Alveolar bone healing using a novel bone substitute material in a sheep tooth extraction model

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“to Him who is able to do superabundantly above all that we ask or think, to Him be the glory…”

Eph. 3:20-21
Abstract
Bone substitute materials placed into tooth sockets after tooth extraction may preserve alveolar bone. It is desirable that these materials be completely resorbed and replaced by patient’s own bone. Electrospun cottonwool-like nanocomposite (ECWN) is a novel synthetic bone substitute that incorporates amorphous tricalcium phosphate nanoparticles into a biodegradable synthetic copolymer poly(lactide-co-glycolide). This has shown encouraging results in a rabbit calvarial defect but has not been tested in a large animal model.

Objectives:
1. To develop a tooth extraction socket model in sheep for bone graft research.
2. To compare ECWN and bovine-derived xenograft (BX) in this model.

Methodology:
Eighteen crossbred female sheep aged four to five years were used. Bilateral mandibular premolars were extracted atraumatically using Piezosurgery® unit. Second and third premolar sockets were grafted (Latin-square allocation) with BX, ECWN or left unfilled. Resorbable collagen membranes were placed over BX and selected ECWN grafted sockets and primary flap closure achieved. Two sheep were sacrificed at baseline and eight sheep each time after eight & 16 weeks. Resin-embedded undemineralised sections were examined for descriptive histology, histomorphometric and histometric analyses.

Results:
At eight weeks, healing was composed mostly of woven bone with no distinct differences among the different sites. At 16 weeks, osseous healing followed a fine finger-like trabecular pattern in ECWN sites. Non-grafted sites showed thick trabeculae separated by large areas of fibrous stroma. In BX grafted sites, residual graft material was encapsulated by newly formed bone or fibrous connective tissue. There were no statistically significant differences in bone formation across the four groups at eight or 16 weeks. However, ECWN sites had significantly less residual graft material than BX sites at 16 weeks ($p = 0.048$). There were significantly more hard tissue bridging formed in ECWN- sites when compared to B+ sites at 16 weeks ($p = 0.024$).

Conclusion:
This first description of a tooth extraction socket model in sheep supports the utility of this model for bone graft research. The results of this study suggested that the novel material ECWN did not impede bone ingrowth into sockets and showed evidence of material resorption. The present study also confirmed previous findings where new bone was formed to encapsulate BX particles.
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List of abbreviations

ATCP  amorphous tricalcium phosphate
B+    bovine xenograft with resorbable collagen membrane
BIC   bone to implant contact
BGM   bone graft/substitute material
BX    bovine xenograft
ECWN  electrospun cottonwool-like nanocomposite
ECWN+ electrospun cottonwool-like nanocomposite with collagen membrane
ECWN- electrospun cottonwool-like nanocomposite alone
EDTA  ethylenediaminetetraacetic acid
FCT   fibrovascular connective tissue
H&E   haematoxylin and eosin
HAp   hydroxyapatite
NB    newly formed bone
NBF   neutral buffered formalin
NO-   no graft
P_2   second mandibular premolar
P_3   third mandibular premolar
PLGA  poly(lactide-co-glycolide)
RG    residual graft material
ROI   region of interest
SEM   scanning electron microscope
Chapter 1 Introduction and literature review

The alveolar process develops with the eruption of teeth. How much is formed is determined by where the teeth are and the tooth angulation (Schroeder, 1986). Following tooth extraction, alveolar bone resorption occurs most rapidly in the first 6 months (Lam, 1960). A common finding 6 months after tooth extraction is a saddle-shaped ridge between the teeth adjacent to the extraction site. Schropp et al. (2003) identified 50% reduction in the width of the alveolar ridge, of which two thirds occurred during the first three months. There is more loss in the bucco-lingual width than the corono-apical height of the alveolar ridge (Lekovic et al., 1998). This can complicate the replacement of the missing tooth, since successful rehabilitation options need adequate bone height and width.

Alveolar bone resorption following tooth extraction may pose problems for clinicians in several ways. The collapsed alveolar bone and soft tissue can lead to unacceptable prosthesis aesthetics. These deformities may result in the use of prosthetic materials to restore the jaw contours (Seibert and Salama, 1996). This frequently causes the prosthesis to appear unnatural. Correct three dimensional positioning of implants may also be compromised due to the lack of hard and soft tissue support (Irinakis, 2006). In addition, irregularities are formed after spontaneous tooth socket healing. This may cause denture construction to be difficult or make the placement of an implant challenging if not impossible.

Ridge preservation refers to procedures that maximise retention of bone at the extraction site. Bone graft/substitute materials (BGM) are placed into the tooth socket immediately after tooth extraction, where they act as a scaffold for the ingrowth of cells and blood vessels and the formation of new bone in the healing tooth socket. It is desirable that BGM be completely reabsorbed during this process and be replaced by the patient’s own bone.

Bone graft/substitute materials are obtained from various sources. The “gold standard” is bone harvested from another site in the patient. However the need for a second surgical site has led to the development of other bone substitutes. Bovine xenograft (BX) is commercially available. The combination of collagen membranes and BX is one of the most researched combinations (Araújo et al., 2008; Araújo et al., 2009; Barone et al., 2008). The common finding from the studies using BX as the
bone graft material was that the elimination of graft material within the graft site may require a long time, possibly years (Artzi et al., 2000; Molly et al., 2008). The non-resorbed graft materials may act as a barrier to bone formation. This may delay socket healing and raise concerns for future implant placement and osteointegration.

In a recent publication by Schneider et al. (2009), the performance of a flexible, mouldable, electrospun cottonwool-like nanocomposite (ECWN) was assessed. This material incorporates amorphous tricalcium phosphate nanoparticles (ATCP) into a biodegradable synthetic copolymer poly(lactide-co-glycolide) (PLGA). This material is prepared through an electrospinning process, which give it the typical cotton wool-like appearance. This characteristic of the material allows easy proportioning, handling and adaption to a bone defect. The in vivo study showed high bioactivity of ECWN four weeks after implantation, with the formation of new bone and increased cell density. Resorption of the graft material as early as 4 weeks was also reported in this same study. However, the authors also highlighted the need for further investigations in animals to evaluate the long-term stability and clinical outcome of this material.

This present study was designed to establish a tooth extraction socket model for bone graft research in sheep and evaluate the extraction socket healing following the implantation of ECWN and bovine xenograft in the sheep extraction socket model.

The following chapter reviews the literature concerning the healing process following tooth extraction, the rational for alveolar ridge preservation, the bone graft/substitute materials used for this procedure, the development of a new material for ridge preservation and the rationale of the development of a new extraction socket model in sheep. A review of the current histometric and histomorphometric analysis techniques reported in the literature is also included in this chapter.
1.1 Alveolar bone remodelling

The alveolar process is the portion of the mandible or maxilla that contains the roots of the teeth. As a tooth dependent structure, it develops with the eruption of teeth. How much is formed is determined by where the teeth are and tooth angulation (Schroeder, 1986). Therefore, its continued preservation also depends on the presence of teeth. Subsequent to tooth extraction, the alveolar process undergoes resorption with marked alterations to the height and width of the alveolar ridge.

1.1.1 Alveolar process

The alveolar process is comprised of three parts: buccal and lingual cortical plates, the central portion of cancellous bone and the alveolar bone proper (Lindhe et al., 2008). Buccal and lingual cortical plates are made up of lamellar bone. They are in continuity with the cortices of the body of the jaws and the alveolar bone proper. The alveolar bone proper, also known as the bundle bone, is the bone that lines the sockets. This is the tissue in which the extrinsic collagen fibre bundles of the periodontal ligament are embedded. The alveolar bone proper and the cortical plates merge at the alveolar bone crest. The cancellous bone is found in between the cortical plates and the alveolar bone proper. It is made of bone trabeculae surrounded by a marrow that is rich in adipocytes and pluripotent, mesenchymal stem cells. These undifferentiated mesenchymal stem cells are able to differentiate into bone forming cells, haematopoietic cells and osteoclasts.

1.1.2 Healing following tooth extraction

Tissue alterations following tooth extraction can be classified into two interrelated processes. These are extraction socket healing and alveolar bone remodeling.

1.1.2.1 Extraction socket healing

The healing process following tooth extraction was first studied using animal models. Using animal models, early studies carried out prior to the 1960s provided valuable overview information of the healing process (Claflin, 1936; Huebsch et al., 1952). In the 1960s, Amler et al. (1960) were the first group to study human extraction socket healing in a detailed time sequence.
Amler (1969) studied the healing in human extraction sockets for up to 50 days following tooth extraction. Biopsies from the healing sockets were taken from volunteers. The socket is initially filled with a blood clot. Within two to four days, granulation tissue begins to form and partially replaces the clot. After one week, the blood clot is completely replaced by granulation tissue. Young connective tissues begin to form in the central and lateral areas of the socket. A vascular network is also formed in the socket with the appearance of osteoblasts and osteoclasts. By three to four weeks, mineralization of the uncalcified osteoid starts to take place. At six weeks, the extraction socket is completely covered by keratinized epithelium and is filled with woven bone. However, this study was only of short duration. The later phase of healing and tissue composition of the fully healed extraction site was not reported.

Dog extraction socket model was developed to study the long-term post-extraction healing (Cardaropoli et al., 2003). Using demineralised histology sections, they demonstrated similar early socket healing events. After seven days of healing, provisional matrix is present, comprising newly formed blood vessels, immature mesenchymal cells, leukocytes and collagen fibres. Several marrow spaces were found within the bundle bone lining the socket harboured osteoclasts. At two weeks, the bundle bone of the extraction socket is absent in most areas and communications exist between the bone marrow spaces of the alveolar bone and the newly formed tissue in the socket. Woven bone that is rich in cells starts to form adjacent to newly formed blood vessels. Therefore, during the early phase of healing, the bundle bone is removed and replaced with woven bone. At 30 days, the woven bone is undergoing osteoclastic resorption. This indicates that modelling/remodelling process of the newly formed woven bone has begun. The woven bone is first resorbed to a certain level and then replaced with new lamellar bone.

However, after 60 days of healing, a hard tissue bridge that separates the marginal mucosa from the extraction socket starts to form. This structure is mainly composed of woven bone. This hard tissue bridge is reinforced by layers of lamellar bone that is deposited on top of the woven bone after 120 to 180 days of healing. Collagen fibres from the lining mucosa are inserted in the new cortical bone, forming a periosteum-like structure. Below the marginal bone bridge, the entire region of the extraction socket is filled with bone marrow and lamellar bone.
1.1.2.2 Alveolar bone remodelling

As bone forms in the extraction socket, resorption of the residual alveolar ridge is also taking place. Following the removal of all teeth in adult patients, bone resorption of residual ridges is a common finding (Atwood and Coy, 1971; Tallgren, 1972). In cases following single or multiple tooth extraction, significant changes in the height and width of the alveolar ridge were also reported (Pietrokovski and Massler, 1967).

Resorption occurs most rapidly in the first three to four months following tooth extraction (Johnson, 1969; Lam, 1960). Schropp et al. (2003) identified 50% reduction in the width of the alveolar ridge over 12-month period. Two thirds of the reduction occurred during the first three months.

There is more loss in the bucco-lingual width than in the corono-apical height of the alveolar ridge (Lekovic et al., 1998). A systematic review on the alveolar bone dimensional changes of human post-extraction sockets included 12 publications (six randomized controlled trials, five clinical trials and one case series study) (Van Der Weijden et al., 2009). The reduction in width of the alveolar ridges was 3.87mm, whereas the mean clinical mid-buccal height loss was 1.67mm.

The buccal surface of the alveolar process undergoes more resorption than the palatal/lingual surface. Using plaster casts models, Pietrokovski and Massler (1967) studied tissue changes after unilateral tooth extraction. They concluded more bone resorption occurred around the buccal bone plates than the corresponding palatal/lingual plates.

Araújo and Lindhe (2005) confirmed these findings using the dog extraction model. They demonstrated the relationship between the buccal and lingual bone crest over 8 weeks period following tooth extraction. At one week post-extraction, the buccal bone crest was coronal to the lingual crest. However, after eight weeks, the buccal bone crest was 1.9mm ± 0.2mm apical to the lingual crest.

Possible explanations for these findings are summarized here. First, the lingual hard tissue wall is substantially wider than the buccal wall. A layer of bundle bone occupies the inner portion of the lingual wall. However, as Araújo and Lindhe (2005) found in their histology sections, the marginal 1-2mm of the crest of the buccal wall is comprised entirely of bundle bone. This tissue is a tooth-dependent tissue that
gradually disappears after tooth extraction. For this reason, alveolar bone loss will be more prominent in the buccal wall.

In addition, the buccal bone is usually thin, frail, and composed mostly of cortical bone. It is more prone to resorption as the result of trauma exerted during extraction procedure (Fickl et al., 2008a). Flap elevation and the separation of the periosteum from the bone tissue will also result in surface resorption (Staffileno, 1974; Wood et al., 1972). This effect may result in more bone height reduction of the thin buccal bone than the wider lingual bone.

1.1.3 Consequences of alveolar bone resorption

Alveolar bone resorption following tooth extraction may pose several problems for clinicians when restoring the edentulous area. Firstly, the collapsed alveolar bone and soft tissue can lead to unacceptable prosthesis aesthetics. This usually occurs in the anterior and premolar region. These deformities may require the use of prosthetic materials to restore the jaw contours (Seibert and Salama, 1996). The pontic teeth or implant-supported crown used to restore this area may need to be longer than the adjacent teeth or incorporate the use of a flange or extension to simulate gingival tissue (Christensen, 1996). This frequently causes the prosthesis to appear unnatural.

Secondly, correct three-dimensional positioning of implants may also be compromised due to lack of hard and soft tissue support. An adequate width and height of the alveolar ridge is important for implant placement. A buccal concavity may form as a result of the resorption pattern. This may lead to an unfavourable condition for implant placement.

The third major problem that may arise from alveolar bone resorption is the irregularities formed after spontaneous tooth socket healing, especially when multiple teeth are extracted. This may cause denture construction to be difficult or make the placement of an implant challenging if not impossible.

1.1.4 Summary

As a tooth dependent structure, the alveolar process undergoes resorption subsequent to tooth extraction. Marked alterations to the height and width of the alveolar ridge are often observed after three months of healing. This poses several aesthetic and functional problems for future rehabilitations of the edentulous area, especially in today’s prosthetically driven implant therapy.
1.2 Alveolar ridge preservation

Alveolar ridge preservation has been defined as “any procedure undertaken at the time of, or following an extraction that is designed to minimize external resorption of the ridge and maximize bone formation within the socket” (Darby et al., 2008). This procedure is also known in the literature as socket augmentation or socket preservation. Socket preservation has been used to describe the treatment of fresh extraction sockets with intact buccal bone walls (Ackermann, 2009). Ridge preservation, on the other hand, was referred to in situations involving deficient buccal bone walls. Ridge preservation will be used to describe this procedure in this thesis.

1.2.1 History of ridge preservation

The concept of ridge preservation was developed around the late 1970s. Residual ridge resorption posed a significant problem for complete denture wearers. Observations were noted where bone resorption did not occur around retained teeth. Therefore, the concept of vital root retention was proposed (Garver et al., 1978). However, this was later abandoned due to soft tissue complications (Garver and Fenster, 1980; Von Wowern and Winther, 1981). The use of synthetic materials, such as polymethylmethacrylate, for this purpose was also reported. However, they were subject to host rejection and fibrous encapsulation (Ashman and Moss, 1977; Ashman and Bruins, 1985). A similar concept using non-resorbable hydroxyapatite root-shaped implants was evaluated in the 1980s (Boyne et al., 1984; Quinn and Kent, 1984). New bone formation over these implants was observed after 6-month of healing. Residual ridge resorption was limited throughout the study periods.

Current methods used for ridge preservation include placement of bone graft/substitute materials into the extraction sockets. The concept of ridge preservation was also advanced from the initial prevention of residual ridge resorption in complete denture wearers to maintaining the functional and aesthetic requirements for fixed and removable prosthesis. An adequate width and height of the alveolar ridge is essential for implant placement. With the increased popularity of endosseous implants, the latest area of interest for ridge preservation is with the implant site development.
1.2.2 Rationale for ridge preservation

If an implant placement is scheduled within six to eight weeks after extraction, ridge preservation using bone graft/substitute materials to encourage bone fill is not necessary. However, if implant placement is to be delayed or even if there is no plan for implant placement to replace the tooth in the near future, ridge preservation should be considered. This not only maximises the possibility of an implant option in the future without major bone graft surgery, but also preserves the aesthetic appearance at pontic sites in conventional fixed prosthodontics or the bony support to removable prosthodontics (Darby et al., 2008).

Other indications summarised by Darby et al. (2008) are as follows: firstly, sites where the buccal plate is less than 1.5-2mm thick and where there has been damage or loss of one or more of the socket walls. These sites may lose a clinically significant amount of buccal plate during healing, which will lead to width reduction of the alveolar ridge. Another indication is for sites close to anatomical structures, such as posterior maxillae and mandible. If the alveolar bone is lost, implant placement in the future may pose additional risk to the surrounding structures, maxillary sinus and inferior dental nerve. Ridge preservation should also be considered when a patient has high aesthetic demands and when many teeth are to be extracted and ridge preservation is crucial for future restoration.

1.2.3 Clinical procedures

Several clinical guidelines have been proposed to carry out ridge preservation procedures (see review by Tischler and Misch, 2004; Wang et al., 2004). However, they vary considerably in the literature and are summarised in this section (Table 1.1).
Table 1.1 Summary of ridge preservation procedures in the literature

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1.2.4 Common practices

1.2.4.1 Atraumatic tooth removal

After local anaesthesia is achieved, the supracrestal attachment apparatus includes the epithelium and connective tissue attachments to the tooth surface is cut by sulcular incisions. Periotomes are applied to sever the subcrestal attachment apparatus circumferentially and moved further down towards the apical one third of the tooth. Elevation of the tooth may be required to achieve tooth mobility. Then dental forceps can be applied to extract the tooth without intruding the forceps into the periodontal ligament space. If the tooth is multi-rooted, it should be decoronated and the roots are sectioned and extracted separately. This will help to preserve the walls of the tooth socket and allow for the placement of graft material.

Raising a full-thickness periosteal flap has been associated with increased alveolar bone resorption (Staffileno, 1974; Melcher, 1976). The cells of peristium make a major contribution to healing of bone wounds. It consists of at least two layers: an outer fibrous layer that does not appear to possess osteogenic potential and an inner osteogenic layer. Following the elevation and replacement of a gingival flap, osteogenesis may be preceded by necrosis and resorption of bone. New bone that is deposited in the site can only take origin from cells of undisturbed periosteum surrounding the flap.
Fickl et al. (2008a) confirmed the above findings using dog extraction socket model. They compared tissue alterations after tooth extraction with and without surgical trauma. Surgical trauma was exerted with the elevation of a muco-periosteal flap with vertical releasing incisions. They reported significantly higher bone resorption rate on the buccal side with the surgical trauma group when compared to the “flapless” group.

1.2.4.2 Debridement and removal of chronic inflammatory tissue

After tooth extraction, the tooth socket should be thoroughly debrided (Tischler and Misch, 2004). Chronic inflammatory tissues should be removed with a curette or other surgical instrument. Adequate blood supply to the extraction socket is necessary as it contains osteoprogenitor cells, proteins and growth factors for bone healing. If there is bleeding from the walls of the alveolar bone after the extraction, the vascular supply to the area is assured. Otherwise, bleeding should be encouraged. This can be done using a round bur to perforate the socket walls in order to gain greater access for blood vessels into the socket (Buser et al., 1993).

However, whether or not the remnant PDL cells contribute to socket healing has been controversial. An experimental study carried out in dog extraction model demonstrated the presence of a large number of periodontal ligament cells close to the coagulum and PDL-like cells to be part of the provisional matrix (Cardaropoli et al., 2003). The authors suggested that PDL cells might contribute to hard tissue formation in the healing socket. Using a cell labelling technique to follow the fate of PDL fibroblasts, Lin et al. (1994) demonstrated that PDL fibroblasts proliferated and migrated into the centre of the extraction socket and differentiated into osteoblasts for the formation of new bone. However, a later study by the first group showed close to identical wound healing characteristics in PDL replete sites and PDL deprived sites (Cardaropoli et al., 2005).

Ridge preservation with grafting materials should be delayed in the event of acute infection (Darby et al., 2009). Infection must be controlled as this may interfere with the osteoprogenitor cells migrating to reach the graft site for bone remodelling.

1.2.5 Variation in practices

Variation in ridge preservation procedures can be summarized under three main areas: bone graft/substitute materials, barrier membranes, and soft tissue coverage.
1.2.5.1 Use of bone graft/substitute materials

To maximize bone formation within the extraction sockets and minimize alveolar ridge resorption, bone graft/substitute materials are placed into the debrided sockets. The use of bone graft/substitute materials is often accompanied by the placement of barrier membrane over the extraction sockets. Many bone grafting and bone substitute materials have been developed and researched for ridge preservation. These include autogenous bone, allografts, xenografts, and alloplastic materials. These materials will be discussed in detail in the following section.

1.2.5.2 Barrier membranes

According to the guided tissue regeneration principle, barrier membranes are used to cover the socket and to prevent the downgrowth of soft tissue into the socket. This may in turn allow maximal bone infill (Darby et al., 2008). They have been reported to be used alone or in combination with a bone graft/substitute material. Flap elevation is required when a barrier membrane is used in ridge preservation. There is a wide range of barrier membranes available. They can be either non-resorbable or resorbable membranes.

Lekovic et al. (1997) demonstrated less alveolar bone loss in height and width associated with the extraction socket covered with expanded polytetrafluoroethylene (ePTFE) membrane alone six months after extraction when compared with controls (Lekovic et al., 1997). However, some notable disadvantages of non-resorbable ePTFE membrane include difficulty in handling and placement, risk of wound dehiscence and infection, and the need for a second surgery for removal (Augthun et al., 1995; Chiapasco et al., 1999). Post-operative complications such as, infection and exposure of the membrane, were also reported as complications (Lekovic et al., 1997).

