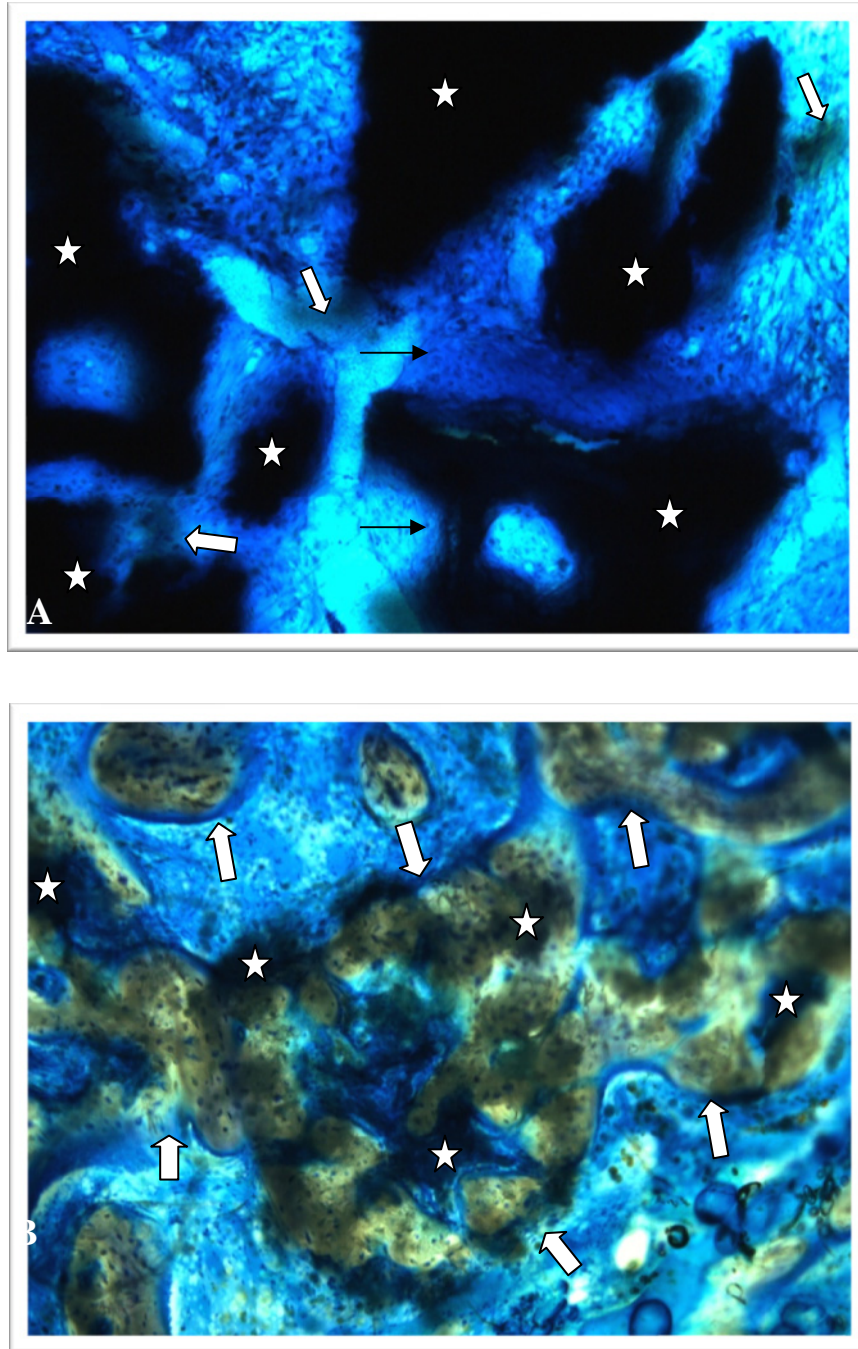
Figure 6. Changes in β-TCP particles from 2 to 4 weeks of healing with new bone growth replacing the particles in PDGF treated defects. (a) β-TCP particles (*) at 2 weeks are mostly surrounded by dense fibrous connective tissue (black arrows) but with small areas of new mineralised bone surrounding the particles (white arrows); (b) At 4 weeks of healing, smaller and fewer β-TCP particles (*) remained with abundant new woven bone (arrows) surrounding the β-TCP particles. Undecalcified ground section, toluidine blue surface stain; original magnification x10.

