Alveolar Ridge Preservation in the Sheep Model

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Abstract

Post extraction remodelling of the alveolar ridge results in significant reduction in the width of the ridge, which may preclude the placement of dental implants.

Alveolar ridge preservation (ARP) procedures have been shown to reduce these changes, and thus are desirable, especially when the buccal plate is partially missing. Bovine-derived xenografts with porcine collagen membrane (BX) are considered the "gold standard" against which novel ARP materials should be compared. Four equine collagen products developed for ARP were tested: membrane (CM), cone with/without biphasic phosphate particles (CC, CO), and cone with integrated membrane (CS).

Objectives

To compare four novel products against BX in a novel sheep mandibular extraction socket model with standardised buccal defect.

Methodology

In 11 animals, mandibular premolars were extracted and standardised 5x2 mm buccal dehiscence defects were created. The sockets were grafted (Latin-square allocation) with BX, CC, CS, CO, CO+CM or ungrafted control (CON). The animals were euthanised after 16 weeks. Socket healing, new bone formation and reduction in the alveolar ridge width were analysed in undemineralised sections.

Results

No distinctive pattern of healing was noted for any of the materials. BX particles were partially resorbed by osteoclast-like multinuclear cells. Remnants of equine collagen-based products were not observed. BX grafted sites, compared to CON, showed a threefold decrease in reduction of the alveolar ridge width ($p=0.002$). Width preservation achieved by equine collagen products compared to non-grafted controls was not statistically significant, however better results were observed in groups CS and CO+CM.

Conclusion

A challenging extraction socket model with buccal defects representative of a "real-life" clinical situation was created. The test materials did not preclude new bone formation and were completely resorbed during the healing period, whereas BX-grafted sites have shown only partial resorption of the graft. The test materials, unlike the "gold standard" BX, were unable to demonstrate significant width preservation, although the results suggested that barrier membranes play an important role in ARP procedures.

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List of abbreviations

AEC	Animal Ethics Committee
β	beta
ARP	alveolar ridge preservation
BCP	biphasic calcium phosphate
BCS	biphasic calcium sulphate
BMP	bone morphogenic protein
BRG	bone replacement graft
BX	bovine xenograft
CBCT	cone beam computed tomography
CC	collagen cone
CM	collagen membrane
CO	Cone Oss
CON	control
CS	Collagen Sombrero
CT	computed tomography
EDTA	ethylenediaminetetraacetic acid
et al.	*et alii* (latin = and others)
GBR	guided bone regeneration

GmbH	*Gesellschaft mit beschränkter Haftung* (German = Limited Company)
HA	hydroxyapatite
H&E	haematoxylin and eosin
HCl	hydrogen chloride
i.m.	intra-muscular
i.v.	intra-venous
kg	kilogram
mm	millimeter
MLA	mandibular left anterior
MLM	mandibular left middle
MLP	mandibular left posterior
MRA	mandibular right anterior
MRM	mandibular right middle
MRP	mandibular right posterior
MMA	methyl methacrylate
NBF	neutral buffered formalin
P_1	first mandibular premolar
P_2	second mandibular premolar
P_3	third mandibular premolar

PBS	phosphate buffered saline
PDL	periodontal ligament
rhBMP	recombinant human bone morphogenic protein
TCP	tri-calcium phosphate

Chapter 1 - Introduction and review of literature

Tooth extraction has always been one of the most common procedures in dentistry. In many of the first world countries unmanaged complete or partial edentulism is considered to be functionally debilitating and aesthetically unacceptable. When an extraction of a tooth is planned, the operating dentist should consider the options for future rehabilitation of the newly created edentulous space. This could be accomplished with fixed prosthodontics, removable dentures, or dental implants.

The shape and volume of the residual alveolar ridge are important for the optimal tooth replacement option. The edentulous ridge undergoes bucco-lingual and corono-apical remodelling, which causes post-extraction loss of width and height. This could have a detrimental effect on the aesthetics and/or the functionality of the fixed and removable rehabilitation options.

ARP procedures aim at preventing, or at least minimising the post-extraction resorption of the alveolus, thus optimising the edentulous ridge for future rehabilitation. This is usually done by grafting the extraction socket with various grafting materials.

This study was designed to evaluate novel grafting materials against negative and positive controls in a sheep model of an extraction socket with a standardised defect in the buccal wall.

In this chapter we will be reviewing the healing process of the extraction sockets, the requirements for successful rehabilitation, the techniques and the materials most commonly used to preserve the alveolar ridge. Furthermore, we will review different animal experimental models and experimental techniques for analysis of the study results.

1.1 Alveolar crest remodelling

1.1.1 Alveolar ridge as a functional bone

Periodontium is a complex system of tissues, surrounding the roots of the teeth. It is composed of the gingiva, alveolar ridge, cementum and the periodontal ligament (PDL). On the histological level, the alveolar bone has two components: the alveolar process, the thickened ridge of bone that contains the tooth sockets (dental alveoli), and the alveolar bone proper, a thin layer of compact bone lining the tooth socket or the alveolus. The alternative name for alveolar bone proper is “bundle bone”, which is a histological term that originated because Sharpey fibers, a part of the fibers of the PDL, are inserted here.

The alveolar bone is a functional one, and its presence is conditioned by the presence of the tooth which is embedded in it (Marks, 1995). The outline of the alveolar ridge is determined by the shape of the teeth and their position within the dental arch. Any changes in tooth position, such as overeruption, tilting or extraction, will lead to subsequent changes in the shape of the alveolar bone.

Following the extraction of the tooth, the periodontium undergoes degeneration due to the loss of attachment apparatus of the extracted tooth, which includes the cementum, PDL fibers and the bundle bone. This process leads to changes in the shape of the alveolar ridge and overlying soft tissues in both horizontal and vertical dimensions (Araujo and Lindhe, 2005; Schropp *et al*., 2003). These changes could compromise the anatomy and aesthetics of the residual ridge to the extent that the successful prosthetic rehabilitation of the edentulous area by fixed/removable prosthodontics or implants is very difficult if not impossible to achieve.

1.1.2 Alveolar bone healing following tooth extraction

The healing of the extraction socket can be described as a series of processes that may be divided for didactic purposes into intra-alveolar and extra-alveolar healing.

1.1.2.1 Intra-alveolar healing

One of the early studies describing the human alveolar socket healing on a histological level was published by Amler nearly half a century ago (Amler, 1969). This study was performed on biopsies obtained from human volunteers.