Bioresorbable collagen membranes should be combined with appropriate bone graft/substitute materials to avoid membrane collapse and to enhance new bone formation in the membrane-protected space. Cross-linked collagen membranes, mostly from porcine or bovine origin, have been shown to have haemostatic function that allows wound stabilization and chemotactic effect over gingival fibroblasts (Rothamel et al., 2004). Membrane permeability also allows nutrient transfer into the healing site.
Current clinical trends support the use of resorbable membranes with bone graft/substitute materials. The combination of collagen membranes and bovine xenograft is one of the most researched combinations (Araújo et al., 2008; Araújo et al., 2009; Barone et al., 2008). Specific combinations of membranes and bone substitute materials that would afford optimal results in ridge preservation have not yet been identified. However, when one aspect of the alveolar wall is missing, a barrier membrane should be used to contain the graft material (Tischler and Misch, 2004).

1.2.5.3 Soft tissue coverage

To prevent the contamination and loss of graft material and barrier membrane, soft tissue closure over the grafted site has been suggested. Several techniques have been discussed in the literature. Soft tissue coverage at the time of extraction can be achieved by splitting the periosteum at the base of a flap and advance the flap coronally across the alveolar ridge (Becker et al., 1994). However, this may cause unsightly appearance in aesthetic zone, such as altering the mucogingival line, and creating a shallow vestibule. These problems will then need to be addressed subsequently by another surgical procedure.

To avoid the complications from coronally advanced flap, Artzi et al. (2000) reported the use of a partial thickness pedicle flap over the preserved site in the maxillary arch. The same group of researchers also reported the use of a free gingival graft harvested from the palate to seal the preserved site (Tal, 1999). However, around 11% of all grafts were non-vital and could be easily removed at 1-week post-operative review. This procedure was modified and simplified by Jung et al. (2004). The free gingival graft was harvested by using soft tissue biopsy punch and sutured unto de-epithelialized surfaces above the extraction sockets. They reported only 0.1% of the surface areas appeared necrotic after 1 week.

Another technique that involves no flap elevation or the use of membrane was first described by Sclar (1999) and later modified by Wang and Tsao (2008). After the selected graft material is placed and condensed into the socket, a collagen matrix, Collaplug® (Zimmer Dental, Carlsbad, CA, USA), is placed over the graft. The grafted site is secured with horizontal mattress suture without the use of barrier membrane. No primary closure is achieved over the socket. Spontaneous
epithelialisation of the socket is allowed to form under a denture tooth or ovate pontic. However, no long-term studies have been conducted to support the use of this technique for ridge preservation.

Whether soft tissue coverage at the time of extraction is required for optimum healing of the socket is still debatable. Using a dog extraction model, Fickl et al. (2008c) showed no difference in vertical buccal bone loss using Bio-Oss® collagen with or without gingival graft. Sockets grafted with Bio-Oss® Collagen alone had more width reduction than those grafted with Bio-Oss® and gingival graft ($p<0.05$). However, the statistical power of this study is weak due to the limited number of sites. Therefore, the results may only represent a trend for further investigations.

1.2.6 Ridge preservation outcomes

Various systematic reviews have been carried out to evaluate surgical protocols and healing after ridge preservation therapies (Ten Heggeler et al., 2011; Morjaria et al., 2012; Vignoletti et al., 2012b). Ten Heggeler et al. (2011) reviewed bone dimensional changes following ridge preservation therapy in non-molar regions. Nine studies were included in this review. No meta-analysis was conducted. The authors reported a mean reduction in width ranging between 2.6 and 4.6mm and in height between 0.4 and 3.9mm following natural healing after extraction. A mean reduction in width and height was also observed in the test groups, which ranged between 1.2 and 3.5mm for width and between 0.4 and 0.7mm for height.

Vignoletti et al. (2012b) evaluated the efficacy in the surgical protocols designed for ridge preservation therapy. Fourteen randomized clinical trials and prospective cohort studies met the eligibility criteria, of which nine studies were included in the meta-analyses. The control groups demonstrated a mean vertical bone loss ranged from 0.3 to 3.8mm and horizontal bone loss from 0.2 to 4.5mm. Results were more heterogeneous in the test groups. The mean vertical bone changes ranged from -2.5mm to 1.3mm and the mean horizontal bone changes ranged from -2.5 to 3.3mm. Results from the meta-analyses showed a greater ridge height reduction for the control groups (weighted mean difference (WMD) = -1.47mm; 95% confidence interval (CI) (-1.98, -0.95). There was also a greater bone width reduction for control groups compared to the test groups (WMD = -1.83; 95% CI (-2.95, -0.73).
Similar results were reported in the systematic review conducted by Morjaria et al. (2012). Although a meta-analysis was not conducted, the review found the mean loss of bone width ranged from 2.46 to 4.56mm in the control groups compared to 1.14mm to 2.5mm in the test sites. The mean loss of height in the control sites was 0.9 to 3.6mm compared to a gain of 1.3mm to a loss of 0.62mm in the test sites.

The variability in these outcomes obtained following ridge preservation therapy may be due to the clinical conditions of the socket site, the surgical protocol utilized, the biomaterial or barrier membrane used and the type of evaluation method used (Vignoletti et al., 2012b). The efficacy of using barrier membranes with or without graft materials in maintaining alveolar ridge dimensions have been demonstrated in a recent systematic review (Vittorini Orgeas et al., 2012). This may be due to the protective and space-maintaining effect of the barriers on the blood clot inside the socket and on the remaining bone walls outside the socket. No conclusions could be made from the current literature on the effect of primary wound closure following ridge preservation therapy. No significant differences were found on the ridge width changes between the test sites with primary closure or control sites (Engler-Hamm et al., 2011). However, greater post-operative discomfort was reported in the group with primary wound closure.

Studies included a histological investigation demonstrated concerns of the large amount of residual graft material and the variation in the amount of vital bone within the test sites (Froum et al., 2002; Iasella et al., 2003; Barone et al., 2008; Fiorellini et al., 2005). Froum et al (2002) demonstrated that almost three times more residual graft material was found in demineralised freeze dried allograft sites when compared to bioactive glass grafted sites. It was speculated that the presence of residual graft material might interfere with the osseointegration process or have like a foreign body and enhancing a developing inflammatory lesion (Morjaria et al., 2012).

One of the objectives of ridge preservation is to maintain adequate alveolar bone dimensions in order to facilitate implant placement in prosthetically driven positions (Tarnow and Eskow, 1996) and avoid the necessity for alveolar ridge reconstruction (Horváth et al., 2013). In the studies selected for the systematic reviews (Horváth et al., 2013; Vignoletti et al., 2012), the majority of the studies did not report differences in the feasibility of implant insertion following ridge preservation procedures. Fiorellini et al. (2005) reported less augmentation had to be performed in the test
group compared to the control group. Two studies (Serino et al., 2003; Serino et al., 2008) reported the placement of dental implants achieved good primary stability in both test and control groups. No studies have reported the long-term success rate of the inserted dental implants.

1.2.7 Immediate implant

The term “immediate implant” refers to dental implants that have been placed into fresh extraction sockets (for review see Mayfield, 1999; Wang and Lang, 2012). This reduces the number of surgical procedures required for the patient as well as reduces the interval between extraction and insertion of the prosthetic restoration (Botticelli et al., 2004). It has also been suggested that immediate implant may preserve the dimensions of alveolar bone. However, controversies exist on this issue. While some clinical studies showed preservation of hard and soft tissue after immediate implant placements (Denissen and Kalk, 1991; Wheeler et al., 2000), other animal and clinical studies revealed opposite results. A study on dog model by Araújo et al. (2005) found that the placement of implants in fresh extraction sockets failed to prevent the remodelling of the socket walls. Similar findings were also reported in a recent animal study with 6-week healing period (Vignoletti et al., 2012a). Botticelli et al. (2004) conducted a prospective clinical trial to study the dimensional changes of hard tissues following immediate implant placements in 18 patients. They also reported a 56% horizontal bone loss from the buccal aspect and a 30% bone loss from the lingual aspect four months after the immediate implants were placed. This finding corresponds to the normal healing pattern of alveolar bone after tooth extraction as reported by Schropp et al. (2003).

1.2.8 Summary

Ridge preservation is carried out to preserve the ridge volume present at the time of tooth extraction. Various clinical procedures have been described and investigated in the literature. Most of these techniques involved atraumatic extraction, removal of inflammatory tissues and placement of graft material into the extraction sockets. The potential benefits of ridge preservation therapies have been demonstrated in these review articles. Overall, ridge preservation therapies did reduce alveolar ridge dimensional changes following tooth extraction. However, the therapies carried out
did not prevent bone resorption. No studies have reported the long term success rate of the inserted dental implants.
1.3 Bone graft/substitute materials

Augmentation of extraction sites with graft materials has been shown to reduce or limit alveolar bone resorption (for reviews see Darby et al., 2009; Vignoletti et al., 2012b). This section will discuss the possible mechanisms of ridge preservation using bone graft/substitute materials (BGMs), their properties and origins.

1.3.1 Mechanism of ridge preservation

The mechanism of ridge preservation using BGMs has not been fully explained in the literature. Several possible mechanisms were summarized by Bartee (2001).

1.3.1.1 Biomechanical stimulation

During normal jaw function, grafting materials within the extraction socket may provide physiologic and bioelectric stimulation to extraction socket wall and alveolar bone (Ortman et al., 1992; Kalk et al., 1993). Compressive, shear and tensile forces may be transmitted from the random orientation of graft particles to bone in a manner similar to the periodontal ligament of a natural tooth. Elevated remodeling activity with increased bone density may result from mismatch in elastic modulus between graft materials and bone (Garetto et al., 1995). Therefore, these indirect forces within the physiologic range transmitted onto the bone-graft interface may contribute to bone preservation.

1.3.1.2 Wound isolation and scaffolding effect

Bone formation within an extraction socket is initiated in the apical region (Amler, 1969). Marginal epithelial and connective tissue proliferation results in soft tissue invagination and a convex bone defect. Therefore, following the principles of guided tissue regeneration, using a barrier membrane has been demonstrated to prevent invagination of the oral epithelium into the healing socket (Lekovic et al., 1998). Excluding soft tissue from the extraction socket may also aid in repopulating the socket with bone-producing cells and increasing the concentration of growth factors and cellular elements necessary for healing.

BGMs can also act as a bioactive framework or scaffold to allow bone formation to be distributed more efficiently within the extraction socket. They may also support osteoblastic cell attachment and proliferation and facilitate bone fill in the socket (Stephan et al., 1999).
1.3.2 Properties of bone graft/substitute materials

BGMs can be classified according one of the following properties: osteogenic, osteoinductive or osteoconductive (for review see Stevenson, 1999).

1.3.2.1 Osteogenic BGM

This refers to the presence of osteoblasts or bone-forming cells within the bone graft that directly form bone. Under proper handling, cells from cortical and cancellous grafts can survive and form new bone.

1.3.2.2 Osteoinductive BGM

Osteoinductive BGMs contain differentiating factors that facilitate the recruitment and differentiation of mesenchymal stem cells and induce them to form osteoblasts (Allegrini Jr et al., 2008). These differentiating factors may include: the family of bone morphogenetic proteins (BMPs), transforming growth factors (TGF-βs), insulin-like growth factors (IGFs), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs).

1.3.2.3 Osteoconductive BGM

Osteoconductive materials act as a biocompatible scaffold to support new bone formation through the in-growth of capillaries, perivascular tissue and osteogenic cells from the recipient sites.

1.3.3 Types of bone graft/substitute materials

1.3.3.1 Autografts

These are bone grafts taken from an adjacent or remote site in the same patient. Common sites to harvest autogenous bone intra-orally are around the surgical site, ascending ramus, chin and tuberosity. Autogenous bone graft is referred to as the “gold standard” grafting material. It is the only osteogenic material available. Bone structure such as minerals and collagen is maintained, as well as viable osteoblasts and bone proteins. However, the percentage of these cells that survive the transplant procedure has been questioned (Allegrini Jr et al., 2008). The disadvantages of autogenous bone graft include: the need for a second surgical site, postoperative discomfort or complications, and the limitations on the quantity of graft material.
Studies carried out using autogenous bone as ridge preservation material are sparse in the literature. Using a dog extraction model, Araújo and Lindhe (2011) compared the effectiveness of autogenous bone and Bio-Oss Collagen® in ridge preservation. Grafting with autogenous bone failed to preserve marginal bone volume. Extraction sockets grafted with autogenous bone chips demonstrated 25% crestal volume loss, which resembled that of the non-grafted sites.

1.3.3.2 Allografts

Allograft is bone graft harvested from one individual and transferred to another individual of the same species (Tischler and Misch, 2004). They are prepared by methods such as freezing (Freeze-Dried Bone Allograft, FDBA) or demineralising and freezing (Demineralised Freeze-Dried Bone Allograft, DFDBA) (Esposito et al., 2009). They are thought to be osteoinductive and osteoconductive. DFDBA is believed to induce bone formation through bone morphogenic proteins (BMPs) exposed during the demineralization process (Schwartz et al., 1996; Wang et al., 2004).

However, some clinical trials have found little evidence of new bone formation around bone allograft (Becker et al., 1994; Froum et al., 2002). DFDBA combined with barrier membranes has shown positive results (Brugnami et al., 1999). These materials have just become available in New Zealand. These are bone grafts harvested from cadavers and there are additional concerns related to religious beliefs and possible transmission of infectious diseases (Allegrini Jr et al., 2008).

1.3.3.3 Xenografts

These grafting materials consist of deproteinised skeletal bone tissue from one species that are transferred to a recipient site of another species (Tischler and Misch, 2004). Xenograft materials are processed to remove the organic component and thus eliminate antigenicity. The remaining structure, hydroxyapatite, provides a biocompatible scaffold for bone formation.

Bovine xenograft is one of the most researched bone graft/substitute materials for ridge preservation. It has been shown to be well tolerated and integrated by the host tissue (Artzi et al., 2001). The same study also reported that there was 82.3% overall bone fill of the augmented socket sites. However, no control site was used in this study. The
histomorphometric analysis in this study was carried out using the transverse cross-section of the trephined bone core.

The common finding from the studies using bovine bone-derived xenograft as the bone graft material was that the graft was not eliminated from the grafted sites. The graft material was still present within the grafted sites after a long time, even up to years (Artzi et al., 2000; Molly et al., 2008). The non-degraded graft materials may act as a barrier to bone formation (Schneider et al., 2009). This may delay socket healing and raise concerns for future implant placement and osseointegration.

In the recent years, studies have used deproteinised bovine bone mineral combined with purified porcine collagen in a 90:10 ratio (Bio-Oss Collagen®) as a bone substitute material (Araújo et al., 2008; Araújo and Lindhe, 2009). According to the manufacturer (Geistlich Pharma, Switzerland), the collagen in this device acts as a cohesive for the granules and enhances the handling characteristics. However, the result from these studies did not show any enhancement in new bone formation. In addition, the use of Bio-Oss®/Bio-Oss collagen® was only able to limit the post-operative alveolar ridge resorption to a certain extent. Fickl et al. (2008b) reported the mean vertical distance between buccal and lingual crest following treatment with Bio-Oss collagen® was 2.8 ± 0.2mm compared, whereas the reported difference at control sites was 3.2 ± 0.2mm. They were not able to prevent tissue alterations entirely after tooth extraction (Fickl et al., 2008b).

1.3.3.4 Alloplastic graft materials

Alloplasts are synthetic materials that are used to replace human bone. They provide a physical framework for bone in-growth. Therefore, they are osteoconductive. Many synthetic materials have been reported in the published literature. To name a few, these are: hydroxyapatite (Nemcovsky and Serfaty, 1996), tricalcium phosphate (Horowitz et al.), biphasic calcium phosphate (Boix et al., 2006), bioactive glasses (Santos et al., 2010), calcium sulphate (Guarnieri et al., 2004), biocompatible composite polymers (Ashman and Bruins, 1985; Froum and Orlowski, 2000). The material of interest in our study belongs to this group. This will be discussed further in Section 1.4.

The main disadvantages for alloplastic graft materials are the tendency for granular migration and unpredictable rate of bioresorption (Schneider et al., 2009). Alloplastic materials are now being developed as carriers or scaffolds for osteoactive agents, for
example, bone morphogenetic proteins (Howell et al., 1997), platelet-derived growth factor, bioactive polypeptides (Neiva et al., 2008) and stem cells.

1.3.4 Summary

Bone graft/substitute materials are placed into the extraction socket for the preservation of alveolar bone. According to the origin of the material, they are classified as autograft, allograft, xenograft and alloplast. Autogenous bone graft, although referred to as the “gold standard” grafting material, failed to preserve the alveolar ridge volume according to the animal study available. Allograft, xenograft and alloplast have been shown to limit but not eliminate post-operative alveolar ridge resorption.
1.4 Development of new bone graft/substitute materials (BGMs)

1.4.1 Properties of an ideal BGM

The criteria for an ideal BGM have been outlined by various researchers. (Allegrini Jr et al., 2008; Ashman, 2000; Darby et al., 2008; Wang et al., 2004). These include: unlimited supply; good handling properties; no possibility of disease transmission to patient; biologically inert with no immunologic reaction; easy to adapt to the recipient site in terms of size and shape; hydrophilic; not prone to infection or migration; osteoconductive with an internal structure compatible with cell attachment and colonization; facilitating revascularization; enabling the formation of dense lamella bone suitable for implant placement; being completely replaced by new bone.

1.4.2 Disadvantages of the commercially available BGMs

1.4.2.1 Handling properties, adaptation, migration

Most of the commercially available bone graft/substitute materials are in granular form. Transferring the materials into the grafted sites and condensing them can be time consuming. They are hydrophobic and prone to migration in bleeding sites. The newly developed bovine bone derived xenograft and porcine collagen matrix composite (Bio-Oss Collagen®, Geistlich Switzerland) is a moldable material. However, perfect adaptation to the size and shape of the graft site is still difficult to achieve.

1.4.2.2 Resorption rate

The resorption rates of the available graft materials are unpredictable. Most of these materials were still present at six months after grafting. Bovine bone derived xenograft in particular is well known for its low resorption rate. Studies have shown graft particles were still present nine months after implantation (Artzi et al., 2000). The xenograft area fraction averaged 31% at nine months in this study. In fact, the graft material was still detectable years after placement (Molly et al., 2008).

A recent study by Mardas et al. (2010) reported the use of a new biphasic ceramic bone substitute (BoneCeramic®, Straumann, Basel Switzerland) in ridge preservation. No active resorption of the graft particles was observed after 8 months healing.
1.4.2.3 Safety and religious concerns

Allografts and xenografts are harvested from cadavers and animals. They are then processed to eliminate the organic component, viruses and antigens by chemical, gamma irradiation, and heat. However, there are still concerns related to religious beliefs and possible transmission of infectious diseases.

1.4.3 Electrospun cottonwool-like nanocomposite

In a recent publication by Schneider et al. (2009), the performance of a flexible, mouldable, electrospun cottonwool-like nanocomposite (ECWN) was assessed. This material incorporates amorphous tricalcium phosphate (ATCP) nanoparticles into a biodegradable synthetic copolymer poly(lactide-co-glycolide) (PLGA). PLGA has been used extensively as scaffolds for cell-seeding in tissue engineering. This material is prepared through an electrospinning process, which gives it the typical cotton wool-like appearance (Figure 1.1). The use of ceramic nanoparticles in bopolymers provides a large surface area for cell attachment, proliferation and mineralization.

![Image](image.png)

Figure 1.1 Macroscopic image of ECWN
(modified from Schneider et al. 2007)

1.4.3.1 Development and production of ECWN

This material incorporates aerosol derived amorphous tricalcium phosphate nanoparticles (ATCP) into a biodegradable synthetic copolymer poly(lactide-co-glycolide) (PLGA) (Figure 1.2). ATCP has exhibited high \textit{in vitro} bioactivity (Meyer and Eanes, 1978) and increased solubility (Brunner et al., 2007). This material used the nanoparticle of ATCP to provide a large surface area for cell attachment, proliferation and mineralization.
PLGA has been used extensively as scaffolds for cell-seeding in tissue engineering (Hsiong and Mooney, 2006), drug delivery (Song et al., 1997), soft tissue engineering (Pattison et al., 2005), nerve regeneration (Bini et al., 2004) and orthopaedics (Middleton and Tipton, 2000). PLGA degrades by hydrolysis of its ester linkages in the presence of water. It has been shown that the time required for degradation of PLGA is related to the monomers' ratio used in production. The higher the content of glycolide units, the lower the time required for degradation. The possibility to tailor the polymer degradation time by altering the ratio of the monomers used during synthesis has made PLGA a common choice in the production of a variety of biomedical devices such as: grafts, sutures, implants. In the development of ECWN, PLGA used had a copolymer ratio of 85:15.

![Figure 1.2](image)

**Figure 1.2 Scanning electron microscopic images of ECWN fibres and fibre surface structure**

(modified from Schneider et al. 2007)

(a) PLGA/ATCP fibres

(b) PLGA/ATCP fibre surface structure

To prepare this material, ATCP nanoparticles and PLGA with a copolymer ratio of 85:15 were dispersed and dissolved in a chloroform/Tween20 (surfactant) mixture. This solution was then fed through a capillary using a syringe pump. A high voltage supply was used to apply voltage to the needle tip. A positively charged jet was formed and sprayed unto a rotating collection tube (Figure 1.3). The materials were dried and stored under vacuum at room temperature. A more detailed description on the production of ECWN may be found in the original paper (Schneider et al., 2007).
1.4.3.2 **Properties of ECWN**

As the electrospun scaffold is manually uncompressed, this material resembles the appearance of cotton wool. This characteristic of the material allows easy proportioning, easy handling and adaption to a bone defect (Schneider et al., 2009). Defects with hindered accessibility and different opening diameters and depths could be filled with ECWN within less than one minute. The material is easy to handle with no preshaping required. Misplacement during operation can be easily corrected by the removal of the entire scaffold in one piece whereas granular bone substitutes are more difficult to remove.