2.4.3 Histomorphometric analysis: quantitative

The results of the percentage of new bone and residual β-TCP particles at 2-weeks of healing are presented in Table 1 and represented in Figures 7 and 8, and the results after four weeks of healing are presented in Table 2 and represented in Figures 7 and 8.

Independent of time there was a significant difference between the three filling materials (P = 0.04). As a result, further post-hoc tests were performed indicating that the percentage of new bone was significantly higher for the combination of PDGF-BB with β-TCP compared to β-TCP alone (P = 0.017) and control (6.1% ± 3.7; P=0.015) at 2 weeks of healing. Defects filled with β-TCP particles showed the lowest average percentage of newly formed bone after 2-weeks of healing, which was significantly less than that seen in the control defects (P=0.007). At 4 weeks of healing, defects filled with the combination of PDGF-BB with β-TCP continued to show the highest percentage of new bone compared to β-TCP alone (P = <0.0001) and control (P = <0.0001). However, there was no significant difference in the percentage of new bone observed between defects filled with β-TCP or with blood clot alone at this time (P = 0.71).

Independent of which material was used, the percentage of new bone formation was higher at 4 weeks than at 2 weeks of healing for all groups, but the highest rate of new bone formation was seen in the combined PDGF-BB with β-TCP group with an increase from 11% to 52% (P< 0.0001).

The percentage of residual β-TCP particles occupying the defects at 2-weeks was significantly higher in the defects filled with β-TCP than in the growth factor treated defects (P = 0.002). However, after 4-weeks of healing, the amount of residual β-TCP particles reduced substantially in the β-TCP defects from 55% to 18% (P = 0.00007), but underwent only limited degradation in the PDGF treated defects from 34% to 31% (P=0.68).

Parameter	Treatment Group	Mean	Standard Deviation	Median
% New bone	Control	6.1	3.7	6.8
	β-TCP	1.1	0.5	1.1
	GEM 21S	11	2.9	12
% β-TCP	Control	0	0	0
	β-TCP	55	7.5	56
	GEM 21S	34	9.8	36

Table 1. Summary of results of each treatment group on percentage of new bone and residual β-TCP within the defects at 2 weeks

Parameter	Treatment Group	Mean	Standard Deviation	Median
% New bone	Control	28	15	27
	β-TCP	25	15	19
	GEM 21S	52	13	48
% β-TCP	Control	0	0	0
	β-TCP	18	11	15
	GEM 21S	31	14	35

Table 2. Summary of results of each treatment group on percentage of new bone and residual β-TCP within the defects at 4 weeks
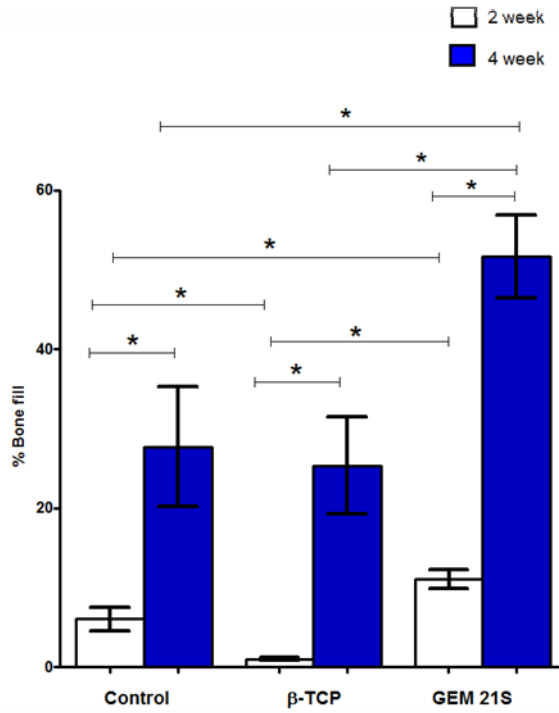
Figure 7. Graphic representation of the percentage of new bone in defects at 2 and 4 weeks of healing. New bone expressed as a percentage of defect area. The bars represent the mean of 6 measurements and the standard deviation. Asterisk, $P < 0.05$ by post-hoc tests (see text for P values).