This study showed that within the first 24 hours following the extraction a blood clot was formed within the socket. Two to three days post-extraction neutrophils, monocytes and fibroblasts migrate into the formed clot, and granulation tissue begins to gradually replace the clot, beginning from the apical portion of the socket. Within four to five days epithelium begins to creep over the aperture of the socket filled with granulation tissue. Osteoclasts can be noticed at the margins of the alveolus, whilst osteoblasts are present in its apical portion. One week after the extraction the biopsies showed that the socket contained granulation tissue that was being replaced by young connective tissue, and the apical portion of the socket showed some presence of osteoid. After three weeks, epithelium should completely cover the wound and the underlying socket should contain connective tissue with mineralising osteoid. Some radiographic evidence of mineralisation was present. Following an additional three weeks, with a total of six weeks of healing, the socket exhibited pronounced bone formation and trabeculae of new bone could be seen.

Another long term study using the dog model (Cardaropoli *et al*., 2003) provides more details on each phase of the healing process, as the biopsies have been taken up to the apical portion of a socket, and the follow up of the healing has been prolonged to 180 days. It is important to mention that as part of the socket filling with woven bone, the hard tissue wall of the socket, named the alveolar bone proper, is being resorbed.

1.1.2.2 Extra-alveolar healing

Post-extraction changes in the dimensions of the alveolar ridge were always of special interest to prosthodontists, as they could affect the aesthetic outcomes of the restorative treatment, and were described in detail by Pietrokovsi and Massler (1967) using cast models of arches with a unilateral missing non-replaced single tooth. The authors noticed that the centre of the ridge had shifted lingually/palatally, meaning greater resorption of the buccal plate, however absolute resorption rates varied between each group of teeth.

Another study using a dog model (Araujo and Lindhe, 2005) examined the dimensional changes of the alveolar ridge following tooth extraction through sequential biopsies. At baseline it was noted that the lingual wall of the extraction socket was wider than its buccal counterpart. The bundle bone (alveolar bone proper, that is a part of the attachment apparatus of the tooth, together with cementum and the PDL) occupied the inner portion of the socket; on the lingual wall, it was present at the tip of the bony wall, however looking at the buccal wall, it was noted that the coronal 2mm of the mineralised tissue is completely composed of bundle bone. One week after the extractions, osteoclasts were present on the inside and the outside of both buccal and lingual walls, representing the resorption of the bundle bone. After eight weeks of healing, while the height of the lingual wall of the socket remained relatively unchanged, the height of the buccal wall had been reduced by 2mm. That was partially explained by the fact that, at least in the dog model, the coronal portion of the buccal wall was completely composed of bundle bone. Another possible explanation is that the flap elevation, used as part of the study protocol to remove the roots of the teeth, had caused an external resorption of the socket walls (Wood *et al*., 1972), and considering the initial thickness of the buccal wall, it led to higher reduction in its height. However, in an experiment in a dog model, comparing the healing patterns of flap versus flapless extraction sites, similar amounts of tissue loss were observed independent of the technique used (Araujo and Lindhe, 2009a).

A non-invasive prospective study in human subjects (Schropp *et al*., 2003) involved clinical measurements, casts and subtraction radiography for up to a year of extraction sites (premolar and molar areas). The authors reported an average of 30% reduction in the alveolar ridge's width (bucco-lingual/palatal dimension) three months after the

extractions, increasing to 50% within a year. The vertical dimension of the buccal plate was reduced by an average of 1.2mm following 12 months of healing.

Similar results were reported by several recent systematic reviews on this subject (Tan *et al*., 2012; Van der Weijden *et al*., 2009). Another finding was the thickening of the soft tissue that occurs during the healing process, suggesting that underlying bony resorption is even more pronounced. The reviewers did not find enough evidence to conclude whether flapless extractions, smoking status, chlorhexidine rinses or immediate prosthesis are of significant influence on the degree of alveolar ridge remodelling.

Using subtraction radiography of standardised radiographs, Schropp and co-workers have shown bone resorption at the alveolar crest region (height) and most of the gain in mineralised tissue within the socket occurring within three months (Schropp *et al*., 2003). Additional gain of bone up to six months, and from 6 to 12 months of healing there was evidence of bone remodelling.

Summarising the above, following a tooth extraction, the edentulous alveolar ridge undergoes adaptive changes of both hard and soft tissues, resulting in resorption, primarily of the buccal plate of the ridge.

1.1.3 Extraction socket with buccal dehiscence

It has already been established that the buccal wall of the socket undergoes significant resorption following tooth extraction. It can also be damaged during the extraction process, resulting in a larger defect. A large-scale retrospective study (Venkateshwar *et al*., 2011) involving over 22000 extractions in almost 15000 patients studied the frequency of exodontia complications. According to the results, cortical plate fractures were the third most common complication (16.2% of all cases). It is worth noting that the extractions were performed in a teaching hospital by interns and undergraduate students, with the incidence of complications being higher with less experienced operators. Nevertheless, even in a more controlled environment of a prospective study, though on a lesser scale, evaluating the alveolar ridge integrity following an minimally traumatic extraction done by experienced periodontists (Leblebicioglu *et al*., 2015), various degrees of damage to the buccal plate (ranging from fracture to complete loss) were noted in up to 40% of cases.

Substantially less experimentally validated data is currently available about the post-extraction alveolar ridge remodelling of sockets with buccal dehiscence, compared to their intact counterparts. It would be reasonable to assume that these defects would undergo greater resorption and will be more challenging to treat at a later stage. Therefore, the potential benefit of ridge preservation procedures would be higher in cases with dehiscence type defects.

The current study is partially intended to improve our level of understanding of the post-extraction healing and resorption of sockets with buccal wall defect, and minimising its effects by ridge preservation strategies, using commercially available products.

1.2 Alveolar ridge preservation

ARP was defined as the procedure of arresting or minimising the alveolar ridge resorption following tooth extraction for future prosthodontic treatment including placement of dental implants (Atieh *et al*., 2015). Another term that is widely used in dental jargon is "socket preservation", however it is a misnomer, as the extraction socket is the defect in the alveolar bone that is created iatrogenically once the tooth is extracted. Obviously, we do not wish to preserve the bony defect created by the extraction, but to keep the original anatomy of the alveolar ridge for successful future prosthodontic rehabilitation.

It is also important to distinguish between "ridge preservation" and "ridge augmentation", the former being volume preservation within the envelope of the extraction site, and the latter being the increase in the bone volume beyond the existing bony envelope at the time of the extraction. Ridge preservation could be attempted immediately following the extraction or be delayed as a separate procedure. Delaying the procedure for a few weeks after the extraction (to allow for soft tissue healing) should be considered in cases of an infected site, or insufficient soft tissue to cover the extraction socket.

1.2.1 Clinical rationale for ARP

Resorbed alveolar ridge following a dental extraction can pose several problems to the restorative clinician, with any type of restoration.