1.4.3.3 **In vitro study**

*In vitro* studies were carried out to test the bioactivity and biocompatibility of the material (Schneider et al., 2007; Schneider et al., 2009). ECWN was cut into rectangles (70x10mm²) for *in vitro* biomineralisation tests. The samples were placed in simulated body fluid and incubated at 37 degrees.

After 45 hours immersion in simulated body fluid, a continuous nano-featured hydroxyapatite layer with a thickness of about 1µm was deposited on the surface of ATCP-containing fibres (Figure 1.4). The mass of ATCP-containing fibres increased by 18% within the same time period. This change in mass is the result of hydroxyapatite formation on the surface of ATCP-containing composites. After 360 hours of immersion, the thickness of the hydroxyapatite layer grew up to 2µm. These findings confirmed the very high *in vitro* bioactivity.
Biocompatibility tests were carried out by seeding human mesenchymal stem cells onto the nanocomposite scaffolds. The detection of normal cell morphology and near identical proliferation was detected both on the nanocomposite scaffolds and pure PLGA scaffolds. Alkaline phosphatase activity and osteocalcin content was comparable between the two scaffolds as well. These findings demonstrated a lack of cytotoxic effects from potential calcium burst in the nanocomposite at early stages of exposition.

1.4.3.4 In vivo study

An in vivo study was carried out in a rabbit calvarial defect model (Schneider et al., 2009). Bone regeneration was evaluated in four 6mm diameter non-critical-size calvarial defects in nine rabbits (Figure 1.5). In each animal, one untreated defect was compared to defects treated with pure PLGA, ECWN and Bio-Oss® (Geistlich Pharma, Switzerland). The animals were sacrificed after four weeks of healing.
Figure 1.5 Surgical procedure in New Zealand white rabbits
(1) pure PLGA, (2) ECWN, (3) bovine xenograft, (4) empty defect as negative control (modified from Schneider et al. 2009)

Microscopic examination of the experimental sites revealed new bone formation originated from the bony borders and directed toward the centre (Figure 1.6). Complete bridging of the defects with mineralized bone occurred in two of the nine test defects. Bone formation around Bio-Oss particles is mainly solid lamellar bones, whereas for the flexible ECWN used in the present study, the newly formed bone showed a more spongiosa-like appearance.

Figure 1.6 Histological sections of the cranial defects in rabbits after four weeks
(modified from Schneider et al. 2009)

The average area fractions of the newly formed bone within the defects were: 28.4 ± 14.9% for the empty defect; 30.8 ± 14.3% for Bio-Oss treated defects and 34.9 ± 17%
for ECWN treated defects. However, no statistically significant differences between the groups could be detected. A large ratio of original Bio-Oss implant material was remaining in the grafted site after four weeks of healing. Resorption of ECWN as early as 4 weeks was reported in this same study.

The authors also highlighted the need for further investigations in animals to evaluate the long-term stability and clinical outcome of this material before the human trials can take place.

1.4.4 Summary

It is desirable that BGM be completely resorbed and replaced by the patient’s own bone. However, the current commercially available BGMs are shown to have low resorption rate. They were found to be present for years after placement. Electrospun cottonwool-like nanocomposite (ECWN) is a novel synthetic bone substitute that incorporates amorphous tricalcium phosphate nanoparticles into a biodegradable synthetic copolymer poly(lactide-co-glycolide). Both the in vitro and in vivo studies showed high bioactivity of ECWN. Resorption of the graft material as early as four weeks was reported in the rabbit calvarial defect study. However, the long-term stability and clinical outcome of this material have not been tested in large animal models.
1.5 Animal models

1.5.1 The need for an animal model

Large animal models have been used in the studies of extraction socket healing and bone graft/substitute materials for ridge preservation by many research groups. This is due to the need of invasive procedures for outcome examination, such as histological and histomorphometric analyses of hard tissue healing in the tooth extraction sockets. It is also important for the biocompatibility, degradation rate and potential toxic effects of any new absorbable biomaterials to be tested in animal models before human clinical trials (An and Friedman, 1999).

An animal model should only be used after in vitro biocompatibility testing in cell culture. It acts as the bridge between in vitro testing and human trials. Animal models are also used to evaluate the potential applications of the material and the process of material degradation and replacement by host tissues. If the material functions well, then a well-controlled human trial may be carried out.

1.5.2 Monkey extraction socket models

Non-human primates were used in extraction socket research in the 1970s and 1980s (Gumaer et al., 1985; Pietrokovski and Massler, 1971; Stanley et al., 1976). Due to cost and availability issues, their application in bone research is now limited to projects for which they are absolutely required, such as osteoporosis research (Vlaminck et al., 2008).

1.5.3 Dog extraction socket models

In recent years, there has been an increasing amount of literature based on dog extraction socket models. An earlier group used three premolar sockets of maxillary and mandibular arch for the investigation of bone graft/substitute materials (Indovina and Block, 2002). A later model developed by Cardaropoli et al. (2003) uses the mandibular premolar extraction sites. The surgical procedures were described in detail in their original papers. Intrasulcular incisions were made in the premolar region. Buccal and lingual full thickness flaps were elevated to expose the alveolar crest. The mesial root canals of the premolar teeth were obturated with gutta-percha. The teeth were then sectioned with fissure burs. The distal roots were removed using elevators. The buccal and lingual flaps were then closed at the extraction site with interrupted
sutures. The sutures were removed 10 days later. This model has the advantage of maintaining the alveolar bone height adjacent to the extraction socket. Assuming the mesial and distal bone heights were equal, they were able to compare the dimensional changes of the alveolar bone from histology. This model was later adopted for investigation of extraction socket healing following the placement of BGMs (Araújo et al., 2008; Araújo et al., 2010b; Araújo and Lindhe, 2011)

Other groups also used the maxillary third incisors (Iibuchi et al., 2010) and mandibular second molars (Rothamel et al., 2008) without root canal therapy or hemisection of the teeth in their models. The healing periods of these studies varied from one week to six months. However, Araújo et al. (2008, 2009) found few changes in the socket healing and marginal ridge resorption from three months to six months. This may indicate the socket healing may be completed by six months in canine models (Araújo and Lindhe, 2009).

Using dogs in animal research has also raised ethical concerns as they are considered as companion animals. The public may find it unacceptable to have them as research animals. In the recent years, sheep have been increasingly used as large animals for bone graft/substitute research.

1.5.4 Sheep in bone graft/substitute material research

An and Friedman (1999) listed the factors to consider when choosing an animal model. These include: ethics, availability, housing requirements, ease of handling, cost and susceptibility to disease.

Domesticated animals such as sheep or pigs are more accepted by the public to be used in animal research than companion animals such as dogs or cats (An and Friedman, 1999). They are available in large numbers. Sheep are similar to humans in size, weight and general physiology (Newman et al., 1995). They also have similar metabolic rate with human (Schmidt-Nielsen, 1997). Reports have shown that they responded well to surgical procedures (Salmon and Duncan, 1997). According to the authors, sheep and goat should always be considered first in a new project. If they are available, there is no specific reason for dogs to be used.

Sheep (Ovis aries) are readily available in New Zealand. The cost and housing of sheep in animal research are not major issues in New Zealand (Duncan, 2005). The Hercus-Taieri Resource Unit (HTRU) at the University of Otago has rich experience
in dealing with sheep surgery in orthopaedic and dental research. HTRU provides excellent facilities for transportation, housing, peri-operative care, specimen collection and disposal.

The use of sheep as animal models in bone graft/substitute material research in dentistry have been reported in the studies of bone healing (Salmon and Duncan, 1997), dental implant research (Duncan, 2005; Vlaminck et al., 2008), periodontitis studies (Duncan et al., 2003) and maxillary sinus augmentation (Haas et al., 1998).

To our knowledge, there has not been any tooth extraction socket model developed in sheep. Vlaminck et al. (2008) have recently investigated immediate postextraction implant placement in a sheep model (Vlaminck et al., 2008). They chose the first two mandibular premolars to be the operation sites due to their accessibility. However, they encountered difficulties such as crown fractures during extraction, post-op premature loosening of the gingival flaps, etc. Therefore, the authors suggested several adjustments to the model to overcome these difficulties such as the use of specially designed periotomes to achieve atraumatic extraction and soft diet in the first week of gingival healing.

1.5.5 The development of tooth extraction socket model in sheep

1.5.5.1 Dental anatomy of sheep

A prominent feature of sheep dental anatomy is that they lack maxillary incisors, having instead a "dental pad" (Figure 1.7). They have three mandibular incisors and one canine on each side. A wide diastema separates the lower incisors and canines from the premolars. Sheep have three premolars and three molars on each side of the maxillary and mandibular arch.
Duncan (2005) studied the anatomical dimensions of the mandibular premolar region (Figure 1.8). The first mandibular premolar (P₁) is small in size with two roots. Some have longer mesial roots and shorter distal roots, and others have similar length roots. The second premolar (P₂) has two roots which are longer than the roots of P₁. However, the anatomy of the distal roots also varies between animals, with some having equal length with the mesial and some are substantially shorter. The third premolars (P₃) have longer mesial and distal roots that are usually of the same length.
1.5.5.2 Dental extractions in sheep

Duncan method

Duncan developed extraction protocol for delayed implant placement research in sheep (Duncan, 2005). Following a crevicular incision around the mandibular premolars, full thickness buccal and lingual flaps were raised. A shallow circum-dental osteotomy was created within the superficial portion of the periodontal ligament. This was done under irrigation using a high speed handpiece and tungsten-carbide straight fissure bur. The first premolar, if present, was usually elevated mesially and removed. The second and third premolars were sectioned vertically along the midpoint between the mesial and distal interproximal surfaces towards the furcation region. The distal and mesial portions were then removed with elevators. The flaps were then closed with 3-0 resorbable sutures.

Vlaminck method

A Belgium group investigated immediate post-extraction implant placement in sheep mandibles (Vlaminck et al., 2008). In the brief methodology section, the authors stated that extraction of the first and second premolars was achieved after longitudinal sectioning and with the use of root elevators and extraction forceps.

Both groups mentioned the brittleness of the teeth and extremely dense inelastic mandibular bone resulted in a high frequency of root fractures during extraction. More aggressive methods were required to remove the root fragments.
1.5.5.3 Sheep healing time

Wound healing times differ between animal and human. Claffin (1936) showed eight weeks post-extraction socket healing in dogs equals 3.5 months in human. Duncan (2005) compared sheep healing time to the healing time of dogs. He found they had similar healing times. Both were faster than human (see Table 1.2, adopted from (Duncan, 2005)).

Table 1.2 Healing times of sheep compared to human

<table>
<thead>
<tr>
<th>Sheep healing times</th>
<th>Human healing times</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hours</td>
<td>8 hours</td>
</tr>
<tr>
<td>5 days</td>
<td>1 week</td>
</tr>
<tr>
<td>1 week</td>
<td>9 days</td>
</tr>
<tr>
<td>2 week</td>
<td>3 weeks</td>
</tr>
<tr>
<td>4 weeks</td>
<td>5 weeks</td>
</tr>
<tr>
<td>6 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td>8 weeks</td>
<td>11 weeks</td>
</tr>
<tr>
<td>12 weeks</td>
<td>16 weeks</td>
</tr>
<tr>
<td>16 weeks</td>
<td>21 weeks</td>
</tr>
</tbody>
</table>

1.5.6 Summary

Large animal models are useful to study the histology and histomorphometry of hard tissue healing in the tooth extraction sockets, as well as the biocompatibility of new bone substitute materials. Dog extraction socket models have been described and applied in the literature. Domesticated animals such as sheep or pigs are more accepted by the public to be used in animal research than companion animals. Developing a sheep extraction socket model is of particular value in New Zealand, as sheep are readily available. The cost and housing of sheep in animal research are also not major issues in New Zealand.
1.6 Techniques for analyzing healing following ridge preservation in animal studies

Various techniques have been reported in the literature to assess healing following ridge preservation procedure in animal studies. These techniques can be divided into ones that measure the dimensional changes of the alveolar ridge following ridge preservation and others that measure bone to tissue ratio within the healing socket.

1.6.1 Techniques measuring changes in ridge dimension

1.6.1.1 Histometric measurements

This technique was originally developed and investigated by Araújo’s group (Araújo and Lindhe, 2005; Araújo et al., 2008; Araújo and Lindhe, 2009) to study dimensional ridge alteration following tooth extraction and ridge preservation procedures in dog studies. This was later adopted by Fickl’s group in their studies (Fickl et al., 2008b).

In their model, Araújo and Lindhe (2005) extracted the distal roots of premolars and obturated the canal of each mesial root with gutta-percha. Assuming the shape and dimension of the alveolar process at the mesial and distal roots to be similar, the cross section area of the tooth site and edentulous portion of the ridge were compared. The dimensional change of the alveolar process was estimated by subtracting the value obtained at the extraction site from the corresponding tooth site.

The authors recognized that the mesial and distal root cones of the premolars projected into the alveolar process with a certain degree of separation and assumed the two roots had similar shape and dimension. This may be a source of error in the measurement outcome. At the same time, this technique requires the precision in histology preparation to identify the apex of the tooth and the centre of the socket.

Rothanmel et al. (2008) described recording the buccal and lingual total bone height from the top of the buccal and lingual alveolar wall to the most apical point of the jaw. They also measured the lingual and buccal alveolar wall bone width and the total bone width at one, three and five mm underneath the top of the respective bone crest perpendicular to the long axis of the extraction socket. The validity of this technique will again be dependant on the angle at which the histology section is prepared.
1.6.1.2 Optical Scanning

To study the dimensional changes of the ridge contour after ridge preservation, Fickl’s group (2009) obtained impressions at baseline and post-operatively. They scanned the casts with a 3D camera (Cerec®3, Sirona Dental Systems, Germany). Because of the limited access for the optical scanner, multiple images captured were composed into one digital image. These images were then superimposed using the buccal surfaces of retained teeth as reference points. The volume difference between the time points was calculated. In their earlier study (Fickl et al., 2008b), the scanning was achieved by detecting the lateral displacement of a laser beam’s reflection on the cast.

The authors recognized the accuracy of the measurements might be influenced by artifacts and dimensional changes of the impression and cast materials. They suggested direct optical impressions in the mouth might overcome this source of error. However, the quality of the scans may be impeded by the limited access to the site and the presence of saliva.

1.6.1.3 Radiographic analysis

Standardized radiographs were also used to measure the bone height and density changes for ridge preservation procedures carried out in animal studies (Oltramari et al., 2007). The films were digitalized and analysed using computer software to determine the bone height and bone density within the extraction socket. This would be the least invasive and most economical technique. However, the authors failed to mention the reproducibility of the placement of x-ray films in this animal model. This may potentially impede the accuracy of the measured outcome. Sources of error may arise from the use of radiographic stents.

1.6.1.4 Micro-computed tomography

More recent studies (Al-Hezaimi et al., 2012; Bashara et al., 2012) also reported the use of micro-computed tomography (micro-CT) for the measurements of alveolar ridge width, bone volume, bone mineral density and vertical bone height. The advantage of using micro-CT in this instance is the specimen reconstruction can be analyzed in three dimensions, the transverse, sagittal and coronal plane. Volumetric measurements are easily conducted. Measurements can also be taken at different
levels below the alveolar bony crests. However, the authors did not mention how the long axis of the tooth socket was determined in their measurements for vertical bone height.

1.6.2 Techniques measuring bone formation within the extraction socket

1.6.2.1 Histological observations

Animal studies that carried out ridge preservation therapy mostly involved histological preparation after euthanasia of the animals. Some of these preparations were demineralised using ethylenediaminetetraacetic acid (EDTA), embedded in paraffin and sectioned into very thin slides (4-6µm). The others were kept undemineralised and prepared as resin embedded blocks. These blocks were then cut and polished to produce 40-100µm sections.

Demineralised sections

Demineralised sections are useful in studies that investigate early healing events in extraction sockets. Araújo et al. (2009; 2010b) studied grafted socket healing from one day to four weeks. Calixto et al. (2007) also employed this technique in their rat study from one to nine weeks. Thinly sectioned, paraffin embedded sections were stained with haematoxylin and eosin. This preparation allowed them to identify cells involved in the healing process. In deparaffinised sections, osteoblasts and osteoclasts maybe identified using histochemical and immunohistochemical markers. Other studies have used alkaline phosphatase and osteopontin markers to identify osteoblast activity and tartrate-resistant acid phosphatase (TRAP) stain for osteoclasts (Araújo et al., 2010a).

Undemineralised sections

Resin-embedded undemineralised sections are prepared to differentiate mineralized bone, graft material, connective tissue and bone marrow. This technique is relatively simple to conduct. It minimises the possible processing artifacts and tissue separation that may occur during specimen decalcification.

1.6.2.2 Histomorphometric analysis

Histomorphometric analysis of the grafted extraction sockets investigates the quantity of different tissue volumes. They are usually expressed in area fractions within the
chosen region of interest. For bone graft/substitute studies, the most commonly identified tissue types are newly formed bone (woven and lamellar bone), residual graft, fibrovascular connective tissue.

1.6.2.3 Region of interest

No consensus has been developed for the selection of region of interest (ROI) in extraction socket healing. Cardaropoli et al. (2005) confined their measurements to the central and apical portions of the extraction socket. They excluded the newly formed cortical bone that sealed the socket from their measurements. However, they did not clearly define how the ROI was selected or how they ensured similar areas were selected between different sites or animals. The irregular margin of the healing extraction socket also makes it difficult to outline the entire defect area. Araújo et al. carried on the same protocol in their subsequent studies (Araújo et al., 2008; Araújo and Lindhe, 2009; 2011). However, they too did not address or define this issue. Calixto et al. (2007) measured bone formation in the cervical alveolar third only. Yet still others (Hong et al., 2012) included the whole socket area in their measurements and analysis. The lack of consensus in the selection of ROI in these studies makes comparisons from the results extracted from different studies difficult.

1.6.2.4 Measurement technique

The most commonly used measurement technique by these aforementioned papers is a light-point counting procedure. A lattice comprising 100 light points is superimposed over the tissue in the healing socket. The total number of points fall on different types of tissue are calculated and expressed as area fraction. This technique is modified from Schroeder et al. (1973). In a recent study by Hong et al. (Hong et al., 2012), different tissue types were selected manually and measured using image analysis software.

1.6.3 Summary

Descriptive histology and histomorphometric analysis are the most common evaluations carried out in the study of extraction socket healing following ridge preservation procedures. However, the selections of regions of interest for histomorphometric analysis reported in the literature are not clearly described.
1.7 Histological outcomes of bovine-derived xenograft in animal extraction socket models

Block section specimens obtained from the animal studies allowed the researchers to carry out histological analyses of healing over the entire extraction socket and residual alveolar ridge. The majority of the animal studies available are from the works in the dog extraction socket model performed by Araújo and Fickl’s groups (Araújo et al. 2008, 2009, 2010a, 2011; Araújo and Lindhe 2009; Fickl et al. 2008b; Fickl et al. 2009). The healing time varied from two weeks to six months in these studies.

1.7.1 Histomorphometric results

Five studies carried out histomorphometric analyses of the extraction sockets grafted with bovine xenografts (Araújo et al. 2008, 2009, 2010, 2011; Araújo and Lindhe 2009). A summary of these studies can be found in Table 1.3. No statistically analysis was carried out in these studies.

All of these studies quantified the mean percentage of new bone formation, which ranged from 15.3 percent to 62.5 percent. Two studies investigated the early healing within grafted extraction sockets at one, two and four weeks (Araújo et al. 2009, 2010). The other three studies studied the healing of grafted extraction sockets after three and six months (Araújo et al. 2008, 2011; Araújo and Lindhe 2009). The amount of new bone formation was the lowest (15.3 percent) after the first week and increased with time.

Residual graft material was not quantified in all of these studies (Araújo et al. 2008, 2009, 2011; Araújo and Lindhe 2009). The mean amount of graft material detected ranged from five percent to 24.6 percent. The smallest amount of graft material (five percent) was detected in the specimens after six months of healing (Araújo and Lindhe 2009).

1.7.2 Histometric results

Four studies reported on the dimensional changes of the ridge contour following ridge preservation (Araújo et al. 2008, 2009; Fickl et al. 2008b, 2009). In all specimens, the crest of the buccal bone wall was located apical to the lingual bone crest. The mean difference between the buccal and lingual bone crests was significantly larger in
grafted extraction sites compared to the tooth site. Placement of xenograft material with in the extraction socket failed to prevent resorption of the buccal crest (Araújo et al. 2008). Similar results were shown by Fickl et al. (2008b, 2009).