Figure 8. Graphic representation of the percentage of residual β-TCP in the defect at 2 and 4 weeks of healing. Amount of β-TCP expressed as a percentage of defect area. The bars represent the mean of 6 measurements and the standard deviation. Asterisk , $P < 0.05$ by post-hoc tests (see text for P values).
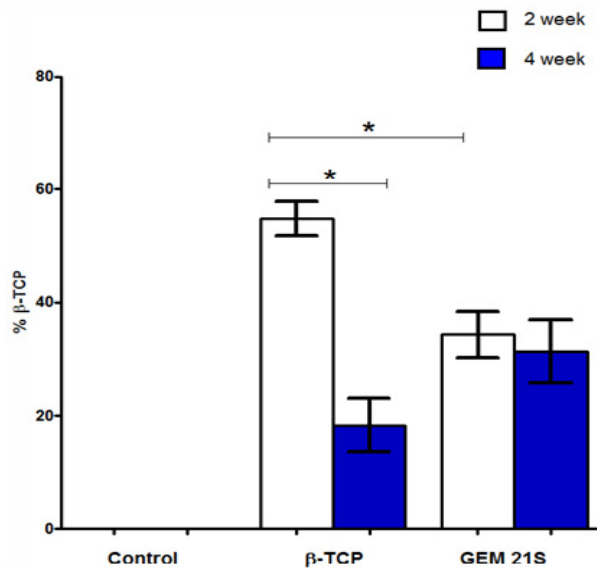
2.5 Discussion

The present investigation is a pilot study primarily designed to evaluate the effect of a growth factor rhPDGF-BB combined with a β-TCP scaffold on bone formation. It is the first study to the authors' knowledge to examine the effect of rhPDGF-BB combined with β-TCP on new bone formation around critical size peri-implant defects in a sheep pelvis model. Previous studies employed a different study design and methodology, which makes comparing results between studies difficult (Jung et al., 2008). These differences include the concentration and dose of PDGF used, whether other growth factors were added to PDGF, carrier types, animal models, and the type and size of bone defects created around the implants.

The sheep is a well established model for studying bone healing and has been frequently used in orthopaedic and dental implant research due to its similar remodelling rate, bone structure, and proportion to humans (Auer et al., 2007, Langhoff et al., 2008; Ferguson et al., 2008). Furthermore, human and sheep PDGF-B appear to be similar with approximately 90% homology (Donnelly et al., 2006). For the present study, the pelvis model was selected as it provided the ideal conditions to investigate our aims. The large size of the pelvis allowed for multiple implants with large critical size defects to be placed simultaneously and access to the site was simple and not complicated by vital anatomical structures. Further, mouth hygiene and post-op complications such as chewing forces on implants, and risk of infection were less of a problem for healing, and variation in healing of sites treated due to different thicknesses of the buccal and lingual walls of the alveolar ridge was minimised. In addition, these animals are relatively easy to handle and manage.

A critical size defect should not heal spontaneously without placement of any graft material given the time allowed for healing (Schmitz and Hollinger, 1986). It is a useful model for evaluating the regenerative potential of bone repair materials (Schmitz and Hollinger, 1986). Studies that do not employ a critical size defect model observe little or no difference in bone healing between test and control sites (Stefani et al., 2000). No difference in bone healing was observed when implants were placed almost or in contact with bone in extraction sockets filled with growth factors rhPDGF/IGF-1 around immediate implants (Stefani et al., 2000). Additionally, it has been demonstrated that dental implants placed in the ilium of sheep without critical size defects healed after two weeks in cancellous bone (Langhoff et al., 2008). Thus, the present study employed a large 3.25 mm wide and 3 mm

deep circumferential defect between the implant and bone to represent a critical size defect. As a result, none of the control defects demonstrated complete bone healing at 4 weeks of healing.

Initial bone healing of the defects was observed to occur at the base and side of the defect wall. This pattern of bony healing occurs because the defect margins are located in cancellous bone, which has exposed bone marrow with good blood supply providing a source of osteogenic and angiogenic cells to the area, and thus allowing new bone to sprout from the base and from the side wall of the defect. Similar observations of healing have been reported in previous studies using a similar defect model (Buser et al., 1998, Botticelli et al., 2003a; Jensen et al., 2007; Jung et al., 2007; Yoon et al., 2008; Araujo et al., 2010).