When restoring the edentulous space with removable prostheses following multiple extractions, the post-extraction ridge resorption can result in irregularities of edentulous spaces, sometimes with undercuts, preventing correct seating of the denture. In severe cases, pre-prosthetic surgery, such as alveoloplasty, may be required (Hillerup, 1994).

In cases where dental bridges are the chosen treatment modality, the resorbed alveolar ridge often impedes the aesthetics of the prostheses. Vertical resorption may require the use of longer pontics, or in more severe cases – pink porcelain. Horizontal deficiency may lead to incorrect bucco-lingual positioning of the pontics. All of the above are compromises enforced on the restorative dentist, and lack the natural appearance (Seibert and Salama, 1996). Nowadays, with dental implants gaining popularity

(considered to be the treatment of choice in many cases) and the aesthetic requirements for rehabilitation options being high (sometimes even at the expense of functionality), post-extraction alveolar ridge resorption may compromise the ability to place the implant in a correct three-dimensional position (Irinakis, 2006).

Therefore, ARP may be necessary in many cases and the surgical procedures involved could be a great challenge for the practitioner.

1.2.2 History of and alternatives for ARP

1.2.2.1 Decoronation

The concept of decoronation for preserving the dimensions of the alveolar ridge is dated to the 1980s, originating in the field of paediatric dentistry dealing with dental traumatology. Decoronation was suggested as a surgical treatment for infra-occluded ankylosed teeth in adolescents (Malmgren *et al*., 1984). In young patients, rehabilitation with dental implants is contraindicated until the skeletal growth is completed (Koch G, 1996). Therefore, there is a genuine need to preserve the alveolar bone until the implant placement becomes possible.

Decoronation of the tooth slightly below the crestal bone, leaving the root in situ allows the soft tissue to heal and cover the buried root, that would preserve the volume of the ridge to support an adequate functional and aesthetic prosthesis. The limitation of this technique is that it can be implemented in a handful of selected cases, and would not be viable if the edentulous area was planned for restoration by a dental implant. However, decoronation might still be considered as a temporary measure, should the area be planned to be restored with an implant, when the implantation needs to be postponed for more than just a few months. Nevertheless, the rationale behind this technique is clear and does take into account the fact that the alveolar bone is functional, and needs the presence of the root in order to maintain its dimensions.

Many studies have shown that this technique has proven to successfully preserve the contour of the alveolar ridge with regards to ankylosed teeth (Cohenca and Stabholz, 2007; Filippi *et al*., 2001; Malmgren, 2000). Other studies expanded this approach to submerge non-ankylosed, but endodontically treated teeth with adequate preservation of the alveolar ridge (Casey and Lauciello, 1980; Dugan *et al*., 1981). Uneventful

healing of decoronated teeth, even without prior root canal treatment, is well documented in Oral and Maxillofacial Surgery literature in cases of lower third molars with increased risk of damage to inferior alveolar nerve if a conventional extraction was attempted (Leung and Cheung, 2009).

In 2007, Salama and co-workers reported that root submergence technique (RST) allows a preservation of alveolar bone frame in pontic areas, thus assisting in the creation of aesthetic results.

The major concern with regards to alveolar bone resorption following an extraction is the limited availability of bone for implant placement or incorrect positioning of the implant within the dental arch. Davarpanah and Szmukler-Moncler (2009a) went further and challenged the widely accepted concept of osseointegration by inserting dental implants through ankylosed or impacted teeth in order to avoid a surgical procedure that could require significant bone removal. An uneventful period of up to four years post loading, was reported by the authors. Nevertheless, this approach, labelled "unconventional" by the authors, did not change the consensus with regards to integrated implant interfaces (Davarpahah and Szmukler-Moncler, 2009a,b).

It can be concluded that submerging of a root of a non-infected tooth could effectively preserve the volume of the alveolar ridge for the purposes of subsequent rehabilitation with fixed/removable partial dentures or with implant borne prostheses.

1.2.2.2 Immediate implants

The concept of immediate implantation has emerged in an attempt to reduce the total number of required surgical procedures and minimise the time from tooth extraction to final restoration. The first mention of immediate implantation in the literature dates back to 1978 (Schulte *et al*., 1978), however being published in German it did not gain popularity until Barzilay and his research group (1991, 1996a,b) published their results more than a decade later. It has been hypothesised that the implant will keep the alveolar bone under functional forces, and thus prevent the post-extraction resorption (Barzilay, 1993).

This hypothesis has been evaluated in a study by Botticelli and co-workers (Botticelli *et al*., 2004). The authors performed 21 immediate implants without simultaneous bone

grafting. The dimensions of the buccal and the lingual walls were measured, as well as the size of horizontal defect between the implant and the socket wall in the most coronal aspect of the extraction socket. At re-entry surgery after four months of healing, only eight out of 52 initial gaps exceeding 3mm were present. The average horizontal resorption of the buccal bone plate was 56%, while the lingual wall lost on average 30% of its width. Vertical resorption was up to 1mm. These results suggested that the marginal gap between the implant and the socket could resolve without grafting. However, the most important conclusion was that post-extraction alveolar ridge resorption is not prevented by immediate implantation.

Another study described the bone morphology at implant insertion and re-entry (after four months) in 93 cases of extractions of maxillary single rooted teeth and immediate implantation (Ferrus *et al*., 2010). A more pronounced fill of horizontal gap was observed in the premolar segment, compared to the incisor-canine area, and the reduction in vertical dimension was significantly smaller. Furthermore, sites with thick buccal bone wall (>1mm) and a large horizontal gap (>1mm), resulted in a more substantial degree of gap fill.

A Cochrane review (Esposito *et al*., 2010) failed to provide clear recommendations for the use of immediate implantation due to insufficient evidence. The authors mentioned the possibility of higher failure risk for the immediate or immediate-delayed implants, however this could be balanced by the increased chances of a better aesthetic outcome. No conclusions could be drawn with regards to the need for augmenting the bone gap or the preferred procedure or material used for this augmentation.

1.2.2.3 Socket shield technique

This technique, which was first described only a few years ago (Hurzeler *et al*., 2010), aims at combining the principles of immediate implantation with submerged root technique. This technique modified existing protocols for immediate placement of implants, in order to compensate for post-extraction alveolar ridge resorption, especially in aesthetic zones.

According to this concept, it is assumed that retaining the root fragment attached to the buccal wall of the extraction socket will prevent the wall's remodelling. In a proof-of-concept study in a dog model, the root was partially removed, and the lingual wall of

the retained fragment was treated with enamel matrix derivate (Emdogain) prior to implant placement. Histological results have shown that a layer of newly formed cement is developed over the exposed dentin, and mineralised tissue has filled the gaps between the threads of the implant following the healing process. Also, absence of any osteoclastic remodelling at the crest of the buccal alveolar was noted.