The relative change of the cross-section area of the alveolar process was reported by Araújo et al. (2008, 2009). At the marginal portion of the grafted sites, a smaller reduction of the marginal hard tissue was observed when compared to non-grafted sites. Fickl et al. (2008b) measured the horizontal distance between the borders of the alveolar ridge at 1mm apical to the lingual bone crest. The mean distance at 1mm apical to the lingual crest was significantly larger in test sites grafted with bovine xenograft when compared to non-grafted sites. The authors also mentioned due to the limited sample size, the results should be evaluated with caution and only used as a trend.
Table 1.3 Summary of histomorphometric results following the placement of xenograft in dog extraction socket model

<table>
<thead>
<tr>
<th>Author</th>
<th>Healing time</th>
<th>New bone Mean% (SD)</th>
<th>Connective tissue Mean% (SD)</th>
<th>Residual graft material Mean% (SD)</th>
<th>Bone marrow Mean% (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Araújo et al. 2010</td>
<td>1 week</td>
<td>15.3 (6.2)</td>
<td>42.7 (6.2)</td>
<td>NQ</td>
<td>NQ</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>29.7 (12.8)</td>
<td>40.1 (2.9)</td>
<td>NQ</td>
<td>NQ</td>
</tr>
<tr>
<td></td>
<td>4 weeks</td>
<td>45.1 (10.1)</td>
<td>37.3 (7.1)</td>
<td>NQ</td>
<td>NQ</td>
</tr>
<tr>
<td>Araújo et al. 2009</td>
<td>2 weeks</td>
<td>14.7 (5.9)</td>
<td>62.7 (12.6)</td>
<td>18.9 (9.8)</td>
<td>NQ</td>
</tr>
<tr>
<td>Araújo et al. 2008</td>
<td>3 months</td>
<td>58.1 (10.7)</td>
<td>NQ</td>
<td>12.2 (9.1)</td>
<td>26.7 (14.4)</td>
</tr>
<tr>
<td>Araújo et al. 2011</td>
<td>3 months</td>
<td>43.1 (10)</td>
<td>NQ</td>
<td>24.6 (3.7)</td>
<td>16 (7.6)</td>
</tr>
<tr>
<td>Araújo and Lindhe 2009</td>
<td>6 months</td>
<td>WB 15.4 (0.2)</td>
<td>NQ</td>
<td>5 (2.4)</td>
<td>14.1 (6.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LB 62.5 (4.9)</td>
<td>NQ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NQ = not quantified; WB = woven bone; LB = lamellar bone
1.8 Summary of literature review

Following tooth extraction, alveolar bone resorption occurs most rapidly in the first six months. Two thirds of the reduction in the width of the alveolar ridge occurs during the first three months. There is more loss in the bucco-lingual width than the corono-apical height of the alveolar ridge. This can complicate the replacement of the missing tooth, since successful rehabilitation options need adequate bone height and width.

Ridge preservation refers to procedures that maximise retention of bone surrounding the extraction site. Bone graft/substitute materials (BGM) are placed into the tooth socket immediately after tooth extraction, where they act as a scaffold for the ingrowth of cells and blood vessels and the formation of new bone in the healing tooth socket. It is desirable that BGM be completely reabsorbed during this process and be replaced by the patient’s own bone.

BGMs can possess different properties and harvested from different sources. Bovine xenograft is one of the most researched bone graft/substitute materials for ridge preservation. It has been shown to be well tolerated and integrated by the host tissue. However, the common finding from the studies using bovine xenograft as the bone graft material was that the graft was not eliminated from the grafted sites. The non-reabsorbed graft materials may act as a barrier to bone formation. This may delay socket healing and raise concerns for future implant placement and osseointegration.

The performance of a newly developed, flexible, mouldable, electrospun cottonwool-like nanocomposite (ECWN) was reported in the literature. This material is prepared through an electrospinning process, which give it the typical cotton wool-like appearance. This characteristic of the material allows easy proportioning, easy handling and adaption to a bone defect. The clinical study showed high bioactivity of ECWN four weeks after implantation, with the formation of new bone and increased cell density. Resorption of the graft material as early as four weeks was also reported in this same study. However, the authors also highlighted the need for further investigations in animals to evaluate the long-term stability and clinical outcome of this material.
Chapter 2 Materials and methods

This section describes the materials and methods used to compare the healing of tooth extraction sockets in a sheep model. Ethical approval for this study was obtained from the Otago Animal Ethics Committee under protocol number AEC 65-11.

2.1 Experimental animals

Eighteen cross-bred ewes aged four to five years were used in this study from flocks sourced by the AgResearch Invermay Breeding Station. The animals exhibited an intact dentition with a healthy periodontium. Following selection, the sheep were transported to the Hercus Taieri Research Unit, where they were treated to control parasites and immunized in preparation for surgery. The animals were individually tagged and maintained on pasture in a secure site until required for surgery.

All surgical procedures were performed in the large animal operating theatre of Hercus Taieri Research Unit, University of Otago. The sheep arrived 48-72 hours before surgery and were starved for 24 hours prior to the administration of the general anaesthesia. This group of experimental animals received treatments to multiple sites. In addition to extraction socket site, the same animals also received surgery to left and right femurs and left and right maxillary sinuses. The results from these sites will be reported elsewhere by different authors.

The sheep were divided into two groups of eight with two additional animals used as baseline. There were two healing periods: eight weeks (n = 8 animals) and 16 weeks (n = 8 animals). As no previous study had been conducted using the current experimental model and material, eight animals were included in each group in an effort to increase the statistical power of the study. There were four treatments provided to each animal: test graft with or without resorbable collagen membrane (ECWN+/ECWN-), bovine xenograft with membrane (B+), and non-grafted socket with no membrane (No-). The study design is summarised in Figure 2.1 and detailed further in Section 2.2. Details of the distribution of the experimental treatments are shown in Table 2.1.
**Figure 2.1 Flowchart for study design**

**Table 2.1 Experimental sites allocation**

<table>
<thead>
<tr>
<th>Sheep no.</th>
<th>Healing time (weeks)</th>
<th>R P2 socket</th>
<th>R P3 socket</th>
<th>L P2 socket</th>
<th>L P3 socket</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>ECWN +</td>
<td>B +</td>
<td>No -</td>
<td>ECWN -</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>ECWN -</td>
<td>ECWN +</td>
<td>B +</td>
<td>No -</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>No -</td>
<td>ECWN -</td>
<td>ECWN +</td>
<td>B +</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>B +</td>
<td>No -</td>
<td>ECWN -</td>
<td>ECWN +</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>ECWN +</td>
<td>B +</td>
<td>No -</td>
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2.2 Surgical and graft materials

2.2.1 Endobon®

Endobon® (BIOMET 3i, USA) is a commercially available osteoconductive bovine xenograft (BX). It is derived from deproteinised cancellous bovine bone. Commercially packaged Endobon® granule size 500-1000µm, 1.0ml were used in the selected surgery sites (Figure 2.2). A new package was opened for each animal.

2.2.2 OsseoGuard® Resorbable Collagen Membrane

Commercially packaged OsseoGuard® Resorbable Collagen Membrane (Biomet 3i, USA) (15mmx20mm) was used in selected surgical sites. A new package was opened for each animal. The membrane was hydrated for 30 minutes prior to placement. A single layer of membrane was placed over all sockets grafted with Endobon® and the selected sockets grafted with ECWN.

2.2.3 Electrospun cottonwool-like nanocomposite

ECWN (Functional Materials Lab, ETH Zurich, Switzerland) was weighed and apportioned into each container and packaged in Eppendorf tubes (Fig 2.3). The material was supplied by the manufacturer after receiving γ-radiation treatment.
Figure 2.2 Endobon, in sterile glass vial

Figure 2.3 ECWN, in Eppendorf tube
2.3 Graft quantity calculation

To determine the amount of graft materials required for the study, calculation was made prior to surgery. Three sets of mandibular premolars (P1-P3) were extracted from three sheep used for other studies in the same research unit.

A three-dimensional analysis of the teeth was performed using a micro-computed tomography scanner (SkyScan 1172, Kontich, Belgium). The x-ray generator of the micro-CT was operated at 80kV. Volumetric measurements of the tooth roots were calculated following 3-D reconstruction using computer software Image J (version 1.47q, NIH, USA) with plug-in BoneJ. The average value of the three tooth samples was taken into consideration when calculating the amount of ECWN required for surgery according to manufacturer’s recommendation (20mg ECWN/0.1ml defect) (FML, Zurich, Switzerland).

Volumetric measurements for the second and third mandibular premolars were obtained. The mean volume for the two roots of the second premolar was close to 240mm$^3$. The mean volume for the third premolar roots was close to 520mm$^3$. The micro-CT scan results are attached in Table 2.2.

**Table 2.2 Volumetric measurements for the second and third mandibular premolars**

<table>
<thead>
<tr>
<th>Tooth (2 roots)</th>
<th>Volume area (mm$^3$)</th>
<th>Mean volume (mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2-1</td>
<td>254.4</td>
<td>243.7</td>
</tr>
<tr>
<td>P2-2</td>
<td>241.2</td>
<td></td>
</tr>
<tr>
<td>P2-3</td>
<td>235.5</td>
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<tr>
<td>P3-1</td>
<td>504.9</td>
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<td>P3-2</td>
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</tr>
<tr>
<td>P3-3</td>
<td>579.3</td>
<td></td>
</tr>
</tbody>
</table>

According to the manufacture recommendation, 20mg of ECWN test material should be used for 0.1ml defect. The approximate amounts used for grafting the second premolars and the third premolars were 50mg and 100mg respectively.
2.4 Surgical protocols

All surgeries were performed in the large animal operating theatre of Hercus Taieri Research Unit, University of Otago. Standard sterile operating techniques were adopted throughout the study.

2.4.1 Anaesthetic management

All sheep received an antibiotic, Trimethoprim (Amphoprim injection 1ml/15kg, Virbac New Zealand Ltd., East Tamaki, Auckland) pre-operatively. General anaesthesia was induced using IV Thiopentone 20mg/kg (Bomac Laboratories Ltd., Manukau City, Auckland) and maintained by Halothane (1-2%) and Nitrous oxide/oxygen (1:2). The sheep were placed on a mobile operating table with the head of the sheep rotated onto either the left or right side. A stomach tube was inserted to decompress the rumen and to allow the contents to drain into a container at floor level.

2.4.2 Surgical sites

The second and third mandibular premolars were selected as the experimental sites, as the roots of these teeth resembled those of the human teeth. Only the premolars were selected because the surgical accessibility is limited past the third premolar without a cheek incision. The surgical sites were cleansed using gauze soaked with 0.2% w/v chlorhexidine gluconate (Savacol®, Colgate-Palmolive, NZ).

2.4.3 Surgical pain control

Buccal and lingual local anaesthetic infiltrations were given around mandibular premolars using 2% Mepivicaine HCl (Scandonest® with 1:100,000 adrenaline, Septodont, UK). A post-operative dose of long acting anaesthetic 0.5% Bupivacaine HCL (Marcaine® with 1:200,000 adrenaline, Carestream Health, UK) was also administered when the wound closure was complete.

2.4.4 Surgical procedures

2.4.4.1 Flap design

The basic flap design involved intra-sulcular incisions from the mesial aspect of the first mandibular premolar to the distal aspect of the third premolar on both the buccal
and lingual aspects (Figure 2.4a). Full thickness mucoperiosteal flaps were raised (Figure 2.4b).

2.4.4.2 Tooth extraction protocols

A surgical piezotome unit (Piezosurgery®, Mectron, Genoa, Italy) with the extraction tip (EX1, EX2, EX3) was used to sever the buccal and lingual periodontal ligament of the premolars under copious irrigation with 0.9% sterile saline (Baxter Healthcare Pty Ltd., NSW Australia)(Figure 2.5a). The first to the third premolars were then elevated mesially using Coupland’s and Cryer’s elevators (Figure 2.5b). The teeth were extracted with considerable care in order not to fracture their roots or to damage the cortical plates (Figure 2.5c). The extraction sockets were curetted and radiographed.
Figure 2.4 Extraction flap design

(a) Intrasulcular incision from mesial of the first premolar to distal of third premolar

(b) Full thickness mucoperiosteal flap raised

Figure 2.5 Atraumatic extraction protocol

(a) Piezotome tip applied to severe the periodontal ligament

(b) Teeth elevated using Coupland’s elevator

(c) Extraction sockets with intact buccal and lingual cortical plates
2.4.4.3 Site allocation

Using Latin-square allocation, the second and third premolar extraction sockets of the eighteen sheep were treated with one of four modalities: no graft material (NO-), bovine xenograft Endobon® (BIOMET. 3i, USA) and resorbable collagen barrier membrane (OsseoGuard® Resorbable Collagen Membrane, Biomet 3i, USA) (B+), ECWN with resorbable collagen barrier membrane (ECWN+), and ECWN alone (ECWN-). Detailed site allocations are described in Figure 2.6 and Table 2.1.

2.4.4.4 Grafting procedure

The sockets were filled with graft material to the marginal bony crest (Figure 2.7a and 2.7b). Resorbable collagen membrane was placed over grafted sockets according to site allocation (Figure 2.7c).

2.4.4.5 Wound closure

Primary closure was achieved at the extraction sites using resorbable suture material (Vicryl 3-0, Ethicon Inc., Somerville, MA, USA).
Figure 2.6 Representative diagram of a sheep mandible, indicating location of surgical sites

Figure 2.7 Placement of grafting materials and collagen membrane

(a) Placement of ECWN into surgical site
(b) Placement of BX into surgical site
(c) Placement of collagen membrane over the grafted site
2.4.5 Post-operative management

Following surgery, the sheep were transferred to a recovery area where they were closely monitored by veterinary staff for three days. During this period, the animals received anti-inflammatory Carprofen 5ml (Rimadyl® injection 50mg/ml, Zoetis, Mt Eden, New Zealand) and antibiotic medication (Trimethoprim 1ml/15kg) once daily. These medications were administered subcutaneously for three days. After three days, the animals were returned to pasture for the rest of the healing period until euthanasia.

2.4.6 Euthanasia and perfusion protocol

After a healing period of eight and 16 weeks, eight animals each were euthanized under general anaesthesia and perfused through the carotid arteries using 10% neutral buffered formalin (NBF) (BioLab Ltd., New Zealand).

General anaesthesia was induced by IV Thiopentone (20mg/kg) and maintained by Halothane (1-2%) and Nitrous oxide/oxygen (1:2). The animal was placed in a supine position with the neck slightly overextended. Bilateral transcutaneous incisions were made to allow blunt dissection to identify the carotid arteries (Fig 2.8a). The arteries were cannulated bilaterally, using 14G indwelling catheters (Optiva TM, Smiths Medical, UK). The catheters were ligated to prevent dislodging of the cannula (Figure 2.8b).

Figure 2.8 Perfusion during euthanasia

(a) Identification of the carotid artery

(b) Cannulation site and ligated carotid artery
The animal received an anaesthetic overdose and was moved to the post-mortem room. The cannulated carotid artery was connected to a one-litre bag of 0.9% normal saline (Baxter Healthcare Ply Ltd., NSW Australia) with 1.5ml of 5000 IU heparin. The external jugular veins were severed, to allow venous outflow. A further two litres of 10% NBF was introduced via the same route.

2.4.7 Specimen harvesting

Following euthanasia and perfusion, the surgical sites were identified. The tissue specimens in the mandible were retrieved en bloc. The dissected block included the edentulous region of the mandible from the mesial of the first premolar to the mesial of the first molar. The block specimen was photographed and placed into a sealed container of 10% NBF.
2.5 Specimen preparation

2.5.1 Fixation

The block specimen was placed into individual containers with 10% NBF for two weeks. The ratio of tissue to fixative volume was 1:50.

2.5.2 Specimen sectioning

The specimens were radiographed to confirm the long axis of the tooth sockets and identify the individual extraction sockets for the second and third premolars (Figure 2.9a). The radiographs were taken using Gendex dental systems (Monza, Italy) on Kodak intraoral dental films (Carestream Health, NY, USA). The films were exposed at 38cm focal length for 0.16 seconds and processed in an automatic processing machine.

The individual extraction sockets were identified on the radiograph and matched on the mandible specimens. The sockets were separated using a manual coping saw (Spear and Jackson, England) (Figure 2.9b). The sectioned specimens were placed into histology cassettes with the corresponding labels (Figure 2.9c).

Figure 2.9 Specimen preparation for histology processing

(a) Taking radiograph for block specimen

(b) Separating extraction sockets using manual coping saw

(c) Placing specimen into histology cassette
2.5.3  Paraffin-embedded specimens

As no hard tissue formation was expected at baseline, the specimens were demineralised and processed for paraffin-embedding. This was carried out by Histology unit, Department of Pathology, Dunedin School of Medicine.

2.5.3.1 Decalcification

The specimens were treated with 10% ethylenediaminetetraacetic acid (EDTA) and placed in rapid microwave labstation at 37°C (KOS Microwave Histostation, Milestone, Italy). EDTA solution was changed every 48 hours. The specimens were radiographed weekly using the same protocol as mentioned in Section 2.5.2 to examine the degree of decalcification. Decalcification was considered complete when no sign of radiopaque tissue could be identified on the radiographs. The decalcification process took approximately 8 weeks to complete.

2.5.3.2 Processing for blocking

Once decalcification was completed, the processing was carried out using an automatic processing machine (Excelsior ES tissue processor, Thermo Scientific, Waltham, USA). The specimens were treated through twelve stations with a graded series of ethanol and xylene, and paraplast wax (56°C melting point).

The specimens were radiographed again after processing to identify and label the long axis and the centre of the extraction socket. The processed specimens were embedded in molten paraffin (56°C).

2.5.3.3 Sectioning and staining

Eight decalcified paraffin-embedded specimens from baseline were sectioned in a buccal-lingual direction along the long axis of the tooth. Two 4µm thick sections were taken from the centre of each extraction socket specimen using a Leica RM 2025 microtome (Leica Microsystems Inc. Deerfield, USA). The sections were stained with haematoxylin and eosin stain.
2.5.4 Resin-embedded specimens

The specimens from the eight and 16 weeks healing point were processed for resin-embedding. The tissues were processed according to the protocol described by Donath and Breuner (1982) and Duncan (2005). The protocol is summarised below and attached in Appendix II-2 and II-3.

The samples were dehydrated in increasing grades of ethanol. The specimens were transferred into xylene (Ajax Finechem Pty Ltd, New Zealand) for 4 days in a fume hood, with two changes of xylene during the time period. Specimens were washed in methyl methacrylate (MMA) monomer and then placed in MMA I and MMA II for two days each.

They were then transferred into glass jars with pre-set bases. These glass jars were prepared one week prior to the time required, by filling the jars with MMA III to one third depth of approximately 8mm. The jars were then filled with MMA III and sealed with the screw top lid. They were placed in the water bath and allowed to set, which took around three to four weeks.

The blocks of resin containing the specimen were retrieved by breaking the glass jars. They were then trimmed and polished to smaller blocks following the outline of the specimen prior to final sectioning. The prepared specimen blocks were then radiographed to identify the long axis of the tooth extraction sockets.

2.5.4.1 Sectioning of resin-embedded specimens

The sectioning machine used was a Struers Accutom precision table-top cut-off machine (Ballerup, Denmark) fitted with a diamond cut-off wheel (MOD 13 127 x 0.4 x 12.7mm). The resin blocks were mounted onto the machine in a buccal-lingual direction along the long axis of the tooth extraction sockets. Sequential 500µm sections were cut and press mounted onto an opaque acrylic base plate using cyanoacrylate glue.

For each specimen, two buccal-lingual sections representing the central area of the socket were selected. They were then further ground and polished using a rotating grinding machine (Tegra-Pol, Struers, Ballerup, Denmark) and Silicon Carbide Paper (grit size #180 to #4000). The final thickness of the sections was around 100µm. This
thickness was measured using a digital micrometer (Digital Indicator, Mitutoyo, Japan).

2.5.4.2 Staining

After superficial etching and decalcification with 20% ethanol and 1% formic acid in an ultrasonic bath, all sections were stained with one part MacNeal’s tetrachrome and two parts toluidine blue. The staining protocol is attached in Appendix II-4. The slides were then rinsed with distilled water and air-dried.
2.6 Histological analyses

2.6.1 Imaging of histological sections

For descriptive histology and histomorphometric analyses, images were obtained and digitalised using a light microscope (Olympus AX70, Olympus Optical Co. ltd, Japan) and an imaging system (Micropublisher 5.0 RTV, Qimaging) at 10x magnification. A series of images were taken using the montaging software Volocity 5.2.0 (Improvision, MA, USA). The individual digital images obtained were combined using Autopano Pro 2.5.2 (Kolor, USA) software to produce an image of the entire healing socket.

2.6.2 Polarised images

The resin-embedded specimens were examined under polarized light microscope (Olympus AX70, Olympus Optical Co. ltd, Japan) to differentiate the orientation of the collagen fibres in the newly formed bone. However, it was not possible to obtain satisfactory polarised images due to the thickness of the mounting acrylic base.


2.7 Histomorphometric analyses

Histomorphometric analyses were carried out for the eight and 16 weeks resin-embedded specimens.

2.7.1 Region of interest

A region of interest (ROI) measuring 4x6mm was identified for each specimen using the following protocol. The coronal margin of the ROI was aligned with the level of the alveolar crest. Effort was made to avoid the alveolar bone proper and the cortical bone to be included in the ROI. These regions were chosen because they represent the portion of the alveolar ridge that most likely to be utilized for implant placement. The sheep mandible contains large areas of bone marrow space. Therefore, the dimension of the ROI was also limited by the anatomy in sheep. A representative ROI is shown in Figure 2.10.

Figure 2.10 Selected ROI measured 4x6mm
2.7.2 Morphometric measurements

For each specimen, the area occupied by newly formed bone (NB), residual bone graft material (RG), and fibrovascular connective tissue (FCT) were measured using full-colour thresholding on a computer-based image analysis system, Image J (version 1.47q, NIH, USA). A semi-automated segmentation technique was used to calculate the different tissue volumes. Threshold values for NB, RG and FCT were selected manually according to the signal intensity in each image (Figure 2.11a, b and c). These segmented tissues were measured and expressed as area percentages within the ROI.

Measurements were taken from the two buccal-lingual sections representing the central area of each socket. The mean value of these two sections was used to calculate the overall mean results for each treatment type.

Bone to residual graft ratio was obtained by dividing the amount of newly formed bone by the amount of residual graft to demonstrate the proportion of bone and residual graft within the selected region of interest.

2.7.3 Intra-examiner reliability test

Intra-examiner reliability for the morphometric evaluation was assessed. Duplicate measurements at two weeks apart were completed using eight randomly selected samples of the specimens. The concordance correlation coefficient for the morphometric measurements ranged from 0.87 to 0.98. The two sets of data are highly correlated to each other. There are no statistically significant differences between each pairs of measurements. The intra-class correlation coefficients are generally high.
Figure 2.11 Representative images showing segmentation values of the tissue of interest using Image J software

(a) Threshold selected for newly formed bone (red)

(b) Threshold selected for residual graft material (red)

(c) Threshold selected for fibrous connective tissue (red)
2.8 Histometric analyses

Histometric analyses were carried out for the eight and 16 weeks resin-embedded specimens using Image J (version 1.47q, NIH, USA).