A healing period of 2 to 4 weeks was chosen for the present study because the effects of PDGF-BB reported in the literature appear to be most significant during the early stages of bone healing (Becker et al., 1991; Lynch et al., 1991; Nociti Jr et al., 2000; Stefani et al., 2000; Sarment *et al* 2006). Nociti Jr *et al* (2000) reported that the most obvious effect of PDGF-BB/IGF-1 was observed at 3 weeks rather than at 8 weeks of healing. Sarment *et al* (2006) reported that the highest bone turnover rate was measured at 6 weeks in PDGF-BB treated intrabony defects of humans compared to controls over a 24 week observational period. Thus, if a longer healing time was allowed, any differences in bone healing with the PDGF treatment may be less obvious compared to the control defects.

The dose level of rhPDGF-BB employed is higher than in previous studies. Similar experiments have used dose levels of 50 µg/ml (Becker et al., 1991; Nociti Jr et al., 2000; Stefani et al., 2000) and 40 µg/ml (Lynch et al., 1991), whereas the present study used a dose level of 300 µg/ml. The rationale for utilising a higher dose level was because PDGF has a high clearance rate *in vivo* (Lynch et al., 1989) and the effects of PDGF-BB on mitogenesis and chemotaxis of osteoblasts appears to be proportional to the concentration administered (Matsuda et al., 1992; Oates et al., 1993). Furthermore, the 300 µg/ml dose level of PDGF-BB is used in the product GEM 21S$^{®}$ which is FDA approved for clinical use in periodontal regeneration as it has shown to be safe in humans even with dose levels of up to 1 mg/ml (Nevins et al., 2005).

The results of the present investigation support the potential of rhPDGF-BB to improve bone formation. Given PDGF-BB has stimulatory effects on angiogenesis (Schwarz et al.,

2009), pronounced angiogenesis could allow for more osteoprogenitor cells to be recruited in the defect area. Considering PDGF-BB is a potent mitogen and chemotactic factor, it could therefore result in more osteoblast recruitment, proliferation, attachment and migration along β-TCP graft particles, as well as on the implant surface, and thus result in more bone fill in the defect. At 2-weeks, we observed almost twice the rate of new mineralised bone in the PDGF filled defects compared to the control defects, and about 10 times more than the β-TCP filled defects. After 4-weeks of healing, the PDGF filled defects revealed almost twice the rate of new bone growth compared to the control and β-TCP filled defects, which showed comparative results. Previous experiments have also reported favourable results in terms of bone healing when PDGF was used around implants (Lynch et al., 1991; Becker et al., 1992; Lee et al., 2000). Lynch *et al* (1991) reported that after 1-week, the difference in percentage of bone fill was almost 10% higher in the growth factor treated group than in the controls, and after 3-weeks of healing, the percentage of bone fill was almost 30% higher than the controls. Becker *et al* (1992) reported achieving almost twice the thickness of bone adjacent to implants in buccal dehiscence defects treated with PDGF/IGF-1 compared to controls after 4 months of healing. Lee *et al*. (2000) showed a substantial amount of new bone growth in 8 mm wide diameter critical size calvarial defects filled with a combination of rhPDGF-BB and chitosan/TCP sponge in rats. In that study, after 2 and 4-weeks of healing, the PDGF-BB filled defects showed more than two to seven times the amount of new bone compared to controls.

Comparative studies evaluating β-TCP report that the presence of β-TCP slows bone healing (Buser et al., 1998, Jensen et al., 2007; Araujo et al., 2010). Our observations corroborate the results of previous studies, as defects filled with β-TCP particles showed the least mean percentage of new bone formation after 2 and 4-weeks of healing. The reason β-TCP may retard bone formation is not well understood but it has been suggested that when the particles disintegrate, it results in the release of calcium and phosphate ions into the extracellular environment, however a high local concentration of calcium and phosphate ions above a threshold may negatively affect osteoblastic function (Yuan et al., 2001). In contrast, sections from defects treated with the addition of the growth factor PDGF-BB showed consistently greater and thicker amount of new bone throughout the defect, suggesting that PDGF-BB enhanced and accelerated bone healing even in the presence of β-TCP. Another interesting observation was the amount of β-TCP particles remaining in the defects at 2 and 4-weeks of healing. Previously, it was observed that almost 75% of β-TCP granules had disappeared in defects filled with β-TCP granules at 4 weeks of healing (Jensen et al., 2007).