The stages of site preparation prior to implant placement have been outlined in detail elsewhere (Glocker M, 2014): the tooth deemed “irrational to treat” is decoronated supragingivally; then, the root is separated vertically in a ratio between 1:3 and 2:3. The smaller, buccal root fragment is retained and the larger lingual root fragment is removed in a manner that spares bone and soft tissue to the greatest possible extent. The height of the buccal socket shield is reduced to the level of the bone.

Furthermore, this technique was modified to retain a lateral fragment of the root in order to preserve the dental papilla in aesthetic areas (Kan and Rungcharassaeng, 2013).

This procedure is highly technique sensitive, and only case series have been published up to date (Glocker *et al*., 2014). Further data in large cohorts of patients are needed to draw any conclusions.

1.2.3 ARP as guided bone regeneration

Since the extraction socket is virtually a bony defect of the alveolar ridge that heals with external resorption of the socket walls, the majority of ridge preservation techniques apply the principles of guided bone regeneration (GBR). Bone regeneration requires adherence to four main principles: stability of the blood clot, space maintenance, exclusion of epithelium and connective tissue, and primary closure of the wound (Wang and Boyapati, 2006).

1.2.3.1 Minimally traumatic extraction

The failing tooth should be extracted in a minimally traumatic way in order to preserve the soft tissue and the bony walls of the alveolus. Applying lateral pressure on the papilla and the bony walls of the socket must be avoided. Instead, the root is to be separated from the socket by using periotomes to cut through the PDL fibers and vertical movements used to sever the rest of them and to deliver the root. Lateral movements during the extraction could damage the walls of the socket (especially the buccal wall) or cause cellular necrosis and thus interfere with the blood supply that is necessary for the healing process (Kubilius *et al*., 2012).

The question whether a mucoperiosteal flap should be raised for the extraction is debatable. For classical ARP procedures, where the grafting material is to be covered by a membrane, raising the flap is required. It can also be necessary in order to obtain sufficient access to the tooth to be extracted, especially when bone deficiencies and/or infection is present. However, it is well described that a full thickness mucoperiosteal flap is associated with alveolar bone resorption (Melcher, 1976; Yaffe *et al*., 1994). The reasons for resorption are attributed to the fact that periosteum is a source of blood supply to the cortical bone, and also has osteogenic capacity, as may be seen in cases of Garre's Osteomyelitis. It has been shown in a dog model that at sites with flap elevation there was more resorption of the buccal plate, compared to sites that had received a flapless extraction (Fickl *et al*., 2008a).

1.2.3.2 Debridement of extraction socket

Following the root delivery, the socket walls should be assessed for fractures, prior to continuing to the alveolar preservation procedure. In the case where the extracted tooth has been periodontally involved, and/or presented with endodontic lesion, the extraction socket should be thoroughly debrided with a surgical curette in order to remove the chronic inflammatory soft tissue (Tischler and Misch, 2004). Bleeding from the walls of the extraction socket should be verified, or induced if absent, as the blood contains the osteoprogenitor cells and growth factors essential for bone healing.

1.2.3.3 Application of bone replacement grafting materials

Bone replacement grafting (BRG) materials are placed into the socket immediately after tooth extraction. The grafting materials are intended to serve as scaffolds for ingrowth of cells and blood vessels and formation of new bone during healing. Gradually, BRGs undergo resorption and replacement by the patient's own bone. A membrane is usually used to cover the grafted material and bony wall defects, and the soft tissue is sutured over the membrane, preferably enabling primary closure. Different types of bone grafting materials will be described in the next section.

1.2.3.3.1 Biological sources of bone grafting materials

BRG materials can be categorised into several groups, based on their origin.

1.2.3.3.1.1 Autologous bone

Autologous bone is harvested from another site of the same patient. It is usually referred to as the "gold standard" of grafting materials, it has the obvious disadvantage of a second surgical site, adding to postoperative discomfort and complications, as well as the possibility of a limited available quantity of graft material (Wang *et al*., 2004) and higher resorption rates.

1.2.3.3.1.2 Allografts

Allografts are materials that were previously harvested from another individual of the same species (Tischler and Misch, 2004). They were believed to induce bone formation through bone morphogenetic proteins (BMPs) exposed during preparation (Schwartz *et al*., 1996; Wang *et al*., 2004). However, clinical trials have found little evidence of that

(Becker *et al*., 1994; Froum *et al*., 2002). These grafts come from processed human cadavers, and therefore may raise some concerns related to religious beliefs and possible transmission of infectious diseases (Allegrini *et al*., 2008).

1.2.3.3.1.3 Xenografts

Xenografts are grafting materials that originate from other species (Tischler and Misch, 2004). Bio-Oss® (Geistlich AG, Wolhusen, Switzerland) is a popular and widely used commercially available bovine (cow) deproteinized bone. Schneider *et al*. (2009) referred to this material as the current "gold standard" in bone substitution. However, its elimination from the graft site is incomplete and may take years (Artzi *et al*., 2000). A study examining human biopsies from patients who underwent a sinus floor elevation procedure using xenograft has found remnants of grafting material four years after the surgical procedure (Piattelli *et al*., 1999). Theoretically, the non-resorbed graft materials may act as a barrier to replacement bone formation. It has been suggested, that non-resorbed xenograft particles may delay healing and impede future implant placement and osseointegration, however there is lack of scientific evidence to substantiate this claim. In a human case report, xenograft material was not found to reduce the bone-to-implant contact (Valentini *et al*., 1998). Similar results have been found in experimental dog models (Berglundh and Lindhe, 1997) and in recent reviews of the literature (Nkenke and Stelzle, 2009).

1.2.3.3.1.4 Alloplasts

Alloplasts are synthetic materials of non-animal origin (Allegrini *et al*., 2008). They provide a scaffold for bone in-growth. Alloplasts can be calcium phosphate-based materials or polymers that may also carry osteoactive agents (Neiva *et al*., 2008). The main disadvantages of alloplastic graft materials are the tendency for granular migration and unpredictable rates of resorption (Molly *et al*., 2008).

1.2.3.3.2 Biological mechanisms of bone regeneration.

The differences between the various bone grafting materials are not only based on their origin, but on their mode of action or interaction with the host. (Darby *et al*., 2008; Darby *et al*., 2009; Darby, 2011)

1.2.3.3.2.1 Osteogenesis

Osteogenesis is the generation of new bone by means of viable bone forming cells (osteoblasts) in the graft, and therefore, the only currently available osteogenic material is the autograft. Osteoblasts from other sources would be immunologically intolerable. For the application of ARP, an autologous bone graft would usually require another intraoral surgical site, such as a mandibular ramus, tori or maxillary tuberosity. A limiting factor for use of these materials can be the donor site morbidity (Silva *et al*., 2006).