2.8.1 Height differences between the buccal and lingual bone crest

The height of the buccal and lingual bone walls was determined in the following way (Figure 2.12): the boundaries between the buccal/lingual cortical walls and newly formed bone were identified (c-c’ and b-b’). The angle formed by c-c’ and b-b’ was bisected, which gave rise to the long axis of the extraction socket (a-a’). The vertical distance between the buccal bone crest and the lingual bone crest was measured as the height difference between the buccal and lingual cortical bone walls (red line). This was expressed in millimetres.

2.8.2 Width of the buccal and lingual bone walls

The width of the buccal and lingual bone walls was measured at 1mm apical to the buccal and lingual bone crest (Figure 2.12). The values were recorded in millimetres.

2.8.3 Hard tissue bridging

The outline of the healed extraction socket was determined following the contour of the newly formed bone. The distance from the buccal bone crest to the lingual bone crest was measured (Figure 2.13). The proportion of hard tissue bridging was calculated by measuring the linear distance of newly formed bone divided by the distance from buccal to lingual bone crest. This was expressed as a percentage.

2.8.4 Coronal overbuilding

The distance from the lingual crest to the most coronal point of the newly formed bone was measured along the long axis of the extraction socket (a-a’) (Figure 2.13). A positive value was obtained when the newly formed bone was found above the lingual crest. A negative value was recorded if the newly formed bone was below the lingual crest. This was measured in millimetres.
Figure 2.12 Histometric analysis to measure the height and width of buccal and lingual cortical bone walls

Red line: height difference between buccal and lingual crest

Blue line: width of the buccal bone at 1mm apical to buccal bone crest (BC)

Green line: width of the lingual bone at 1mm apical to lingual bone crest (LC)

a-a’, b-b’, c-c as referenced in section 2.8.1
Figure 2.13 Histometric analysis to measure the hard tissue bridge and coronal overbuilding

Red line: the linear distance of newly formed bone at the most coronal part of the extraction socket

Green line: the outline of the newly formed bone at the most coronal part of the extraction socket between the lingual crest (LC) and buccal crest (BC)

Blue line: the distance between the lingual crest and the most coronal part of the extraction socket along the long axis of the extraction socket

Scale bar = 1mm
2.9 Statistical analysis

Mean values and standard deviations were calculated for all variables using the sheep as a statistical unit. The differences between the test and control groups were analyzed by using a mixed model analysis with repeated measures for the treatment factor. A compound symmetry covariance structure was assumed for the treatment group factor. This model of analysis takes into account both the fixed effect (treatment groups and healing time) and the random effect (between the experimental animals) and allows for missing values in the dataset. Differences were considered statistically significant when $p$ was $<$0.05. Pairwise comparisons with Bonferroni adjustment were conducted for the groups with statistically significant differences. The statistical analysis was performed with SPSS statistics software for Mac (version 20.0, IBM corporation, Somers, USA).
Chapter 3 Results

3.1 Material handling properties

3.1.1 ECWN vs Bovine xenograft

The handling properties of ECWN and BX were very different in transferring the materials to the surgical site and the management of the materials within the extraction sockets.

ECWN was very similar to cotton wool. The material was easily picked up by tweezers. Transferring the material from the container to the surgical site was simple. It was flexible, moldable and hydrophilic. Therefore, adapting ECWN into the extraction socket was easy. Once the material was in place, it was lightly packed. It adapted well into the defect with no tendency for migration.

BX, on the other hand, was granular. The BX granules were wetted with blood in the provided dish. Transferring the granules into the surgical site required a syringe carrier. This procedure was repeated multiple times in order to graft sufficient amount of the graft material into the extraction socket. Therefore, placing BX into the surgical site required longer operating time. Profuse bleeding was seen after extraction. Small granules of BX often migrated due to the blood flow from within the extraction sockets. Condensing the BX granules within the extraction socket was more difficult to achieve compared to ECWN material.

3.1.2 OsseoGuard® membrane

The OsseoGuard® Membrane is made from purified bovine type I collagen. When used for our study at extraction socket sites, we found the membrane did not adapt well over the alveolar ridge. It was time-consuming to place the membrane and fold it over the alveolar ridge due to the rigidity and memory of the membrane. Extending the hydration time of the membrane in sterile saline did not improve the handing property.
3.2 Post-operative recovery

No post-operative complications were encountered in relation to healing of the extraction socket sites during the study. However, infections were observed in some of the animals around the sinus and femur sites. They were treated with antibiotics Trimethoprim (Amphoprim injection 1ml/15kg, Virbac New Zealand Ltd., East Tamaki, Auckland) and recovered within two to three weeks.

3.3 Site harvesting

Four extraction socket sites were harvested from each experimental animal. A total of 64 sites were processed for resin embedding. Two extraction sockets from the left hemi-mandible of one animal did not heal. Bone sequestrum was detected within the sockets disrupting the complete epithelialisation over the bony defects. All other extraction sockets healed uneventfully. Retained root tips were detected from the radiographs taken prior to sectioning in four extraction sockets. Therefore, six of the specimens were not included in the reporting, of which five were from the eight weeks group and one from the 16-week group.
3.4 Radiographic examination of specimens

Radiographs were taken after tooth extractions and placement of graft materials using intraoral radiographic films (Figure 3.1a-b, 3.2a-b). Post-mortem radiographs were taken prior to sectioning and resin embedding. Bovine xenograft (BX) appeared as radio-opaque granules in the extracted tooth sockets (Fig 3.1d). ECWN material was radio-lucent (Fig. 3.1c). The ECWN grafted sockets appeared similar to the non-grafted sockets on the radiographs.

3.4.1 Eight weeks healing time

Figure 3.1 Radiographic comparison of harvested specimens at eight weeks

(a)-(b) Taken after extraction of 2\textsuperscript{nd} & 3\textsuperscript{rd} premolars

(c)-(d) Taken after grafting procedures

(e)-(f) Taken after 8 weeks of healing
After eight weeks of healing, the extraction sockets are partially filled with bone (Fig 3.1e and f). ECWN grafted sites had similar bone density to non-grafted sites. BX grafted sites appeared more radio-opaque than the rest of the extraction sockets. The inter-radicular bone was still detectable and appeared more radio-opaque than the extraction sockets.

3.4.2 16 weeks healing time

Figure 3.2 Radiographic comparison of harvested specimens at 16 weeks

(a)-(b) Taken after extraction of 2\textsuperscript{nd} & 3\textsuperscript{rd} premolars

(c)-(d) Taken after grafting procedures

(e)-(f) Taken after 8 weeks of healing

After 16 weeks of healing, bone fill appeared homogenous in ECWN and non-grafted sites (Fig. 3.2e and f). BX particles could still be identified on the radiographs (Fig. 3.2f). The inter-radicular bone was not visible in the radiographs at 16 weeks.
3.5 Descriptive histology

3.5.1 Paraffin embedded specimens at baseline

3.5.1.1 Non-grafted (No-) sites

The non-grafted extraction site had intact buccal and lingual cortical bone walls (Figure 3.3a). The extraction socket was filled with blood coagulum to the level apical to the buccal and lingual bone crest. Remnants of the severed periodontal ligament that was rich in collagen fibres and blood vessels were visible in the apical portion of the extraction socket. Under higher magnification (Figure 3.3b), the coagulum was comprised mainly of erythrocytes and platelets. Isolated inflammatory cells were also detected.

![Figure 3.3 Demineralised specimen of non-grafted site at baseline](image)

(a) Overview of a buccal-lingual section from the non-grafted extraction socket. Intact buccal (B) and lingual (L) cortical bone walls with severed periodontal ligament (PDL) found at the apical portion of the extraction socket. H&E staining. Scale bar = 1mm

(b) Blood coagulum containing red blood cells and platelet with isolated inflammatory cells. H&E staining. Scale bar = 100µm
3.5.1.2 *Bovine xenograft with membrane (B+) sites*

The BX grafted extraction sites had intact buccal and lingual cortical bone walls. The buccal bone crest appeared higher than the lingual crest. The extraction socket was grafted to the level of the buccal and lingual bone crest (Figure 3.4a). Collagen membrane (CM) was intact and extended over the buccal and lingual bone walls. The BX granules were not visible from the demineralised specimen. However, the space occupied by BX granules could be identified from the section. When examined under higher magnification (Figure 3.4b), the outline of BX particles could be identified. Strands of fibrin with erythrocytes occupied most of the space between the graft particles.

![Figure 3.4 Demineralised specimen of BX grafted site at baseline](image)

(a) Overview of a buccal-lingual section from the BX grafted extraction socket. CM: collagen membrane extending over the buccal and lingual bone walls. H&E staining. Scale bar = 1mm

(b) Erythrocytes (blue arrows) trapped in a fibrin network occupied the spaces between BX particles (BX). H&E staining. Scale bar = 100µm
3.5.1.3 ECWN with membrane (ECWN+) and ECWN (ECWN-) sites

The ECWN grafted extraction sites had intact buccal and lingual cortical bone walls. ECWN graft material occupied the extraction sockets to the level apical to the buccal and lingual bone crest (Figure 3.4a). The collagen membrane was not visible in the specimens from ECWN+ sites, as the membrane was lost during processing of the tissue blocks. Remnant of the severed periodontal ligament that were rich in collagen fibres and blood vessels was visible in the apical portion of the extraction socket. When examined under higher magnification (Figure 3.4b), the graft material contained fibres orientated in random directions and pores of various dimensions. Erythrocytes were closely associated with the graft material and occupied the pores within the graft material.

![Figure 3.5 Demineralised specimen of ECWN grafted site at baseline](image)

(a) Overview of a buccal-lingual section from the ECWN grafted extraction socket. ECWN graft material outlined in green dotted line. B: buccal bone wall; L: lingual bone wall; PDL: periodontal ligament. H&E staining. Scale bar = 1mm

(b) Grafted extraction site contained ECWN fibres and pores of various dimensions (black asterisks) and erythrocytes (blue arrows). H&E staining. Scale bar = 100µm
3.5.2 Resin-embedded specimens

In both the grafted and non-grafted extraction sites, the healed sockets were covered by thick, keratinized stratified squamous epithelium with sharp rete ridges (Figure 3.6). The epithelial rete ridges over the extraction bony defects were more irregular with hyperchromatism of the basal cells, indicative of epithelium that was recently regenerated. Scattered inflammatory cells were observed in the connective tissue adjacent to the epithelium.

The bone crest of the original buccal cortical plate was found to be located apically to the bone crest of the lingual cortical plate in all specimens (Figure 3.7). The newly formed bone within the bony defect was in direct continuity with the buccal and lingual socket walls. In some of the specimens, newly formed bone was also detected lateral to the original buccal and lingual cortical plates (Figure 3.8).
Figure 3.6 Oral mucosa over the healed extraction sites

Scale bar = 1mm

Figure 3.7 Microphotograph of a buccolingual section of healed extraction site

Red dotted line separating buccal (B) and lingual (L) cortical plates from newly formed bone. Green arrows: buccal and lingual bone crests. Scale bar = 1mm

Figure 3.8 Buccal bone overgrowth at the healed extraction site

Red dotted line: newly formed bone found lateral to the original buccal (B) cortical plate. Scale bar = 1mm
3.5.3 Eight weeks healing time

3.5.3.1 ECWN with membrane (ECWN+) sites

Extraction sockets healing at the sites grafted with ECWN and resorbable membrane was composed of vital newly formed bone with irregular trabeculae, fibrovascular connective tissue and residual graft materials (Figure 3.9a). A dome-shaped portion of the newly formed bone occupied the entrance of the extraction socket. The marginal portion of the newly formed bone was located coronal to the original buccal and lingual bone crests.

New bone formed within the extraction sockets was predominantly woven bone (Figure 3.9b). The orientation of collagen fibres within the newly formed bone was irregular. The fibrovascular connective tissue was immature and vascular with inflammatory cell infiltrate. The cells found were mainly plump fibroblasts, lymphocytes and macrophages. The residual ECWN graft material was intimately associated with the newly formed bone. Some of the ECWN graft materials were incorporated into the osteoid.

3.5.3.2 ECWN without membrane (ECWN-) sites

Extraction sockets grafted with ECWN alone healed in a similar pattern to ECWN+ sites. Irregular trabecular bone was formed in the defects, mostly of woven bone (Figure 3.10a). In contrast to that found in ECWN+ sites, the margin of the newly formed bone did not exceed that of the buccal and lingual bone crests. Residual graft material was observed in close proximity with newly formed bone (Fig 3.10b).
Figure 3.9 Resin-embedded histology for ECWN+ site, at eight weeks

(a) Buccal-lingual section of the extraction socket grafted with ECWN and collagen membrane. B, buccal bone wall; L, lingual bone wall; BM, bone marrow space. Scale bar = 1mm

(b) Newly formed bone (NB) intimately associated with residual bone graft materials (G, red arrows). FCT, fibrovascular connective tissue. Scale bar = 100µm
Figure 3.10 Resin-embedded histology for ECWN- site, at eight weeks

(a) Irregular bony trabeculae within extraction socket grafted with ECWN without collagen membrane. B, buccal bone wall; L, lingual bone wall; BM, bone marrow space. Scale bar = 1mm

(b) Newly formed bone (NB) surrounded by residual graft materials (G, red arrows). FCT, fibrovascular connective tissue. Scale bar = 100μm
3.5.3.3 Bovine xenograft (B+) sites

In the eight weeks extraction socket specimens grafted with BX, most of the graft particles were found in the coronal portion of the extraction sockets (Fig. 3.11a). BX particles were surrounded by woven bone and connective tissue (Fig. 3.11b). There was no bone tissue bridging detected across the coronal margin of the defect in most of the specimens.

3.5.3.4 Non-grafted sites

In the non-grafted sites, islands of loose trabecular bone were seen in the extraction sockets. Trabecular bone was also seen across the coronal margin of the defect, connecting the buccal and lingual cortical plates (Fig. 3.12a). Newly formed bone was mostly immature woven bone, surrounded by immature fibrovascular connective tissue (Fig. 3.12b).
Figure 3.11 Resin-embedded histology for B+ site, at eight weeks

(a) Healing of the extraction socket grafted with BX and collagen membrane. BX materials found in the coronal portion of the extraction sockets. B, buccal bone wall; L, lingual bone wall; BM, bone marrow space. Scale bar = 1mm

(b) BX graft particles (G) surrounded by newly formed bone (NB) and fibrovascular connective tissue (FCT). Scale bar = 100μm
Figure 3.12 Resin-embedded histology for NO- site, at eight weeks

(a) Extraction socket healing at non-grafted site with trabecular bone bridging the buccal and lingual cortical plates. B, buccal bone wall; L, lingual bone wall; BM, bone marrow space. Scale bar = 1mm

(b) Newly formed bone (NB) resembled that of woven bone. FCT: fibrovascular connective tissue. Scale bar = 100µm
3.5.4 16 weeks healing time

3.5.4.1 ECWN+ sites

Healing within the extraction sockets continued to mature by 16 weeks. At the ECWN+ sites, finger-like bone trabeculae were observed within the grafted sites. The newly formed bone sealed the extraction socket entrance and bridged across the buccal and lingual cortical plates (Figure 3.13a).

The new bone formed had developed Haversian systems (Figure 3.13b). Woven bone was being replaced by lamellar bone with parallel collagen fibres. The fibrous connective tissue stroma became less vascular with less inflammatory cells. Fibroblasts became more spindle-like in shape, indicating maturity of the connective tissue. ECWN graft material was almost undetectable.

3.5.4.2 ECWN- sites

In ECWN- sites, healing appeared similar to ECWN + sites. New bone formation across the coronal portion of the extraction sockets was observed in the 16-week specimens (Figure 3.14a). As in the ECWN+ sites, the newly formed bone had thin finger-like trabeculae, extending from the buccal and lingual cortical plates towards the middle of the bony defects.

Newly formed bone consisted of an increased proportion of lamellar bone compared to woven bone (Figure 3.14b). There were numerous fibrous stroma spaces amongst the newly formed bone. Small amounts of residual graft materials were observed in some portions of the extraction sockets.
Figure 3.13 Resin-embedded histology for ECWN+ site, at 16 weeks

(a) Finger-like bone trabeculae extending from the buccal (B) and lingual (L) cortical plates in extraction socket grafted with ECWN and collagen membrane. BM, bone marrow space. Scale bar = 1mm

(b) Newly formed bone (NB) consisting of woven bone (WB) and lamellar bone (LB). G, residual graft material (red arrows); FCT, fibrovascular connective tissue. Scale bar = 100µm
Figure 3.14 Resin-embedded histology for ECWN- site, at 16 weeks

(a) Thin finger-like bone trabeculae extending from the buccal (B) and lingual (L) socket walls towards the middle of the bony defect. BM, bone marrow space. Scale bar = 1mm

(b) Small amount of residual graft material (G, red arrows) amongst the newly formed bone (NB). FCT, fibrovascular connective tissue. Scale bar = 100µm
3.5.4.3 BX sites

Healing within BX grafted extraction sockets appeared to be different at 16 weeks, when compared to ECWN grafted sites. New bone could be detected throughout the different levels of the extraction sockets (Figure 3.15a). A hard tissue bridge formed at the coronal margin of four out of the eight extraction sockets contained BX graft particles. In the other half of the specimens, no hard tissue bridge could be detected. The coronal margins of the entry to these extraction defects were occupied by BX particles surround in connective tissue matrix.

The graft particles could be identified in the bony defects. Most of the particles were surrounded by a mixture of woven bone, lamellar bone or connective tissue (Figure 3.15b). Some of the BX particles, especially in the coronal and apical portion of the defects, were surrounded by connective tissue only. Newly formed bone was mainly found adjacent to the graft particles.

3.5.4.4 Non-grafted sites

At 16 weeks, healing at non-grafted sites was characterised by the formation of thick bony trabeculae extending from the buccal and lingual cortical plates into the bony defects (Figure 3.16a). These trabeculae were separated by larger fibrous stroma space when compared to ECWN grafted sites.

The newly formed bone had parallel collagen fibres, resembling that of lamellar bone (Figure 3.16b). This healing pattern was distinctly different from that found in ECWN and BX grafted sites.
Figure 3.15 Resin-embedded histology for B+ site, at 16 weeks

(a) Healing of extraction socket grafted with BX material. B, buccal bone wall; L, lingual bone wall; BM, bone marrow space. Scale bar = 1mm

(b) BX particles (G, red asterisks) surrounded by newly formed bone (NB) and fibrovascular connective tissue (FCT). Scale bar = 100µm
Figure 3.16 Resin-embedded histology for NO-site, at 16 weeks

(a) Thick bony trabeculae extending from the buccal (B) and lingual (L) cortical plates towards the middle of the bony defect. BM, bone marrow space. Scale bar = 1mm

(b) Newly formed bone (NB) mainly consisting of lamellar bone. FCT, fibrovascular connective tissue. Scale bar = 100µm
3.5.5 Summary of descriptive histology

Baseline demineralised specimens for non-grafted extraction socket revealed aggregation of erythrocytes and scattered inflammatory cells within the blood coagulum. Erythrocytes within a fibrin network occupied the spaces between BX graft particles in the BX grafted sites. Collagen membrane was intact and extended over the buccal and lingual bone walls. ECWN graft material contained fibres oriented in random directions as well as pores of different dimensions. Erythrocytes were closely associated with the ECWN graft material.

The extraction socket healing at eight weeks had no distinct differences across the four different treatment modalities. Irregular trabecular bone formed in all extraction sockets. The newly formed bone was predominantly woven bone and surrounded by unmineralised osteoid. The ECWN graft material was intimately associated with the newly formed bone. BX was encapsulated with bone and connective tissue.

Healing continues to mature at 16 weeks. A hard tissue bridge was present at the coronal portion of the extraction socket in ECWN and non-grafted sites. However, BX graft particles were found in the coronal portion of the extraction sockets, interrupting the continuity of the hard tissue bridge. In ECWN sites, osseous healing followed a fine, finger like trabecular pattern. In comparison, the non-grafted extraction sockets were filled with thick trabeculae separated by large areas of fibrous stroma. At bovine xenograft sites, new bone was formed directly adjacent to the graft granules. At 16 weeks, woven bone was being replaced by lamellar bone, with the development of Haversian systems. ECWN graft materials were almost undetectable after 16 weeks. BX particles were present amongst bone and connective tissue.
3.6 Histomorphometric analysis

Histomorphometric analysis was carried out for two buccal-lingual sections representing the central area of each socket. The percentages of the newly formed bone, residual graft material and fibrovascular connective tissue were identified for each section. The mean value for each parameter from these two sections was used to calculate the overall mean results for each treatment type.

3.6.1 Eight weeks healing time

Examples of the selected region of interest used for histomorphometric analysis are shown in Figure 3.17. Overall mean results for each type of treatment after 8 weeks of healing are presented in Table 3.1. The mean percentages of newly formed bone and residual graft materials in grafted and non-grafted sites at eight weeks are illustrated in Fig. 3.18 and 3.19. Mean results for the two sections analysed for each extraction socket are listed in Appendix III.

The amount of new bone formed in ECWN grafted sites was 36.5 ± 17.3% for ECWN+ sites and 40.7 ± 16.9% for ECWN- sites. The percentage of new bone detected in the eight weeks extraction sockets was the highest among the NO- sites (47.9 ± 5.0%). The amounts of new bone formed in the grafted sockets were similar, with BX sites showing the greatest variation (38.6 ± 18.1%). Comparison of new bone formation in extraction sockets treated with four different modalities did not reveal statistically significant differences ($F_{3, 17.8} = 0.937, p = 0.444$).

Comparison of residual graft materials found in the grafted sites revealed no significant differences ($F_{2, 12.5} = 1.89, p = 0.192$). Small amounts of residual graft (<5%) were detected in all grafted sites.