By comparison, we observed a similar percentage with almost 70% of β-TCP granules degraded at 4 weeks in the β-TCP filled defects. However, defects filled with PDGF underwent only slight degradation of the β-TCP particles at 4-weeks of healing. The reason for this observation is unknown, but could suggest that the rate of dissolution of β-TCP particles may be modified in the presence of PDGF-BB. The reason calcium phosphate material is highly soluble in surrounding tissues is because it has a higher pH (Kamitakahara et al., 2008), and so it may be possible that the presence of PDGF could modify the pH of the surrounding tissues, limiting the degradation of the β-TCP granules, and thus allow the concentration of extracellular calcium ions to be maintained at a level that promotes, rather than inhibit bone formation. This phenomenon may explain why defects filled with PDGF-BB had a higher average percentage of residual β-TCP particles at 4-weeks of healing, as well as a greater percentage of new mineralised bone formed in the defects compared to defects filled with β-TCP, which had undergone a higher rate of resorption of the graft particles by 4-weeks, and resulted in a lower percentage of new bone formed.

Although β-TCP particles seem to retard bone regeneration, the presence of the bone graft substitute served two purposes: (i) provided support to maintain the defect area; and (ii) acted as an osteoconductive scaffold for new bone to form on the graft particles. All sections in the control defects showed partial collapse of the defect area which reduced the total area available for bone regeneration. In contrast, both PDGF and β-TCP-filled defect area was maintained completely, which allowed bone to form to the top of the defect. The osteoconductive effect of β-TCP was clearly demonstrated as the particles were frequently surrounded by new bone after 4-weeks of healing, confirming similar observations from previous experiments (Gatti et al., 1990, Lee et al., 2000; Jensen et al., 2007; Araujo et al., 2010). The osteoconductive effect of β-TCP may be due to dissolution of β-TCP particles, which releases calcium ions into the extracellular environment. An increase in concentration of calcium ions inhibits osteoclastic bone resorption, and promotes bone formation (Yamada et al., 1997).

There were advantages and disadvantages to the study design chosen for the current experiment. Each sheep received the same treatment for all three defects to avoid contamination of PDGF-BB into adjacent defects, since contamination of adjacent sites with the growth factor is possible (Becker et al., 1992). However, this study design prevented the evaluation of how all three treatments would respond in each sheep. Additionally, bone quality can vary between individual implant sites, as the ilium has a broad variety of bone

qualities with almost purely cancellous (0.5 mm cortical thickness) to compact cortical bone (up to 3 mm thickness) (Ferguson et al., 2008). Although similar sites of the ilium were chosen for implant placement between all the sheep, this variation in bone quality may have resulted in differences in the healing response, which may contribute to the diverse histological measurements obtained at the three implant sites within the same sheep. Lastly, even though all the sheep were genetically dissimilar, it may be possible that the healing response of each sheep is similar, considering that all the sheep used in the current study were taken from the same flock, were similar in age, and exposed to the same environmental factors.

2.6 Conclusion

In conclusion, the results of the present study show promise to the approach of using a combination of rhPDGF-BB and β-TCP particles to accelerate bone healing when used in contained critical sized peri-implant defects. However, it must be emphasised that these results cannot be extrapolated to the human clinical situation since it is a pilot study, in which only a very small number of animals were used to demonstrate proof of principle. Additional studies to confirm the results of the present study will be necessary, however, future trials may benefit with the use of a bilateral approach to greatly enhance statistical power and interpretation of the data when a small number of animals are used. Future investigations should also evaluate the potential of rhPDGF-BB combined with β-TCP to enhance bone regeneration in other clinical applications including different bony defect models or in subjects with disease situations involving a compromise in bone healing such as in diabetes. More recent studies on rhPDGF-BB have explored combinations with other carriers, as well as a gene delivery approach where the growth factor could be released over a prolonged time to enhance bone regeneration in larger defects (Schwarz et al., 2009b, Chang et al., 2010).

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