1.2.3.3.2.2 Osteoinduction

Osteoinduction might be described as induction of osteogenesis, where bioactive molecules stimulate the differentiation of pluripotent cells into the bone-forming lineage. The most studied osteoinductive agent is BMP and its addition to different bone grafting materials has been studied both clinically and experimentally (Acil *et al*., 2002; Fiorellini *et al*., 2005; Oryan *et al*., 2014). Although recombinant human BMP is already commercially available (rhBMP-2) in some countries for both labelled and off-label use, the limiting factor is its high price, and therefore it is not commonly used in ARP.

Platelet rich plasma (PRP) has also been suggested as a "concentrated" source of bioactive molecules, however, its added value is still controversial, and usage of this technique requires additional equipment and venepuncture (Hallman and Thor, 2008; Trombelli and Farina, 2008). Allograft materials have been claimed to have some osteoinductive properties (Wei *et al*., 2015), as the harvested bone from the human subject will by definition contain some bioactive molecules embedded in it. However, the concentration of these molecules is probably scarce, and the osteoinductive capacity may vary between the products due to different protocols of each bone bank (Behfarnia *et al*., 2012), and even between different batches of product from the same manufacturer, due to lack of homogeneity in the source of processed bone.

1.2.3.3.2.3 Osteoconduction

Osteoconduction in its original definition means that bone grows on a surface (Albrektsson and Johansson, 2001). In the broader understanding, osteoconductive materials are highly biocompatible with bone, and provide a 'scaffold' which allows bone growth on its surface, or down into pores and channels within the material. This is the main principle of action (POA) of the vast majority of the bone substitutes used in the oral cavity. They serve as "space maintainers" that allow for consecutive bone fill, and thereby are gradually replaced by the native bone (Darby, 2011).

1.2.3.4 Membranes

The principles of GBR are to selectively block epithelial and connective tissue cells, thus promoting bone healing inside the bony defect by osteogenic cells that have a slower proliferation rate. This was described in pioneering studies over 30 years ago (Melcher, 1976; Nyman *et al*., 1982). Additionally, the available/created space must be maintained for subsequent bony fill, and therefore bone substitutes are used for that purpose. Membranes are a crucial component of GBR, as they serve as a barrier in both directions: they allow for both exclusion of connective tissue and epithelium, and for containment of the bone substitute materials inside the defect, thus preventing its spill-over (Hammerle and Jung, 2003).

1.2.3.4.1 Non-resorbable membranes

The non-resorbable membranes were the first generation of barrier membranes used for GBR. The membranes included in this subgroup can be categorised according to the material, such as polytetrafluoroethylene (PTFE), expanded PTFE (ePTFE), high density PTFE (dPTFE), titanium reinforced PTFE and titanium mesh. Originally, ePTFE membranes were the most popular type in GBR procedures, with Gore-Tex® being the known brand (Liu and Kerns, 2014).

The success of the ePTFE membranes was related to the high stability of the polymer, resistance to biological breakdown by both the host tissues and microbiota, and being immunologically inert (Hammerle and Jung, 2003).

Effectiveness of ePTFE membranes was shown in animal experiments in rats (Dahlin *et al*., 1988) and primate models (Dahlin *et al*., 1990) of surgically created defects of

jawbones. In a human study including 10 patients requiring extraction of at least two anterior teeth, when one of the extraction sockets was left to natural healing as a negative control, it was shown that non-resorbable ePTFE membranes can maintain their shape and are able to promote GBR or assist with ARP without the use of bone substitutes (Lekovic *et al*., 1997).

Non-resorbable membranes, however, have significant disadvantages that limit their use (Murphy, 1995a,b). The most common complication of using non-resorbable membranes is wound dehiscence and membrane exposure. When exposed to the oral cavity, they can be easily infected, and therefore need to be prematurely removed. That in turn, can have a negative effect on the outcomes of GBR procedures (Machtei, 2001). This can be more problematic in ARP procedures, when achieving primary closure over the extraction socket without tension can be challenging. Being non-resorbable, these membranes require a second stage surgery for removal, even if good outcomes of the GBR have been accomplished, incurring additional discomfort and increased costs to the patients. For these reasons their use is mostly limited to specific procedures that require high structural stability and larger bone fill. They have been widely replaced in ARP procedures by resorbable membranes, which do not result in major complications when exposed to the oral cavity.

1.2.3.4.2 Resorbable membranes

The aforementioned disadvantages of the non-resorbable membranes have been overcome with the introduction of the resorbable alternatives. Higher tolerance to exposure, abolished need for second stage surgery and lower patient morbidity have contributed to higher acceptance of resorbable membranes as a barrier of choice for a wide variety of GBR procedures, including ARP (Hammerle and Jung, 2003). The weakness of the resorbable membranes most commonly used today is that they lack structural stability, and require the support of bone substitute materials to prevent them from collapsing into the defect (Lundgren *et al*., 1994). This can be of high importance in cases where a large portion of the extraction socket wall is missing due to periodontal involvement or traumatic extraction.

The resorbable barrier membranes can usually be subdivided by material into collagen membranes of various origins, and biodegradable polymers.

In a split-mouth design human study, it has been shown that ARP is achievable with a resorbable polymeric membrane (Lekovic *et al*., 1998). The use of polymeric membranes has resulted in decreased post-extraction dimensional changes of the alveolar ridge, and increased socket fill, compared to control sites at re-entry surgery after six months.

The biodegradable polymer membranes were introduced earlier for clinical use, and mostly include polyglycolides (PGAs), polylactides (PLAs) and their co-polymers that are resorbed by hydrolysis (Hutmacher *et al*., 1996). Another study has evaluated the response of PLA/PGA membranes upon their exposure (Simion *et al*., 1997). The authors have found that the exposure speeds up the resorption of these membranes. Premature resorption may reduce their effectiveness as a barrier, and compromise the anticipated outcomes of the GBR.

Most of the commercially available resorbable membranes are composed of type I collagen of various animal origins, or a combination of type I and type III collagens. For a detailed review see Bunyaratavej and Wang (2001). The degradation of the collagen membrane is achieved by enzymatic cleavage by macrophages and polymorphonuclear leukocytes. The resorption rate of collagen may be decreased by inducing physical or chemical cross-linking (Rothamel *et al*., 2005) to avoid premature loss of barrier function, however it is balanced by decreased vascularisation and increased inflammation in the grafted site (Schwarz *et al*., 2006). The use of collagen membranes with grafting material has been established as a good alternative to previously used ePTFE membranes in many clinical applications of GBR (Zitzmann *et al*., 1997).