3.6.1.1 Newly formed bone to residual graft ratio

The overall mean values for bone to graft ratio are summarized in Table 3.2. Detailed analyses for each extraction socket are listed in Appendix III. Comparable results were obtained for all three types of treatment. No statistically significant differences were identified ($F_{2, 12.7} = 0.235, p = 0.794$).
Figure 3.17 Selected regions of interest measured 4x6mm for eight weeks specimens
(a) ECWN+ site; (b) ECWN- site; (c) B+ site; (d) No- site. Scale Bar = 1mm

Table 3.1 Volumetric data describing the composition (%) of tissues in the grafted and non-grafted extraction sites at eight weeks

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>No. of specimens</th>
<th>Newly formed bone (%)</th>
<th>Residual graft (%)</th>
<th>Fibrovascular connective tissue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Mean ± SD)</td>
<td>(Mean ± SD)</td>
<td>(Mean ± SD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(95%CI)</td>
<td>(95%CI)</td>
<td>(95%CI)</td>
</tr>
<tr>
<td>ECWN+</td>
<td>6</td>
<td>40.7 ± 16.9</td>
<td>3.0 ± 1.8</td>
<td>57.7 ± 16.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(23.0-58.5)</td>
<td>(1.1-4.8)</td>
<td>(40.6-74.7)</td>
</tr>
<tr>
<td>ECWN-</td>
<td>5</td>
<td>36.5 ± 17.3</td>
<td>1.6 ± 0.8</td>
<td>61.7 ± 18.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(15.0-57.9)</td>
<td>(0.6-2.5)</td>
<td>(39.3-84.1)</td>
</tr>
<tr>
<td>B+</td>
<td>8</td>
<td>38.6 ± 18.1</td>
<td>2.5 ± 0.9</td>
<td>59.7 ± 18.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(23.5-53.8)</td>
<td>(1.8-3.3)</td>
<td>(44.7-74.8)</td>
</tr>
<tr>
<td>NO-</td>
<td>8</td>
<td>47.9 ± 5.0</td>
<td></td>
<td>52.9 ± 4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(43.7-52.0)</td>
<td></td>
<td>(49.0-56.9)</td>
</tr>
</tbody>
</table>

Table 3.2 Bone to graft ratio at eight weeks

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>ECWN+</th>
<th>ECWN-</th>
<th>B+</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of specimens</td>
<td>6</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Bone to graft ratio (Mean ± SD)</td>
<td>14.9 ± 10.3</td>
<td>15.9 ± 5.8</td>
<td>12.7± 8.2</td>
</tr>
</tbody>
</table>
Figure 3.18 The percentage of newly formed bone in grafted and non-grafted extraction sites at eight weeks

Figure 3.19 The percentage of residual graft materials in grafted extraction sites at eight weeks
3.6.2 16 weeks healing time

Examples of the selected region of interest used for histomorphometric analysis are shown in Figure 3.20. Overall mean results for each type of treatment after 16 weeks of healing are presented in Table 3.3. The mean percentages of newly formed bone and residual graft materials in grafted and non-grafted sites at 16 weeks are illustrated in Figure 3.21 and 3.22. Mean results for the sections analysed for each extraction socket are listed in Appendix III.

New bone formation within the ECWN- sites (45.2 ± 6.8%) was similar to NO- sites (45.7 ± 10.7%). ECWN+ sites revealed the highest percentage of new bone (49.8 ± 12.5%), whereas BX sites showed the lowest amount of new bone (41.1 ± 11.1%). However, comparison of new bone formation in extraction sockets treated with four different modalities did not reveal statistically significant differences ($F_{3, 19.4} = 0.902$, $p = 0.458$).

The amount of residual graft materials found were 1.2 ± 0.7% for ECWN+ sites, 1.1 ± 0.7% for ECWN- sites and 5.4 ± 5.6% for BX sites. Comparisons of residual graft materials found in the ECWN grafted sites revealed no significant differences. However, there were significant differences between treatment groups ($F_{2, 13.8} = 4.76$, $p = 0.027$). Significant differences were found between ECWN grafted sites without membrane and BX sites ($p = 0.048$). The differences between ECWN sites with membrane and BX sites were marginally significant ($p = 0.055$). Residual graft materials detected within BX sites also showed greater variation between the experimental animals (5.4 ± 5.6%).

3.6.2.1 Newly formed bone to residual graft ratio

The overall mean values for bone to graft ratio at 16 weeks are summarized in Table 3.4. Detailed analyses for each extraction socket are listed in Appendix III. ECWN treated sites with or without membrane had higher bone to graft ratio than B+ sites. To homogenize the variance of the sample data, logarithmic transformations were carried out for bone to graft ratio obtained for 16 weeks. Statistically significant differences were observed between ECWN+ and B+ sites ($p = 0.029$), as well as ECWN- and B+ sites ($p = 0.042$).
Figure 3.20 Selected regions of interest measured 4x6mm for 16 weeks specimens
(a) ECWN+ site; (b) ECWN- site; (c) B+ site; (d) No- site. Scale Bar = 1mm

Table 3.3 Volumetric data describing the composition of tissues in the grafted and non-grafted extraction sites at 16 weeks

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>No. of specimens</th>
<th>Newly formed bone (%)(Mean ± SD) (95%CI)</th>
<th>Residual graft (%)(Mean ± SD) (95%CI)</th>
<th>Fibrovascular connective tissue(%)(Mean ± SD) (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECWN+</td>
<td>8</td>
<td>49.8 ± 12.5 (39.4-60.3)</td>
<td>1.2 ± 0.7 (0.6-1.8)</td>
<td>49.0 ± 12.1 (38.9-59.1)</td>
</tr>
<tr>
<td>ECWN-</td>
<td>8</td>
<td>45.2 ± 6.8 (39.6-50.9)</td>
<td>1.1 ± 0.7* (0.5-1.7)</td>
<td>53.7 ± 6.4 (48.3-59.1)</td>
</tr>
<tr>
<td>B+</td>
<td>7</td>
<td>41.1 ± 11.1 (30.8-51.4)</td>
<td>5.4 ± 5.6 (0.2-10.61)</td>
<td>53.5 ± 10.4 (43.9-63.2)</td>
</tr>
<tr>
<td>NO-</td>
<td>8</td>
<td>45.7 ± 10.7 (36.7-54.6)</td>
<td></td>
<td>54.3 ± 10.7 (45.4-63.3)</td>
</tr>
</tbody>
</table>

* Significantly different from B+ (p<0.05)

Table 3.4 Bone to graft ratio at 16 weeks

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>ECWN+</th>
<th>ECWN-</th>
<th>B+</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of specimens</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Bone to graft ratio (Mean ± SD)</td>
<td>61.8 ± 65.5*</td>
<td>59.4 ± 57.9*</td>
<td>16.1 ± 19.2</td>
</tr>
</tbody>
</table>

* Significantly different from B+ (p<0.05)
Figure 3.21 The percentage of newly formed bone in grafted and non-grafted extraction sites at 16 weeks

Figure 3.22 The percentage of residual graft materials in grated extraction sites at 16 weeks
3.6.3 Summary of histomorphometric analysis

At eight weeks, the amount of new bone formed within the grafted and non-grafted extraction sockets was similar. Residual graft materials were detected in all grafted sites. Histomorphometric analysis did not reveal statistically significant differences for the amount of new bone formation and residual graft materials in extraction sockets treated with different modalities after eight weeks of healing.

At 16 weeks, there were statistically significant differences in the amount residual graft materials found in treated sites. There was less residual graft material detected in ECWN treated sites than BX sites. Comparison of new bone formation in extraction sockets treated with four different modalities did not reveal statistically significant differences. Bone to graft ratio was significantly higher in ECWN treated sites when compared to B+ sites.
3.7 Histometric analyses

Histometric analyses were carried out for eight and 16 weeks specimens. The height differences between the buccal and lingual bone crest were identified and expressed in millimetres. Buccal and lingual bone wall thickness was also recorded at the level 1mm apical to the buccal and lingual bone crest. Hard tissue bridge formation was measured by calculating the proportion of newly formed bone along the most coronal outline of the healed extraction socket and expressed as percentages. The distance between the lingual crest to the most coronal portion of the healed extraction socket along the long axis of the extraction socket was recorded as the coronal overbuilding.

3.7.1 Eight weeks healing time

3.7.1.1 Crestal height differences and buccal and lingual bone widths

Overall mean results for each type of treatment after eight weeks of healing are presented in Table 3.5. The results for the specimens analysed for each extraction socket are listed in Appendix IV. The height differences between the buccal and lingual bone crest varied from $1.8 \pm 0.6$mm to $2.4 \pm 0.8$mm. The buccal bone crest was always found to be apical to the lingual bone crest. The grafted sockets showed greater differences than the non-graft sites. The height differential detected was the lowest among the NO- sites ($1.8 \pm 0.6$mm). The ECWN+ sites showed highest mean buccal and lingual bone thickness ($5.6 \pm 1.8$mm and $5.9 \pm 1.5$mm respectively). However, comparison of the height ($F_{3, 17.8} = 0.467, p = 0.709$), buccal wall ($F_{3, 16.9} = 0.453, p = 0.719$) and lingual wall ($F_{3, 19.0} = 0.965, p = 0.429$) width differences treated with four different modalities did not reveal statistically significant differences.

3.7.1.2 Hard tissue bridging and coronal overbuilding

Overall mean results for hard tissue bridging and coronal overbuilding after eight weeks of healing are presented in Table 3.6. The results for the specimens analysed for each extraction socket are listed in Appendix IV. The formation of the hard tissue bridging varied from $62.3 \pm 20.0\%$ to $73.0 \pm 13.5\%$. At eight weeks, the non-grafted sites and the ECWN grafted sites with no membrane showed the highest mean hard tissue bridging. All of the experimental sites showed comparable coronal overbuilding measured from the lingual crest to the most coronal point of the healed extraction sockets. Comparison of the hard tissue bridging ($F_{3, 17.7} = 1.078, p = 0.384$) and coronal overbuilding ($F_{3, 178.3}$...
= 0.624, \( p = 0.609 \) at sites treated with four different experimental modalities did not reveal statistically significant differences.

Table 3.5 Crestal height difference and width of the buccal and lingual walls of the grafted and non-grafted extraction sites at eight weeks

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>No. of specimens</th>
<th>Crestal height differences (mm) (Mean ± SD)</th>
<th>Buccal bone thickness (mm) (Mean ± SD)</th>
<th>Lingual bone thickness (mm) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECWN+</td>
<td>6</td>
<td>2.4 ± 0.8</td>
<td>5.6 ± 1.8</td>
<td>5.9 ± 1.5</td>
</tr>
<tr>
<td>ECWN-</td>
<td>5</td>
<td>2.3 ± 0.8</td>
<td>4.9 ± 1.1</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>B+</td>
<td>8</td>
<td>2.2 ± 1.3</td>
<td>5.1 ± 1.5</td>
<td>5.1 ± 1.0</td>
</tr>
<tr>
<td>NO-</td>
<td>8</td>
<td>1.8 ± 0.6</td>
<td>5.1 ± 1.2</td>
<td>5.0 ± 0.5</td>
</tr>
</tbody>
</table>

Table 3.6 Hard tissue bridging and coronal overbuilding at grafted and non-grafted extraction sites at eight weeks

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>No. of specimens</th>
<th>Hard tissue bridging (%) (Mean ± SD)</th>
<th>Coronal overbuilding (mm) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECWN+</td>
<td>6</td>
<td>62.3 ± 20.0</td>
<td>0.2 ± 0.7</td>
</tr>
<tr>
<td>ECWN-</td>
<td>5</td>
<td>72.8 ± 9.0</td>
<td>-0.2 ± 0.5</td>
</tr>
<tr>
<td>B+</td>
<td>8</td>
<td>65.6 ± 10.5</td>
<td>-0.3 ± 1.0</td>
</tr>
<tr>
<td>NO-</td>
<td>8</td>
<td>73.0 ± 13.5</td>
<td>0.1 ± 0.8</td>
</tr>
</tbody>
</table>
3.7.2 16 weeks healing time

3.7.2.1 Crestal height differences and buccal and lingual bone widths

Overall mean results for each type of treatment after 16 weeks of healing are presented in Table 3.7. The results for the sections analysed for each extraction socket are listed in Appendix IV. The height differences between the buccal and lingual bone crest were similar amongst all the treated sites, which ranged from 1.1 ± 0.6mm to 1.5 ± 0.6mm. The buccal bone crests were always found to be apical to the lingual bone crest. The ECWN- sites showed thickest mean buccal bone wall (5.7 ± 1.1mm). Comparisons of the height (F<sub>3, 19.8</sub> = 0.748, p = 0.536) and buccal wall (F<sub>3, 120.2</sub> = 0.604, p = 0.620) and lingual wall (F<sub>3, 19.0</sub> = 0.965, p = 0.429) width differences treated with four different modalities revealed no statistically significant differences.

3.7.2.2 Hard tissue bridge and coronal overbuilding

Overall mean results for hard tissue bridging and coronal overbuilding after eight weeks of healing are presented in Table 3.8. The results for the specimens analysed for each extraction socket are listed in Appendix IV. The formation of the hard tissue bridging varied from 54.3 ± 8.8% to 77.9±10.4%. At 16 weeks, the non-grafted sites and the ECWN grafted sites with or without membrane showed similar hard tissue bridging, of which ECWN+ sites revealed the highest hard tissue bridging at 77.9 ± 10.4%. B+ sites resulted in the lowest hard tissue bridging at 54.3 ± 8.8%. There was a significant difference between treatment groups (F<sub>3, 20.0</sub> = 3.88, p = 0.025). Significant differences were found between ECWN- sites and B+ sites (p = 0.024). Both of the ECWN and BX grafted sites with collagen membrane showed the highest mean coronal overbuilding of 0.5 ± 0.8mm and 0.6 ± 0.5mm at 16 weeks. However, comparison of the coronal overbuilding (F<sub>3, 18.6</sub> = 0.258, p = 0.854) treated with four different modalities did not reveal statistically significant differences.
Table 3.7 Crestal height differences and width of the buccal and lingual bone walls of the grafted and non-grafted sites at 16 weeks

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>No. of specimens</th>
<th>Crestal height differences (mm) (Mean ± SD)</th>
<th>Buccal bone thickness (mm) (Mean ± SD)</th>
<th>Lingual bone thickness (mm) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECWN+</td>
<td>8</td>
<td>1.5 ± 0.5</td>
<td>5.2 ± 1.0</td>
<td>5.1 ± 1.0</td>
</tr>
<tr>
<td>ECWN-</td>
<td>8</td>
<td>1.2 ± 0.7</td>
<td>5.7 ± 1.1</td>
<td>5.4 ± 1.2</td>
</tr>
<tr>
<td>B+</td>
<td>7</td>
<td>1.1 ± 0.6</td>
<td>5.2 ± 0.9</td>
<td>5.4 ± 1.1</td>
</tr>
<tr>
<td>NO-</td>
<td>8</td>
<td>1.4 ± 0.7</td>
<td>5.3 ± 1.0</td>
<td>5.0 ± 0.8</td>
</tr>
</tbody>
</table>

Table 3.8 Hard tissue bridging and coronal overbuilding at grafted and non-grafted extraction sites at 16 weeks

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>No. of specimens</th>
<th>Hard tissue bridging (%) (Mean ± SD)</th>
<th>Coronal overbuilding (mm) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECWN+</td>
<td>6</td>
<td>73.4 ± 17.7</td>
<td>0.5 ± 0.8</td>
</tr>
<tr>
<td>ECWN-</td>
<td>5</td>
<td>77.9 ± 10.4*</td>
<td>0.3 ± 0.8</td>
</tr>
<tr>
<td>B+</td>
<td>8</td>
<td>54.3 ± 8.8</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>NO-</td>
<td>8</td>
<td>70.3 ± 18.1</td>
<td>0.3 ± 0.8</td>
</tr>
</tbody>
</table>

*Significantly different from B+ (\(p<0.05\))
3.7.3 Between eight and 16 weeks healing time points

When the data from all analysed specimens were combined according to the healing time points of eight and 16 weeks, the crestal height differences and width of the buccal and lingual bone walls are presented in Table 3.9. The differences between the buccal and lingual crestal bone height were significantly larger at eight weeks (2.1 ± 0.9mm) when compared to the data collected for 16 weeks (1.3 ± 0.6mm) \((p = 0.007)\).

**Table 3.9 Crestal bone height and width of buccal and lingual bone walls at eight and 16 weeks**

<table>
<thead>
<tr>
<th>Healing time points</th>
<th>No. of specimens</th>
<th>Crestal height differences (mm) (Mean ± SD)</th>
<th>Buccal bone thickness (mm) (Mean ± SD)</th>
<th>Lingual bone thickness (mm) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 weeks</td>
<td>27</td>
<td>2.1 ± 0.9*</td>
<td>5.2 ± 1.3</td>
<td>5.3 ± 1.0</td>
</tr>
<tr>
<td>16 weeks</td>
<td>31</td>
<td>1.3 ± 0.6</td>
<td>5.3 ± 1.0</td>
<td>5.2 ± 1.0</td>
</tr>
</tbody>
</table>

*Significantly different from 16 weeks \((p<0.05)\)

3.7.4 Summary of histometric analyses

The buccal bone crest was always found apical to the lingual bone crest at both eight and 16 weeks of healing. No statistically significant differences were detected between the height differences between the buccal and lingual bone crest heights amongst the different treatment groups at eight and 16 weeks. However, when the data from different treatment groups were combined for eight and 16 weeks, the crestal height was significantly larger at eight weeks than at 16 weeks. There were significantly more hard tissue bridging formed in ECWN- sites when compared to B+ sites at 16 weeks \((p = 0.024)\).
Chapter 4 Discussion

4.1 Introduction

The present study was set out to establish a tooth extraction socket model for bone graft/substitute research in a large, ethically acceptable, non-companion animal (sheep). The second objective of the current study was to evaluate the extraction socket healing following the implantation of ECWN and bovine xenograft in the sheep extraction socket model. Protocols and techniques were developed for the atraumatic extraction of sheep mandibular premolars. Histological, histomorphometric and histometric analyses of the healed extraction sockets were undertaken.

The extraction socket healing at eight weeks had no distinct differences across the four different treatment modalities. Irregular trabecular bone formed in all extraction sockets. The newly formed bone was predominantly woven bone surrounded by unmineralised osteoid. The ECWN graft material was intimately associated with the newly formed bone. BX was encapsulated with bone and fibrous connective tissue.

However, different healing patterns were observed at 16 weeks. Woven bone was being replaced by lamellar bone, with the development of Haversian systems. A hard tissue bridge was present at the coronal portion of the extraction socket in ECWN and non-grafted sites. BX graft particles were found in the coronal portion of the extraction sockets, interrupting the continuity of the hard tissue bridge. In ECWN sites, osseous healing followed a fine, finger-like trabecular pattern. In comparison, the non-grafted extraction sockets were filled with thick trabeculae separated by large areas of fibrous stroma. At BX sites, new bone appeared to encapsulate the graft granules. ECWN graft materials were almost undetectable after 16 weeks of healing, whereas BX particles were present amongst bone and connective tissue.

Histomorphometric analysis did not reveal statistically significant differences for the amount of new bone formation and residual graft materials in extraction sockets treated with different modalities after eight weeks of healing. After 16 weeks, statistically significant differences in the amount residual graft materials were found in the treated sites. There was less residual graft material detected in ECWN sites than BX sites. The ratio of newly-formed bone to residual graft ratio was significantly higher in ECWN grafted sites when compared to B+ (bovine xenograft with membrane) sites.
Comparison of new bone formation in extraction sockets between the four different treatment modalities did not reveal statistically significant differences.

The buccal bone crest was always found to be apical to the lingual bone crest at both eight and 16 weeks. No statistically significant differences were detected for the height differences between the buccal and lingual bone crest amongst the different treatment groups at eight and 16 weeks. However, the crestal height differences were significantly larger at eight weeks than at 16 weeks, when the data from different treatment groups were combined. There was significantly more hard tissue bridging in ECWN- sites than B+ treated sites at 16 weeks.

4.2 On materials and methods

4.2.1 The experimental model

The use of sheep as animal models in bone graft/substitute material research in dentistry have been reported in the studies of bone healing (Salmon and Duncan, 1997), dental implant research (Campbell and Duncan, 2013; Duncan, 2005; Vlaminck et al., 2008), periodontitis and periodontal defect studies (Baharuddin, 2010; Duncan et al., 2003) and maxillary sinus augmentation (Haas et al., 1998). To our knowledge, the current study is the first study investigating extraction socket healing following grafting procedures in sheep.

Sheep healing time was previously studied by Duncan (2005). Wound healing in sheep is faster than that in human. The healing time points chosen for this study were eight and 16 weeks. This corresponded to 11 and 21 weeks of wound healing in humans. Clinically, dental implants are usually placed into grafted extraction sockets after three to seven months of healing (Barone et al., 2012; De Coster et al., 2011; Gholami et al., 2012). Therefore, the chosen time periods in our study reflected the extraction socket healing at the time of implant placement in humans.

Mandibular second and third premolar extraction sockets were chosen in our study. The size of the second and third premolar roots in sheep is similar to human teeth (Duncan 2005). Surgical accessibility also restricted us from including mandibular molar tooth sockets into our study design.

Different techniques for tooth extraction in sheep have been described in different studies (Duncan, 2005; Vlaminck et al., 2008). Duncan (2005) created a shallow
circum-dental osteotomy within the coronal portion of the periodontal ligament following raising full thickness buccal and lingual flaps. The second and third premolars were sectioned vertically towards the furcation region. The distal and mesial portions were then removed with elevators. This technique involved removing the coronal portion of the alveolar bone, which may interfere with and alter the subsequent healing of the extraction socket.

Vlaminck et al. (2008) carried out tooth extraction in sheep as part of their investigation of immediate implant placement. The technique described was similar to the previous study. The authors stated that extraction of the first and second premolars was achieved using root elevators and extraction forceps after longitudinal sectioning. However, both groups mentioned the brittleness of the teeth and extremely dense inelastic mandibular bone resulted in a high frequency of root fractures during extraction. In the case of root fracture, more aggressive methods were required to remove the root fragments. Vlaminck and co-workers (2008) suggested the use of specially designed periotomes to achieve atraumatic extraction.

Modifications to extraction technique were made in our study. Following full-thickness periosteal flap on the buccal and lingual aspects, the periodontal ligament around the three premolars was severed using a Piezosurgery® unit with Piezosurgery® extraction tips. This technique was effective in mobilising the premolar teeth. We were also able to avoid sectioning the premolar teeth and removing the alveolar bone. The premolars could be elevated mesially and removed without ostectomy.
4.2.2 Histomorphometric analysis methodology

In this study, a semi-automated segmentation technique was used to calculate the different tissue volumes. Threshold values for NB, RG and FCT were selected manually according to the signal intensity in each image (Figure 2.11a, b and c). These segmented tissues were measured and expressed as area percentages within the ROI.

Different authors have measured the tissue volumes in a variety of ways in the literature. The majority of prior studies investigating bone graft materials in alveolar ridge preservation carried out a light-point counting procedure (Araújo et al., 2008; Araújo and Lindhe, 2009; Artzi et al., 2000). A lattice comprising 100 light points was superimposed over the tissue in the healing socket. The percentage area occupied by different types of tissues were calculated. Others used image analysis software to select and measure the tissue proportions (Heberer et al., 2008; Heberer et al., 2011; Hong et al., 2012).

Duncan (2005) compared the results of bone to implant contact percentage (%BIC) using stereology analysis and computer-assisted analysis. The author reported significant differences between the techniques. The result in %BIC using stereology was significantly higher than the computer-assisted analysis. Another earlier study (Leichter et al., 1998) also noted a consistently higher percentage of bone was identified using stereology when compared to computer-assisted histomorphometric analysis after surgically created defects in the furcation of mandibular second premolars in sheep. Only demineralised H&E stained sections were included in this study. To carry out the computer-assisted histomorphometric analysis, the area of bone was measured manually by using the polygon tool in NIH Image software. The authors were faced with issues such as distortion, limited pixel capture ability of the camera, and restricted image magnification due to the size of computer monitor.

The computer assisted semi-automated segmentation technique is a common procedure in analyzing medical magnetic resonance images and microscopic images (Al-Attar et al., 2006; Bae et al., 2009; Imelinska et al., 2000). It has been suggested the use of semi-automated approach to a large dataset would greatly reduce effects of intra-reader and inter-reader variability compared to manual segmentations (Swanson et al., 2010). At the same time, this technique is less time and resource consuming.
The semi-automated segmentation technique has been compared with the classical point-counting stereology in its application in histomorphometric analysis and immunohistochemical cell counting (Amenábar et al., 2006; Montgomery et al., 2008). The authors compared the intra-reader and inter-reader variability and reported no statistically significant differences between the two methods.

However, this technique is not without its limitations. As with all computer-assisted analysis, the results depend on the quality of the digitalised images. Technology development in the recent years has allowed researchers to overcome issues related to image resolution. However, care must be taken in image capturing to ensure good illumination and focus of the microscope. The utility of this method also depends on the methods of histological preparation. For example, it would be difficult to differentiate different tissue types with hematoxylin and eosin stain, as all tissue structures will appear pink and make the threshold determination impossible.

4.2.3 Histometric analysis methodology

As the first study carried out using the sheep extraction socket model, studying the extraction socket healing in grafted and non-grafted sites was the primary objective when designed this preclinical animal study was designed. We were able to carry out some basic histometric measurements using similar protocols reported by other research groups (Araújo and Lindhe, 2005; Rothamel et al., 2008).

The crestal height differences between the buccal and lingual bone walls were determined by measuring the vertical distance between the buccal and lingual intersections with the long axis of the extraction sockets. The long axis of the extraction socket was identified by bisecting the angle formed by the borders of the extraction socket (Figure 2.12). Prior studies have not specified the methodology in identifying the long axis of the root (Araújo and Lindhe, 2005; Rothamel et al., 2008). These studies calculated crestal height differences using a parallel line to the long axis of the root in the centre of the socket or measuring the distance from the buccal/lingual bone crest to the most apical point of the mandible. In developing the methodology for histometric analysis, we introduced the bisecting angle technique to ensure reproducible measurements from the harvested specimens and took consideration of the variability of sheep mandibles.
The buccal and lingual bone widths were measured at 1mm apical to the crestal bone level. The distance from the border of the buccal/lingual bone perpendicular to the previously identified long axis of the extraction socket was recorded as the buccal and lingual bone widths.

We did not attempt to measure the dimensional changes of the alveolar ridge. As this was the first animal study carried out for extraction socket healing in sheep, we did not include volumetric changes of the alveolar ridge into our study design. Our emphasis was on the feasibility of this model in studying grafted extraction socket healing. However, the volumetric changes in the grafted extraction sockets may be considered in future studies.

As described in the literature review, several techniques have been developed for measuring alveolar ridge dimensional changes following ridge preservation in dog studies. In the series of studies carried out by Araújo’s group (2008, 2009, 2011), the third and fourth premolars were hemisectioned and only the distal roots were extracted. The canal of each mesial root was filled with gutta-percha. In assuming the shape and dimensions of the alveolar process at mesial and distal roots being equal, the cross-sectional areas of the alveolar process obtained at the edentulous sites were compared to the tooth sites.

This technique was developed further in recent studies to study the effect of grafting materials on ridge preservation (Al-Hezaimi et al., 2012; Bashara et al., 2012). Both studies adopted the same surgical procedures, extracting the distal roots of the premolar and root filling the mesial roots. Instead of the histometric analysis, micro-CT images were used to compare the cross-sectional area of the alveolar ridge and calculate the dimensional changes.

However, the above-mentioned technique was not suitable to be applied to sheep studies. The anatomy of the mandibular premolars was studied by Duncan (2005), in which the author found diverse differences in the shape and dimensions between the mesial and distal roots. To carry out hemisection and root canal therapy in sheep may also be difficult to achieve due to surgical access and the anatomy of sheep teeth.

Scanning the study models of treated and untreated alveolar ridge using a 3D camera has been reported by other groups (Fickl et al., 2008c; Fickl et al., 2009; Iibuchi et al.,
The dimensional changes in the alveolar ridge were calculated by superimposing the two scanned images. This may be feasible in future studies in sheep.

4.3 On the experimental results

4.3.1 Differences in extraction socket healing at grafted and non-grafted sites

4.3.1.1 Non-grafted sites

Extraction socket healing has been studied extensively in animal and human studies (Amler, 1969; Cardaropoli et al., 2003). However, to our knowledge, this is the first report of extraction socket healing in sheep. After eight weeks of healing, islands of loose trabecular bone were seen in the extraction sockets. Newly formed immature woven bone was detected across the coronal margin of the defect, connecting the buccal and lingual cortical plates (Fig. 3.9a). Healing at 16 weeks revealed the formation of thick bony trabeculae extending from the buccal and lingual cortical plates into the bony defects (Fig. 3.16a). The newly formed bone resembled that of lamellar bone (Fig. 3.16b).

The healing events of the current study are consistent with those described by Cardaropoli et al. (2003) in a study carried out in the dog model. They reported healing at 60 days (around eight weeks) was characterised by the formation of hard tissue bridge separating the marginal mucosa from the extraction socket and composed mainly of woven bone. By 120 days (around 16 weeks), the marginal hard tissue bridge was reinforced by layers of lamellar bone. It is therefore likely that the healing times in sheep and dog are similar as reported by Duncan (2005).

4.3.1.2 ECWN grafted sites

The present study is the first report in the use of ECWN for extraction socket grafting in large animals. The histology sections harvested after eight weeks of healing revealed no distinctive difference in healing with that found in non-grafted extraction sockets. New bone formed within the extraction sockets was predominantly woven bone. Woven bone was being replaced by lamellar bone within the grafted extraction sockets after 16 weeks of healing.

However, the pattern of healing within the ECWN grafted sites was distinctly different from that was found in non-grafted sites. Fine finger-like bone trabeculae were
observed within the grafted sites. The striae of bone trabeculae were close to each other and extending from the buccal and lingual cortical plates towards the middle of the extraction sockets.

Our current findings are in agreement with previous research reporting the histology of osseous healing following grafting with ECWN material (Schneider et al., 2009). In their study, ECWN was grafted into rabbit calvarial defects. After 4 weeks of healing, the authors also reported newly formed bone within the ECWN grafted defects showed a finer, more spongy appearance. The authors also reported small round pores were observed in the newly formed bone tissue in the ECWN grafted defects.

A possible explanation for this distinct healing pattern might be due to the osteoconductive property of ECWN. As revealed by the baseline H&E sections, the ECWN graft material acts as scaffold for the initial attachment of erythrocytes. A previous in vitro study (Schneider et al., 2007) demonstrated that the ECWN scaffold supported the formation of a continuous hydroxyapatite (HAp) layer unto the graft material. As HAp particles were deposited onto the graft material, the mineralization process led to the formation of fine striae of trabeculae within the grafted extraction sockets or bone defects.

4.3.1.3 Bovine xenograft grafted sites

Commercially available bovine xenograft (BX) granules are hydroxyapatite ceramic derived from cancellous bovine bone. The osteoconductive properties of BX have been reported in various animal and human studies (Araújo et al., 2009; Fugazzotto, 2003; Mardas et al., 2010). A common finding from previous research was that the material was not resorbed or eliminated from the grafted sites (Artzi et al., 2000; Molly et al., 2008; Smith, 2011). Smith (2011) reported that BX particles grafted into a sheep maxillary sinus model were surrounded by new bone after four weeks, but after 12 weeks there was no evidence of resorption of the BX particles.

The results of our study showed that BX particles were surrounded by woven bone and connective tissue at eight weeks. The newly formed bone was increasingly lamellar in structure. After 16 weeks of healing, most of the BX particles were surrounded by a mixture of woven bone, lamellar bone and connective tissue. In the coronal and apical portion of the defects, a small part of the BX particles were surrounded by connective
tissue only. A hard tissue bridge was observed in only half of the 8 extraction sockets contained BX graft particles.

Very little was found in the literature on the histological extraction socket healing in animal models after eight weeks. Early bone formation was detected around BX particles located close to the socket walls as early as two weeks in a dog extraction socket model (Araújo et al., 2009; Araújo et al., 2010a). Newly formed immature woven bone formed around the graft particles in most parts of the extraction sites after four weeks of healing. The findings from our study at eight weeks are comparable with the human clinical studies after 12 weeks of healing (Heberer et al., 2011), which showed two thirds of the sampled specimens contained BX particles surrounded by immature woven bone and connective tissue.

The present histological evaluation of the 16 weeks specimens is corroborated by the findings reported in other animal studies (Araújo et al., 2008; Fickl et al., 2008b). These authors reported that most of the graft particles were in direct contact with woven and lamellar bone after three to four months of healing. A small part of the biomaterial was also surrounded by connective tissue, especially those found at the most coronal part of the extraction defect. Histological sections harvested from clinical studies prior to implant placement also demonstrated similar findings (Artzi et al., 2000; Carmagnola et al., 2003; Heberer et al., 2011; Mardas et al., 2010). They reported the BX graft particles are surrounded in different degrees by connective tissue, woven bone and lamellar bone. In the coronal portion of the specimens, the BX particles were surrounded by connective tissue. Hard tissue bridge formation was observed in four out of the eight extraction sockets containing BX graft particles in our study. This finding was consistent with a recent clinical study of BX grafted extraction socket, where only five of the 11 test sites demonstrated a layer of mineralized hard tissue bridge (Lindhe et al., 2013). It was suggested that augmenting the extractions socket with BX particles to achieve bone regeneration was unpredictable. However, this procedure may stabilize the soft tissue contour and prevent it from collapsing into the socket (Fickl et al., 2008b).
4.3.2 Differences in the histomorphometric analyses at grafted and non-grafted sites

4.3.2.1 New bone formation

The percentage of new bone detected in the eight weeks extraction sockets was the highest among the non-grafted sites (47.9 ± 5.0%). This value remained similar in the 16-week specimens (45.7 ± 10.7%). These findings seem to be consistent with other research carried out in a dog extraction socket model, which found 50.5 ± 7.3% mineralized bone in non-grafted sites after three months of healing (Araújo et al., 2008). A greater percentage of new bone formation of 62.1 ± 17.11% has been reported by Hong et al. (2012). However, the outline of the extraction socket was not identifiable at six months (Araújo 2009). Therefore, no comparisons could be made between the three and six months specimens. The amount of newly formed bone remained stable in our study from eight weeks to 16 weeks. A possible explanation for this might be that bone formation was close to the maximum at eight weeks. From eight weeks to 16 weeks, there was the remodeling of the immature woven bone to a more mature bone morphology as discussed in Section 4.3.1.1.

In the current study, the amount of new bone formed in ECWN grafted sites at eight weeks was 36.5 ± 17.3% for ECWN- sites and 40.7 ± 16.9% for ECWN+ sites. New bone formation within the ECWN- sites (45.2 ± 6.8%) was similar to NO- sites at 16 weeks. ECWN+ sites revealed the highest percentage of new bone (49.8 ± 12.5%) amongst the four treatment modalities after 16 weeks of healing. As this study was the first animal study to investigate ECWN material in extraction socket healing, no comparison with previous histology was possible. However, in the study where ECWN was tested in rabbit calvarial defects (Schneider et al., 2009), the area percentage of newly formed bone was measured to be 34.9 ± 17.0% after 4 weeks of healing. This value was higher than new bone formation in the empty defects (28.4 ± 14.9%) and BX grafted sites (30.8 ± 14.3%).

New bone formation in BX sites showed the greatest variation (38.6 ± 18.1%) at eight weeks and the lowest amount of new bone (41.1 ± 11.1%) at 16 weeks in the current study. These findings were similar to earlier studies in dogs reported healing following 1 and 3 months (45.1 ± 10.1% and 43.1 ± 10% respectively) (Araújo et al., 2010a; Araújo and Lindhe, 2011). However, the results from another study from the same
group (Araújo et al., 2008) following 3 months of healing was 58.1 ± 10.7%, which was much higher than the later study in 2011 (43.1 ± 10%).

In the current study, the differences reported in the percentage of new bone formation within the grafted and non-grafted sites were not found to be statistically significant. In reviewing the literature, very few animal studies conducted statistical analysis to compare the mean value obtained from different treatment groups. To our knowledge, the histomorphometric analyses from animal studies carried out for extraction socket healing have only reported the mean and standard deviation of newly formed bone. One recent clinical study carried out histomorphometric analysis for BX grafted and non-grafted extractions sockets in 48 teeth (41 patients) (Cardaropoli et al., 2012). They also found no significant difference in the fraction of mineralised and non-mineralised tissues between the grafted and non-grafted groups. However, the authors included BX graft particles (18.46 ± 11.18%) as mineralised tissue in their analysis, which was not included in the current study.

The lack of statistical significant differences in our study may be due to the large variations for the percentage of newly formed bone obtained from the 16 sheep. These variations may be sheep dependant and caused by the different healing capacities between sheep. The considerable variation in bone formation within the sockets evaluated might be due to a difference in individual factors influencing bone physiology. This effect was less obvious at eight weeks as demonstrated by Figure 4.1. We plotted the values obtained from the ECWN+ group against No- group using the sheep as a statistical unit. There were no obvious association between new bone formation with the particular sheep. However, there was a more linear relationship between the new bone formation in ECWN+ and No- groups and the particular sheep at 16 weeks (Figure 4.2).
Figure 4.1 New bone formation in ECWN+ and No- group with the sheep as a statistical unit at eight weeks

Another possible explanation for the lack of statistical significant differences is the limited sample size and study power. The paired sample t-test showed the differences in the mean value of new bone formation between the different treatment modalities at eight weeks and 16 weeks (Table 4.1).

For the data obtained from eight weeks specimens, the minimum mean difference between the test and control sites was the value in Pair 3 between ECWN+ and No-, which was 6.33% with a standard deviation of 17.69%. Post-hoc power analysis carried
out using Minitab 16.1 (Minitab inc., State College, Philadelphia, PA, USA) for this difference with sample size \( n = 8 \) was only 20%. For the data obtained from 16 weeks specimens, *post-hoc* power analysis calculated for a difference of 4% with a standard deviation of 6.27 was 34%. In order to achieve 70% power for this study design, 56 sheep would be required for the eight weeks analysis and 18 sheep would be required for the 16 weeks analysis.

**Table 4.1 Paired sample \( t \)-test for new bone formation at eight and 16 weeks**

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>8 weeks Mean ± SD (%)</th>
<th>16 weeks Mean ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1</td>
<td>ECWN+ - B+</td>
<td>-1.58 ± 28.13</td>
</tr>
<tr>
<td>Pair 2</td>
<td>ECWN+ - ECWN-</td>
<td>-4.68 ± 12.09</td>
</tr>
<tr>
<td>Pair 3</td>
<td>ECWN+ - No-</td>
<td>-6.33 ± 17.69</td>
</tr>
<tr>
<td>Pair 4</td>
<td>B+ - ECWN-</td>
<td>0.99 ± 20.47</td>
</tr>
<tr>
<td>Pair 5</td>
<td>B+ - No-</td>
<td>-9.22 ± 15.97</td>
</tr>
<tr>
<td>Pair 6</td>
<td>ECWN- - No-</td>
<td>-11.89 ± 17.26</td>
</tr>
</tbody>
</table>

**4.3.2.2 Residual graft**

Comparison of residual graft materials found in the grafted sites revealed no significant differences at eight weeks. Small amount of residual graft (<5%) was detected in all grafted sites. However, there were significant differences between ECWN- sites and B+ sites \((p = 0.048)\) at 16 weeks. The differences between ECWN+ sites and BX sites were marginally significant \((p = 0.055)\). The amount of residual graft materials found in ECWN grafted sites were \(1.2 ± 0.7\%\) for ECWN+ sites and \(1.1 ± 0.7\%\) for ECWN- sites. Residual graft material detected within BX sites was \(5.4 ± 5.6\%\).

The amount of residual BX graft material was very low at eight weeks in the current study, measuring only \(2.5 ± 0.9\%). This result differed from other published animal studies. After three months of healing, Araújo et al. (2009, 2011) found the residual BX graft material occupied \(12.2 ± 9.1\%\) to \(24.6 ± 3.7\%\) of the measured regions of interest. These studies were conducted using the same animal model at different times. However, there were also great variations between the two studies. The amount of residual BX graft materials reported in the later study almost doubled that of the earlier study. BX residual graft detected after 16 weeks of healing was \(5.4 ± 5.6\%\). This finding was similar to that reported after six months of healing in the dog model, which was \(5 ± 2.4\%\) (Araújo and Lindhe, 2009). Previous histomorphometric analyses of residual BX graft material carried out in clinical studies were mostly carried out from the trephined
bone core prior to implant placement. The amount of residual graft varied from 10-20% with standard deviations of 10-20% (Cardaropoli et al., 2012; Carmagnola et al., 2003; Heberer et al., 2008; Heberer et al., 2011).

A possible explanation for the discrepancy between our result and those reported in the literature may be due to the differences in the anatomy of sheep teeth. As we have discussed in the literature review and observed in the decalcified section at baseline, the periodontal ligament around sheep teeth is very wide. After tooth extraction, there was profuse bleeding from the extraction socket. Some authors have speculated that the ensuing blood flow into the cone-shaped extraction socket may have forced BX graft particles towards the coronal direction (Araújo et al., 2009). Therefore, the apical portion of the extraction socket was void of graft particles and healed in the same way as the non-grafted sites. The degree of compression at the time of grafting may also influence the amount of graft particles within a defined space (Heberer et al., 2008). As the graft material is granular, material migration or displacement may occur. This may account for the low amount of graft remnants found in this study. BX granule migration was also reported in the previous in vivo study comparing the defect healing between ECWN and BX (Schneider et al., 2009). The authors pointed out the granular BX material being more challenging to apply and fix in the bony defect as the small granules were easily washed away from the defect.

The amounts of residual graft materials found in ECWN grafted sites at eight weeks were 3.0 ± 1.8% and 1.6 ± 0.8% for ECWN+ and ECWN- sites respectively. The area fraction of the graft material decreased to 1.2 ± 0.7% for ECWN+ sites and 1.1 ± 0.7% for ECWN- sites after 16 weeks of healing. The amount of residual ECWN was not reported in a previous in vivo study (Schneider et al., 2009). Therefore, no comparison could be made with any previous studies.

Significantly more residual graft materials were observed in BX sites than ECWN grafted sites with or without membrane ($p = 0.055$ and $p = 0.048$ respectively) at 16 weeks. This may be explained by the different resorption rate and mechanism of the two graft materials. The degradation of ECWN and ECWN-like material was tested in in vivo studies (Loher et al., 2006; Schneider et al., 2007). ECWN graft material incorporates aerosol derived amorphous tricalcium phosphate nanoparticles (ATCP) into a biodegradable synthetic copolymer poly(lactide-co-glycolide) (PLGA) in a 40:60 ratio. PLGA is a biocompatible and bioresorbable polymer applied in the area of drug
delivery (Song et al., 1997), soft tissue engineering (Pattison et al., 2005), nerve regeneration (Bini et al., 2004) and orthopaedic surgery (Middleton and Tipton, 2000). ATCP nanoparticles possess enhanced solubility compared with β-TCP (Schneider et al., 2007). After the material was placed simulated body fluid for two weeks, a continuous HAp layer with a thickness of about 2µm was detected. Two processes were reported to be occurring simultaneously, PLGA degradation through polymer hydrolysis and the deposition of hydroxyapatite on the surface of the fibres. Although no study has been conducted to define the time taken for ECWN to be resorbed and replaced, a previous in vivo study reported partial degradation after four weeks of healing in rabbit calvarial defects (Schneider et al., 2009). Another degradation and bioactivity study was carried out by the same group on a similar co-polymer prepared by solvent casting (Loher et al., 2006). They reported the degradation of around 7% of the composite mass after six weeks immersion in simulated body fluid.