1.2.4 Treatment variations

According to the principles of GBR, a membrane serves as a barrier, thus preventing the ingrowth of connective tissue and oral epithelium into areas that are to be filled with bone. Insertion of a barrier membrane dictates elevation of the mucoperiosteal flap during the ARP procedure.

Alternative variations of ARP techniques, not fully utilising the principles of GBR have been published. Some of the published techniques are summarised in Table 1.1.

Table 1.1. Treatment variations for ARP.

Authors	Technique	N.	Controls	Follow-up	Outcomes (ridge width reduction)
(Nemcovsky and Serfaty, 1996)	Hydroxyappatite graft, soft tissue coverage, no membrane.	23	Nil	18months	0.6mm
(Lekovic *et al*., 1998)	No grafting materials, only resorbable membrane, covered by the soft tissue	16	Split-mouth Natural healing	6 months (re-entry)	1.3mm vs 4.56mm in control group
(Camargo *et al*., 2000)	Alloplast graft, no coverage – "open socket" technique	16	Split-mouth Natural healing	6 months (re-entry)	3.48mm test 3.06mm control
(Tal, 1999)	"Socket seal" – sealing the BRG with gingival graft from a donor site	24	Allograft vs Xenograft	1 month	Not measured. (vitality of soft tissue coverage was measured)
(Fiorellini *et al*., 2005)	Collagen sponges with or without bioactive substances	80	rh-BMP2 vs placebo or natural healing	4 months	3.27mm vs 0.82mm width gain; adequacy of bone for implants twice as great

There is lack of consistency in the dental literature regarding the materials, delivery systems, and technique modifications for ARP. Some researchers compare the grafted sockets against negative controls (natural healing), while others use widely accepted techniques and materials as benchmarks. Several systematic reviews have attempted to compare the available information in order to provide the dental practitioners with recommendations. Due to large heterogeneity of the methodology, most of them have failed to perform a meta-analysis on a large number of cases. However, most of the reviewers came to similar conclusions (Horowitz *et al*., 2012; Vignoletti *et al*., 2012; Avila-Ortiz *et al*., 2014; Atieh *et al*., 2015):

1. There is a benefit for ARP. The majority of the studies reported diminished resorption of the alveolar ridge, when compared with natural healing, however preservation procedures could not completely prevent the resorption. While natural healing results in an average of more than a 3mm decrease in alveolar ridge width and around 1mm in height, preservation procedures show a horizontal resorption around 1mm, and a relative preservation of the vertical dimension.

2. No single grafting material has been proven to be superior to others.

3. No surgical technique has been shown to provide better results with regards to ARP.

It must be remembered that the vast majority of the extractions are performed by general dental practitioners, and ARP procedures are still limited in general dental practices due to their being time consuming, requiring advanced surgical skills and the significant cost of materials. Therefore, the market driven by the manufacturers will probably tend to adopt the techniques and materials that are more affordable, and less demanding and time consuming.

1.3 Animal models

1.3.1 The need for an animal model

Animal models are a necessary link between *in vitro* studies and human trials, whenever a new medicine, implantable device or a novel surgical technique is introduced. It is important that biocompatibility and the possible adverse effects are tested in animal models before proceeding to human subjects (An and Friedman, 1998). Another reason for using animal models is the invasive nature of the required measurements (disease infliction, subsequent biopsies, histological analyses, etc.) that would be impossible to perform in human beings.

The choice of an appropriate animal model for a study must be made by thoroughly considering the following aspects:

1) Large or small animals.

2) Ability to create a disease/surgical defect model in an animal.

3) Availability of appropriate animal and/or surgical facilities.

4) Comparability with human anatomy/physiology/pathophysiology.

5) Number of subjects required to have enough statistical power.

6) All of the above in relation to cost per animal and available funds.

Various research groups have used different large animal models to investigate the healing process of the extraction socket, and test various grafting materials. Even though non-human primates were initially used for research in this field (Pietrokovski and Massler, 1971), their high cost and limited availability, combined with ethical issues, restrict their use to a handful of fields of research where another animal model would not suffice. Therefore, the most commonly used non-human models for bone grafting materials and ARP are dogs.

1.3.2 Dog extraction socket models

Significant portion of *in vivo* experiments published in periodontal literature, including studies of guided tissue regeneration, implantology and ARP utilised a dog animal model (Pearce *et al*., 2007). Bone composition and physiology in this large animal dog model have shown similarity to human subjects (Aerssens *et al*., 1998). Moreover, dogs are easier to handle, compared to other large animal species.

Different tooth extraction models were developed in dogs, differing in levels of complexity. The more demanding protocols (Cardaropoli *et al*., 2003; Indovina and Block, 2002) used the premolar sites: the teeth were hemisected, and distal roots removed while obturating the mesial roots in order to preserve the bone height adjacent to the extraction sites. Simplified protocols utilised extraction of either maxillary third incisors (Iibuchi *et al*., 2010) or mandibular second molars (Rothamel *et al*., 2008) without prior hemisection.

However, canine animal models have recently encountered ethical concerns, as public opinion demands restriction in the use of companion animals (such as dogs or cats) for research. An additional reason for the decrease in the use of dogs for *in vivo* research is the high cost of these experimental animals.

1.3.3 Sheep extraction socket model

1.3.3.1 Sheep in bone grafting materials research

Sheep or pigs are domesticated animals, and as such are readily accepted by the public to be used for research (An and Friedman, 1998). Specifically in New Zealand, sheep (*Ovis aries*) are available in numbers, and are widely used for agricultural research, thus driving down the cost and housing of these animals for scientific use (Duncan, 2005). They resemble humans in size, physiology and metabolic rate (Newman *et al*., 1995; Schmidt-Nielsen, 1997). A review published by the AO Foundation, one of the market leaders in devices for orthopaedic and maxillo-facial surgery, evaluating the possible animal models for biomaterial research in bone also ranks sheep amongst the suggested options (Pearce *et al*., 2007) (Table 1.2):

Table 1.2. Relative similarity between animal and human bone (Adapted from Pearce, 2007).

	Rabbit	Pig	Dog	Sheep
Microscopic structure	+	++	++	+
Macroscopic structure	+	++	++	+++
Composition of bone	++	+++	+++	++
Turnover rate	+	+++	++	++

Levels of similarity compared to human bone: + low, ++ moderate, +++ high

Invermay Agriculture Research Centre and the University of Otago have rich experience and collaboration with regards to sheep surgery in orthopaedic and dental research. Invermay provides a well-equipped surgical theatre, as well as excellent facilities for postoperative care, housing, specimen collection and disposal. Therefore, researchers from the University of Otago have accumulated vast expertise in periodontal and implant research in sheep animal model (Duncan *et al*., 2003; Duncan, 2005; Salmon and Duncan, 1997).

1.3.3.2 Dental anatomy of ruminants

The anatomy of the jaws and dental anatomy of the ruminants (including sheep) is quite unique. They lack upper incisors. Three mandibular incisors and one canine on each side operate against an upper "dental pad" (Figure 1.1). The lower anterior segment is separated by a wide diastema from the posterior one, which includes three premolars and three molar teeth. The teeth that are available for extractions during survival surgery are the premolars, as the anterior teeth are essential for grazing, and the access to molar teeth is limited by the mouth opening. Adequate access to the molar areas would necessitate a relieving incision from the corner of the mouth caudally, which is neither acceptable ethically, nor required, as once the premolars are extracted, the animals would need intact molars for grazing.

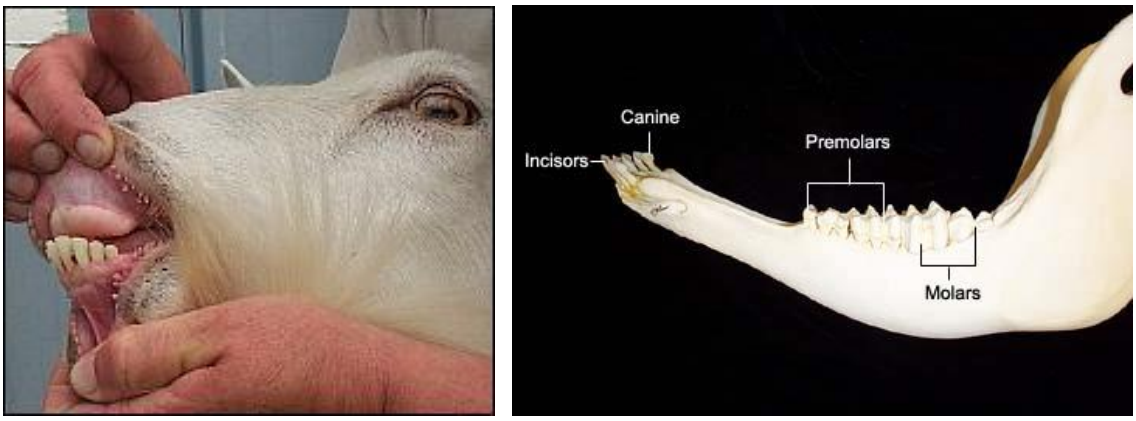

Figure 1.1. Dental anatomy in ruminants. (Adapted with permission from Colorado State University website http://www.vivo.colostate.edu/hbooks/pathphys/digestion/pregastric/cowpage.html)

The mandibular premolar region in sheep was previously described by Duncan in 2005. All the posterior teeth have contact surfaces rather than contact points. The first premolar (P_1) is usually small in size, and sometimes might be exfoliated due to periodontal disease. All three premolars (P1-P3) have two roots that increase in size from P1 to P3. The roots of the third premolar are usually similar in length. P1 and P2 have variable root sizes: while in some teeth both roots are of similar size, in others the mesial root is longer than the distal one. There is a thick cortical plate, while the inner portion of the mandible has a relatively big marrow space with scarce trabeculations (Figure 1.2).

Figure 1.2. Sagittal view of the mandibular premolars. (Adapted from Duncan, 2005)

The presence of the inelastic mandibular bone may result in a higher frequency of root fractures during the extractions, and should they happen – a need for aggressive methods for removing the root fragments.

1.3.3.3 Sheep healing time

When planning survival surgery in an animal model, it is important to be aware of the different healing times between the species, in order to allow adequate time before re-entry surgery and/or euthanasia. In an early study investigating post-extraction sockets it was shown that an 8-week healing period in dogs is equal to 3.5 months of healing in human subjects (Claflin, 1936). Further study by Duncan (2005) found that dogs and sheep have similar healing times, both being shorter than humans (Table 1.3).

Table 1.3. Healing times of sheep compared to human subjects. (Adapted from Duncan, 2005)

Sheep healing times	**Human healing times**
6 hours	8 hours
5 days	1 week
1 week	9 days
2 week	3 weeks
4 weeks	5 weeks
6 weeks	8 weeks
8 weeks	11 weeks
12 weeks	16 weeks
16 weeks	21 weeks

1.3.3.4 Previously described sheep extraction socket model

Liu *et al* (2015) were the first and to date, the only authors to examine the healing of BRG materials and membranes using a tooth extraction model in sheep. In their study, only the second and third premolar sockets were used (due to the differences in the sizes of the three premolar teeth). Eight animals were used for two different time points (8 and 16 weeks) and two different grafting materials were examined, with and without resorbable collagen membranes. There were no statistically significant differences in bone formation across the four groups at the two time points. Data from her thesis (Liu 2013) also suggested that there was greater vertical loss in bone at the buccal versus lingual alveolar plate. Although these authors concluded that "this first description of a tooth socket model in sheep supports the overall utility of this model for bone graft research", further modification to the model could be considered, including the standardization of the size of the tooth sockets and the inclusion of the first premolar site, allowing the comparison of up to six test sites. Liu (2013) conducted post-hoc analysis and suggested that up to five-fold increase in animal numbers was required to achieve statistical significance using her study design; however, modification of the study design could reach statistical significance with fewer sheep which satisfies one of the 3 Rs of animal research - replacement, reduction and refinement (Russell and Burch, 1959).

1.4 Techniques for measurement of alveolar ridge dimensions

The measurements of alveolar ridge dimensions are of use in the fields of implant dentistry and in research of ARP. Both clinical and research fields are interconnected, as currently the primary indication of ridge preservation procedure is planned or potential implant placement at the extraction site.

One of the most cited studies in the field of ARP (Schropp *et al*., 2003) studied the changes of a single tooth extraction in a premolar and molar area within 12 months.

According to their results, the average width of the alveolar ridge, which was 12mm (range 8.6–16.5mm) immediately after the extraction, decreased to only 5.9mm (range 2.7–12.2mm) after a year. This is barely enough for a predictable placement of a standard 3.5-4mm wide implant. Many of the studied sites would probably require augmentation procedures prior to implant placement.

1.4.1 Cast models

Cast models were utilised almost 50 years ago to study the post-extraction resorption of the alveolar bone (Pietrokovski and Massler, 1967). The cast models were also used in more recent studies (Schropp *et al*., 2003), sometimes with additional modifications. The obvious advantages with this technique are the ease of use, as most dentists are well trained in taking impressions, low cost and non-invasiveness. However, the drawbacks include a lack of reliable information about the true dimensions of the bony ridge, as the impression represents the whole jaw, with hard and soft tissues. Therefore, simple casts may only provide limited information about gross trends without being diagnostic. Another argument against using cast models was the added errors of impression and casting materials, which can now be overcome by use of direct intra-oral optical scanning.