On the other hand, the complete resorption of BX particles has been questioned in the literature. Some studies have reported the partial or complete resorption of BX particles (Fugazzotto, 2003; Thaller et al., 1994), while others showed negligible resorption (Artzi et al., 2000; Smith, 2011). BX particles were still detectable after 11 years in a human maxillary sinus study (Mordenfeld et al., 2010). The incorporation of BX particles in extraction socket healing involved a series of processes (Araújo et al., 2010a). The biomaterial was initially exposed to surface resorption by osteoclasts. It was proposed that these osteoclasts found adjacent to BX particles functioned like macrophages to clean and prepare the graft surface for the deposition of new bone (Jensen et al., 2006). However, the biomaterial was not engaged in the processes of resorption or degradation, which may explain the higher proportion of residual graft in BX sties compared to ECWN sites.
4.3.3 Differences in the histometric analyses at grafted and non-grafted sites

4.3.3.1 Height differences between the buccal and lingual cortical plates

The buccal bone crest was always found to be apical to the lingual bone crest in the 8 and 16 weeks specimens, which corroborated previous research (Araújo et al., 2008; Fickl et al., 2008b; Fickl et al., 2009; Rothamel et al., 2008). The buccal and lingual bone heights of the grafted sockets showed greater differences than the non-graft sites at eight weeks, although the differences were not statistically significant. The height difference detected was the lowest among the non-grafted sites. After 16 weeks of healing, the height differences between the buccal and lingual bone crest were similar amongst all the treated sites. No clear trend was observed comparing the grafted and non-grafted techniques. These findings of the current study were comparable with those from the previous research. Studies carried out using similar methodology with the current study reported the similar height differences between non-grafted and grafted sites (Araújo et al., 2008; Fickl et al., 2008b).

The lack of a clear trend between the grafted and non-grafted sites may indicate ridge preservation procedures do not eliminate alveolar ridge remodeling, They may only limit the ridge resorption as suggested by other studies in this field (see review by Ten Heggeler et al., 2011). Another possible explanation may be due to the difficulties in determining the long-axis or centre of the extraction socket, which may affect the measurement of buccal and lingual crestal height. Other techniques used to measure alveolar ridge dimensional changes were discussed in Section 4.2.3. These techniques were not attempted in the current study.

When we compare the height differences obtained from 8 and 16 weeks specimens, the differences for grafted sites became smaller and non-grafted sites stayed similar. This finding was not supported by the literature, because as healing occurs, the distance between the buccal and lingual bone crests was expected to increase (Araújo et al., 2005; Rothamel et al., 2008). The opposite was found in our study, where the height differences became smaller.

One possible explanation is the placement graft materials delayed alveolar ridge remodeling at eight weeks as the three grafting modalities showed larger crestal differences than non-grafted sties. This difference disappeared as healing matured to 16 weeks, where all treatment modalities revealed similar values. Another speculation may
be related to the buccal bone overgrowth in the sheep mandible during healing (Figure 3.8). This may have masked the real difference between the buccal and lingual crestal bone heights. When we combined the data from all analysed specimens according to the healing time points of eight and 16 weeks, the differences between the buccal and lingual crestal bone height were significantly larger for eight weeks specimens than that in 16 weeks ($p = 0.016$). This may represent the remodeling and healing process in sheep occurring between eight and 16 weeks.

4.3.3.2 Widths of buccal and lingual bone wall

The grafted and non-grafted sockets revealed comparable buccal and lingual bone thickness at eight and 16 weeks. No statistically significant difference was found in the treatment groups. Rothanmel et al. (2008) also failed to demonstrate significant differences between the width of buccal and lingual bone wall amongst the grafted or non-grafted sites. Contrary to our study, the buccal wall was always found to be thinner than the lingual wall in non-grafted sites in dog (Araújo & Lindhe 2005). Again, this rather contradictory result may be due to the buccal overgrowth in the sheep mandible (Figure 3.8). Duncan (2005) also reported an increase in the thickness of the cortex following the healing of critical size defects and implant placement. One possible explanation given by the author was that these changes occurred to compensate for the bending forces during grazing and mastication following wounding.

4.3.3.3 Hard tissue bridging and coronal overbuilding

Although eight weeks specimens showed similar hard tissue bridging, B+ sites had significantly less hard tissue formation at the coronal margin compared to ECWN- sites at 16 weeks. No other studies have quantified hard tissue bridging across tooth extraction sockets histometrically. In studying extraction socket healing in the dog model, Cardaropoli et al. (2003) reported the formation of a hard tissue bridge in specimens after 60 days of healing. Araújo et al. (2008, 2009) described the healing of non-grafted extraction sites after three and six months of healing. They observed the formation of a bridge of mineralized bone at the entrance of the socket after three months. In extraction sites grafted with BX particles, graft particles were surrounded by fibrous capsules adjacent to the bone compartment of the ridge. Fickl et al. (2008) also observed the presence of BX particles within the newly formed bone at the coronal portion of the extraction sockets.
The findings in the current study are in agreement with previous research. At 16 weeks, there was less hard tissue bridging in BX grafted sites compared to ECWN- sites. This can be explained by the presence of BX particles in the coronal portion of the newly formed bone. The presence of residual particles may have prevented hard tissue formation at the coronal margin of the extraction sites. The differences in hard tissue bridging were not significant among the four experimental groups at eight weeks. This may be explained by less BX graft particles observed in the eight weeks specimens.

In the current study, ECWN+ and ECWN- sites revealed similar patterns of extraction socket healing at eight and 16 weeks. After eight weeks of healing, gross histological observations described a dome-shaped outline of the newly formed bone over the bony defects that appeared to be more obvious at ECWN+ sites than ECWN- sites. More ECWN- sites showed the overlying mucosa to be below the buccal/lingual bony crest and invading the extraction defect space. The use of a membrane with the test graft material appeared to create a protected supracrestal space that permitted osteoconduction in a vertical direction. However, this difference was not statistically significant when measured histometrically.

Coronal overbuilding was measured from the lingual crest level to the most coronal point of the healed extraction sites along the long axis of the sockets. There were less coronal overbuilding observed in the non-grafted sites and sites that had no collagen membrane at 16 weeks. No study was found to have compared the histometrical differences in the coronal apical direction in the healing of the grafted extraction socket with or without resorbable membrane. It may be speculated that the current finding in the morphological differences at eight weeks was due to the wound isolating effect of the collagen membrane. Soft tissue was excluded from the extraction socket at the initial stage of healing.
4.4 Clinical significance of the research

An adequate width and height of the alveolar ridge is important for implant placement and prosthesis aesthetics. Alveolar ridge preservation is carried out to preserve the ridge volume within the bony envelope existing at the time of extraction (Hämmerle et al., 2012). Various techniques have been proposed to carry out this procedure (Table 1.1). They almost always involve placing a bone graft/substitute material into the extraction sockets.

Various animal and human studies have shown that some of these bone substitutes were able to limit but not eliminate the post-extraction alveolar ridge resorption to a certain extent (Ten Heggeler et al., 2011). However, the quality of the new tissue formed within the socket varied broadly. The different bone substitute materials had different resorption rates and produced different healing patterns within the alveolar socket. Not only is the amount of the newly formed bone is important in these grafted sites, but also the quality of osseous tissues in the socket area is essential, especially when the justification of ridge preservation is to facilitate the placement of a dental implant (Horváth et al., 2013).

The novel bone substitute material ECWN combined the flexibility of PLGA polymer fibres with the bioactivity of tricalcium phosphate ceramic. This flexible cottonwool-like material had superior handling properties and was less prone to migration compared to granular materials used in our study. As the demand for flexible and easy-to-apply implant material for the repair of complex-shaped bone defects is increasing, the current study will serve as a base for applying this novel material in future studies.

Other properties determine the success of a bone graft/substitute material include material degradation and new bone formation. The current study also demonstrated the biocompatibility and resorption of ECWN. Its osteoconductive property was revealed through the pattern of histological healing within the extraction sockets. The in vivo performance of the material did not impede bone formation within the extraction defects. There was also significantly more bone formation in coronal margin of ECWN grafted sites compared to BX grafted sites. These findings add substantially to our understanding of this material and warrant further investigations of ECWN in bony defects in periodontal, implant and peri-implant research.
4.5 Confounding factors and other issues with the investigation

A number of important limitations need to be considered for the current study.

4.5.1 Variation in animals

The eighteen sheep chosen for this study were purchased from a commercial flock of cross-bred ewes. The availability of animals was limited at the time of experiment. The number of sheep required for the current study was relatively large. Although initial screening was conducted by the research unit, there was not much room to match the age or flock of all the selected animals in this study. Therefore, the results obtained may be influenced by variations in the healing physiology from the different sheep. Further studies may desire to purchase the experimental animals from the same flock to minimize the variations from the healing capacities caused by the heterogeneity of the flock.

4.5.2 Experimental design

As this was the first study conducted in the tooth extraction socket model in sheep, there was a steep learning curve in carrying out the surgical procedures. Atraumatic extraction, placement of BX granules, manipulation of the collagen membrane and suturing techniques required clinical skills which the primary investigator did not fully possess at the beginning of the experimental stage. Operator experience has been shown to be an important factor for the outcome of non-surgical and surgical periodontal treatment (Brayer et al., 1989; Chambrone et al., 2010; Kocher et al., 1997; Lambert et al., 1997). However, the skills were developed rapidly and the experimental time was reduced markedly after the first week of experiments.

Some of the sheep developed infections from the femur and maxillary sinus operation sites, which required treatment with systemic antibiotics. Post-operative infection may have influenced initial osseous healing. However, this effect could not be quantified in this study.

The extraction socket chosen for grafting resembled the size of tooth root in human. Therefore, the first mandibular premolar was excluded from the study due to the length of the roots. The extraction sockets from the second and third mandibular premolars were chosen as the experimental sites. However, due to the proximity of the distal root
of the second premolar and the mesial root of the third premolar, migration of the graft material or influence of one graft material on the other experimental site may have occurred. This could have been avoided by root filling the distal root of the premolars and extract only the mesial root, as proposed by Araújo and Lindhe (2005). This technique was not attempted in this study. Access difficulties as well as surgical time constraints prevented adopting the protocol developed in the dog study (Araújo and Lindhe, 2005) into this first study in sheep. This potential influence from neighbouring tooth sockets may be eliminated in future studies by applying only one treatment modality to each hemi-mandible.

There were less residual BX particles found in eight weeks specimens were less than the 16 weeks specimens and the previous studies published in dog models. Two possible reasons for lower residual graft material in the region of interest were identified. Firstly, inexperienced operator in handing granular graft materials may have lead to inadequate BX particles placement at the time of surgery. Another possible explanation for this might be related to the profuse bleeding after tooth extraction, which may lead to granule migration in the coronal direction and subsequently lost during healing.

4.5.3 Excluded specimens

In this study, six out of 64 experimental sites were excluded from analysis. Two extraction sockets did not heal in one sheep. Retained root tips were identified in the coronal portion of four experimental sites. Five of these excluded sites were in the eight weeks specimens. Decreased sample size may have increased the standard deviation and decreased the power of the study.

4.5.4 Examiner blinding

The primary investigator carried out both the animal surgery and the outcome examinations. Blinding at the time of surgery was impossible to achieve because the graft materials used at the experimental sites were distinctly different. Blinding was also difficult to achieve during the histomorphometric and histometric analysis. Graft materials used had distinctly different morphology and were easily differentiated in the histological images. Intra-examiner reliability was tested by repeating the outcome examination for 10 specimens two weeks later.
4.6 Conclusion and recommendations for future research

4.6.1 Conclusions

This present study was set out to establish a tooth extraction socket model in sheep. The second objective was to evaluate the extraction socket healing following the implantation of a novel bone substitute material ECWN and bovine xenograft in the sheep extraction socket model. We carried out histological, histomorphometric and histometric analyses to examine grafted and non-grafted extraction socket healing in sheep.

Non-grafted extraction sockets were filled with thick trabeculae separated by large areas of fibrous stroma after 16 weeks of healing. At BX sites, new bone was formed to encapsulate the graft granules. In comparison, osseous healing followed a fine, finger-like trabecular pattern in ECWN grafted sties. This may suggest osteoconduction where new bone was formed around the test material. The newly formed bone was predominantly woven bone at eight weeks. At 16 weeks, woven bone was being replaced by lamellar bone, with the development of Haversian system.

Histomorphometric analyses did not reveal statistically significant differences for the amount of new bone formation in extraction sockets treated with different modalities after eight and 16 weeks of healing. However, there were less residual graft materials detected in ECWN treated sites than BX sites after 16 weeks of healing.

The buccal bone crest was always found to be apical to the lingual bone crest at both 8 and 16 weeks. No statistically significant differences were detected for the height differences between the buccal and lingual bone crest amongst the different treatment groups. However, when the data from different treatment groups were combined for eight and 16 weeks, the crestal height differences were significantly larger at eight weeks than at 16 weeks. There were significantly more hard tissue bridging formed in ECWN- sites when compared to B+ sites at 16 weeks.

This first description of a tooth socket model in sheep supports the utility of this model for bone graft research. The results of this study suggested that the novel material ECWN did not impede bone ingrowth into sockets and showed evidence of material resorption. The present study also confirmed previous findings where new bone was formed to encapsulate BX particles.
4.6.2 Future directions

4.6.2.1 Further development of the current extraction socket model in sheep

Our study did not investigate the volumetric changes that occurred during extraction socket healing. Further development of the current sheep model is required to estimate the dimensional changes of the alveolar ridge. Optical scanning of the impressions taken at baseline and harvesting may be desirable in investigating the alterations of the alveolar ridge. This will allow us to determine the effectiveness in maintaining the ridge dimensions following the placement of bone graft/substitute material into the extraction sockets.

4.6.2.2 Investigation of ECWN material in different format

The current study only reported on the osseous healing of the grafted and non-grafted extraction socket at eight and 16 weeks. We examined resin-embedded specimens at these time points. Further research might explore the effect of ECWN on early osseous healing and bone formation. The use of immunohistochemistry for the identification of bone lineage cells and proteins will contribute to the further understanding of the osteoconductive property of ECWN.

4.6.2.3 Investigation concerning dental implants

The ultimate goal of ridge preservation is for the successful placement of dental implants. Extension of this work should include dental implant placement into grafted extraction sockets after healing. Bone to implant contact within grafted extraction socket is also crucial in evaluating the properties of the tested bone graft/substitute material. From the findings of the current study, we may speculate that implants placed at the ECWN sites may have more bone-to-implant contact compared to those that are placed at the BX sites due to less residual graft material.

4.6.2.4 Investigation in other periodontal and peri-implant bony defects

The flexible and easy-to-apply properties of ECWN are ideal for the repair of complex-shaped bony defects. Further trials should be carried out to investigate the regenerative outcome of ECWN in other periodontal and peri-implant bony defects. When ECWN is placed into bony defects around immediate implants or peri-implant lesions, it may act as an osteoconductive scaffold for bone formation from the socket wall onto implant
surfaces, i.e. ECWN may facilitate peri-implant “gap-crossing”. As more bone formation was found in the coronal margin of ECWN grafted sites compared to BX grafted sites, the application of ECWN should also be explored in periodontal defects. This material may have the potential to support bone healing in periodontal infrabony defects, possibly even in a vertical and coronal direction.
References


### Appendices

#### Appendix I

1. **List of medication used on sheep**

<table>
<thead>
<tr>
<th>Medication</th>
<th>Route</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiopentone</td>
<td>Intravenous</td>
<td>20mg/kg</td>
</tr>
<tr>
<td>Halothane</td>
<td>Inhalation</td>
<td>1-2% (to effect)</td>
</tr>
<tr>
<td>Nitrous Oxide</td>
<td>Inhalation</td>
<td>1:2 (to effect)</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>Intramuscular</td>
<td>1ml/15kg</td>
</tr>
<tr>
<td>Carprofen</td>
<td>Intramuscular</td>
<td>5ml once/day for 3 days</td>
</tr>
<tr>
<td>Mepivicaine HCL (1:20,000 adrenaline)</td>
<td>Local infiltration</td>
<td>2x2.2ml cartridges around surgical site prior to surgery</td>
</tr>
<tr>
<td>Bupivicaine HCL (1:200,000 adrenaline)</td>
<td>Local infiltration</td>
<td>5ml around surgical site post-operatively</td>
</tr>
</tbody>
</table>
2. Chemical reagents used

Distilled Water, (purified via reverse osmosis unit, RiOs™ unit, Millipore Intertech, USA)

Xylene, C6H4(CH3)2, (Ajax Finechem Pty Ltd, New Zealand)

Ethanol, C2H5OH, (High grade, Absolute Ethanol, Thermo Fisher Scientific, USA)

Haematoxylin, (Surgipath®, Gill II Hematoxylin, Leica Microsystems, USA)

10% Natural Buffered Formalin (NBF), (BioLab Ltd, New Zealand)

Methyl methacrylate 99% (MMA), (Sigma Aldrich, USA)

10% Ethylenediamine tetraacetic acid (EDTA) solution, (supplied by Histology Unit, University of Otago, New Zealand)

Scotts water, (supplied by Histology Unit, University of Otago, New Zealand)

3. Equipment used

Piezosurgery® 3 unit, (Mectron, Genoa, Italy)

Piezosurgery® Insert EX1, EX2 and EX3, (Mectron, Genoa, Italy)

Gendex dental systems, (Monza, Italy)

Rapid microwave labstation, (KOS Microwave Histostation, Milestone, Italy)

Excelsior ES tissue processor, (Thermo Scientific, Waltham, USA)

Leica RM 2025 microtome, (Leica Microsystems Inc. Deerfield, USA)

Tegra-Pol, polishing machine, (Struers, Ballerup, Denmark)

Silicon Carbie Paper, Grades 180-4000 (Struers, Ballerup, Denmark)

Accutom, cutting machine, (Struers, Ballerup Denmark)

Incubating/shaking machine, (Multitron®, Infors HT, Switzerland)

RiOs™ wall mounted water distillation unit, (Millipore Intertech, USA)

Olympus AX70 upright compound microscope, (Olympus optical co. ltd, Japan)

Montaging software Volocity 5.2.0, (Improvision, MA, USA)

Montaging software Autopano Pro 2.5.2, (Kolor, USA)
Image J software version 1.47q, (NIH, USA)

SPSS statistics software for Mac version 20.0, (IBM corporation, Somers, USA)

Minitab 16.1 (Minitab inc., State College, Philadelphia, PA, USA)
Appendix II

1. Decalcification protocol

Transfer specimens into histology cassettes with label.

Decalcify in 10% EDTA solution in rapid microwave labstation at 37°C

Change EDTA solution every 48 hours.

Radiograph specimens weekly, until no radiopaque tissue identified

2. Resin for embedding

Ingredients

Methyl methacrylate, (M55909, Sigma Aldrich, USA)

Benzoyl peroxide, (517909, Sigma Aldrich, USA)

Dibutylphthalate, (524980, Sigma Aldrich, USA)

Xylene, (Ajax Finechem Pty Ltd, New Zealand)

Method for MMA I

4 parts Methyl methacrylate

1% Benzoyl peroxide

1 Part Dibutylphthalate

Method for MMA II

4 parts Methyl methacrylate

0.5% Benzoyl peroxide

1 part Dibutylphthalate

Method for MMA III

4 parts Methyl methacrylate

1% Benzoyl peroxide

1 part Dibutylphthalate
3. Resin embedding protocol

Transfer specimens to ethanol in cassettes with label.

Place specimens in 20% ethanol for 4 days, change solution after 2 days.

Place specimens in 40% ethanol and then 75% ethanol for 2 days each.

Place specimens in 95% ethanol for 4 days, change solution after 2 days.

Place specimens in 100% ethanol for 6 days, change solution every 2 days.

Immerse specimens in xylene for 4 days in fume cupboard on rotating platform, change solution after 2 days

Wash specimens in methyl methacrylate MMA monomer

Transfer specimens to MMAI for 2 days in fume cupboard on rotating platform.

Fill glass jars to one third depth with MMAIII, and place in plastic light-proof container part-filled with water. Leave undisturbed until set.

Immerse specimens in MMAII for 2 days in fume cupboard on rotating platform.

Place specimens in glass jars with pre-set bases and cover with MMAIII.

Place glass jars in water bath in light-proof container, at room temperature. Leave undisturbed until set.
4. Staining with MacNeal’s Tetrachrome/Toluidine Blue solution

Solution A (supplied by Histology Unit, University of Otago, New Zealand)

0.5g Methylene blue
0.8g Azur II
0.1g Methyl violet 2B
250ml Methanol
250ml Glycerol

Mix together. Stir with magnetic stirrer
Leave for 12 hours at 50°C then 3 days at 37°C.

Solution B (supplied by Histology Unit, University of Otago, New Zealand)

Toluidine blue in 100ml distilled water +1.0g borax.

Combine 10ml Solution A and 5ml Solution B
Stir and make up to 100ml using distilled water

Staining protocol

Place slides in 20% ethanol in Coplin jar.
Place in ultrasonic bath for 5 minutes.
Replace ethanol with 0.1% formic acid for 5 minutes in ultrasonic bath.
Wash with tap water.
Cover section on slide with diluted combination of Solution A+B for 5 minutes.
Rinse with distilled water for 5 minutes before air-drying.
Appendix III Details of histomorphometric analyses

1. Percentage of New bone formation for each experimental site

<table>
<thead>
<tr>
<th>Weeks of healing</th>
<th>Sheep no.</th>
<th>ECWN+</th>
<th>B+</th>
<th>ECWN-</th>
<th>NO-</th>
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</table>

2. Percentage of residual graft for each grafted site

<table>
<thead>
<tr>
<th>Weeks of healing</th>
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Appendix IV Details of histometric analyses

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