1.4.2 Ridge mapping

Ridge mapping is a technique that has been developed for the purpose of measuring the width of the bony envelope as a diagnostic and planning measure prior to implant placement (ten Bruggenkate *et al*., 1994). The concept of intraoral bone sounding is not new, and was utilised several decades ago for gingivectomy procedures (Goldman, 1951), when the level of the incision was established by probing the periodontal pockets with excessive pressure to identify the location of the coronal portion of alveolar bone. Ridge mapping does not require any use of radiographic methods, and is reliable. A plaster model is used to produce a stent prior to the ridge mapping, to ensure reproducibility and correct spatial allocation of the measurement. For an animal model, this would require an additional general anaesthetic session to take impressions prior to the surgical session, in order to produce the stent. This technique can then be used to measure the ongoing post-extraction changes in the width of the alveolar ridge. According to a study comparing ridge-mapping with direct caliper measurements of the

surgically exposed alveolar ridge, 89-94% (depending on the examiner) of pair measurement deviations were within 1mm (Chen *et al*., 2008). The disadvantages of this technique are that it is time consuming and, being invasive, the area of interest must be anaesthetised prior to bone sounding.

1.4.3 Imaging

Diagnostic imaging is a useful technique for indirect measurement of bony dimensions. Most medical and dental practitioners are well trained to interpret radiographs. The main advantage of radiography is that it is non-invasive. With recent advancements in digital radiography, the time-consuming image developing process is largely obsolete and high-resolution images that use less radiation are now available.

1.4.3.1 Plain radiography

Plain imaging is still widely accepted, as it is simple to perform and does not require advanced and costly equipment, as well as minimising the patient's exposure to radiation. The use of radiographic stents (to allow for reproducibility) and standardisation of the source-to-tissue distance, exposure time and intensity, and the radiographic receptor used, allow changes in bone density to be tracked. Furthermore, theoretically speaking, the width of the alveolar ridge may be evaluated by an intra-oral periapical radiograph in occlusal projection (Desai *et al*., 2013), but this would be impractical and time consuming in modern dentistry, not to mention the various sources of error, such as cone angulation, superimposition, magnification, etc.

1.4.3.2 Cone beam computed tomography (CBCT)

Computed tomography (CT) has been increasingly used within the last two decades as a means of indirect non-invasive hard tissue measurements in the fields of Periodontology and Maxillo-Facial Surgery, mainly because of its high accuracy, and three-dimensional visualisation capability. Many consider it to be the clinical gold standard in Oral Surgery and Implant Dentistry. The major drawbacks of this technique are the relatively high cost and the considerable radiation dose to which the patient is exposed (Monsour and Dudhia, 2008).

1.4.3.3 Micro-computed tomography

Even though histology remains the gold standard and provides evidence on a cellular and microscopic level, imaging technologies gain more popularity in research of hard tissues, including ARP. As summarised in recently published reviews (Schambach *et al*., 2010; Vanderoost and van Lenthe, 2014), micro-CT allows for detailed and accurate measurements and visualisation of the hard tissues in a chosen plane of interest, as well as bone mineral density and volumetric calculations. The obvious advantage of using micro-CT as compared to histology is its non-invasiveness, and ability to reposition the planes and re-measure without destroying the specimen. The use of micro-CT also allows the use of small animals in *in vivo* experiments with radiographic follow-up in different time points. In many of the studies, three-dimensional imaging and histology are not mutually exclusive, but rather increase the total body of evidence that may be obtained from a single animal/specimen.

1.5 Histological analysis following ARP

Despite the interconnection of the clinical practice with research, some of the analytical techniques are only applicable in animal models and may not be utilised in human studies for obvious ethical reasons. Studies in the field of ARP usually compare between different surgical techniques, and various bone grafting materials. The aim of these studies is two-fold: firstly, to compare the absolute values and percentage of dimensional changes of grafted extraction sockets with negative and/or positive controls; and secondly, to test bone grafting materials, and to measure their resorption times and osteoconductivity.

Therefore, the techniques can be divided into those that measure dimensional changes of the alveolar ridge, and those that measure new bone formation and residual graft-to-tissue ratios within the healing sockets.

1.5.1 Histometry - measuring changes in alveolar ridge dimensions

This measurement technique was described and used by well-known research groups (Araujo and Lindhe, 2005; 2009a; Fickl *et al*., 2008b) in a dog model of ARP research. In this study model, one of the roots of a premolar was extracted in a minimally traumatic way, while the other was retained as the control. The measurements were done comparing the cross-sections of the remaining root and the edentulous ridge.

This technique is based on the assumption that both the mesial and distal roots of dogs' premolars are similar in shape and dimensions, as well as the correlating alveolar bone. Another potential technical problem that might affect the results is the non-parallelism of the two roots, and the requirement of precise identification of the tooth/socket apex and axis prior to performing histological cross-sections.

Another technique of measuring the alveolar ridge changes in a dog model was described by Rothamel and colleagues (2008). The vertical changes of the buccal and the lingual alveolar walls were measured to the most apical point of the jaw; the horizontal dimensions of these walls and the total width of the alveolar ridge were measured at set distances below the crest, perpendicular to the long axis of the extraction sockets. Therefore, once again, their technique was highly dependant on the precise angulation of the specimens for histological sections.

1.5.2 Histomorphometry - measuring bone formation in the extraction socket

1.5.2.1 Histological observations

As previously mentioned, histology is still considered to be the gold standard of evidence. In the research involving hard tissues and bone grafting materials, there are two options of histological preparations of specimens – demineralised and undemineralised, based on the primary purpose of the study.

1.5.2.2 Demineralised sections

The specimens are demineralised using ethylenediaminetetraacetic acid (EDTA), embedded in paraffin and very thinly (4-7μm) sectioned. The basic histological staining

with haematoxylin and eosin (H&E) allows visualisation of the cellular component of the specimen, as well as the non-mineralised extracellular structures. Additional staining techniques with histochemical and immunohistochemical markers can help with identifying specific cells types or cellular activity.

Cardaropoli and co-workers (2003) used demineralised histological sections to study the healing process in extraction sockets at nine time-points, ranging from one to 180 days, in a dog model. Araújo and Lindhe (2005) used a similar model to study healing dog sockets after one, two, four and eight weeks of healing.

Demineralised sections have also been used in grafted sockets (Araujo *et al*., 2010). The researchers have identified osteoclast cells by staining the demineralised slides for tartrate-resistant acid phosphatase activity (TRAP), and osteoblasts by marking them with alkaline phosphatase and osteopontin.

1.5.2.3 Undemineralised sections

Undemineralised sections are prepared after embedding specimens in resin blocks. The blocks are then cut to size and grinded to create sections 40-100μm thick. These sections are used for histomorphometric analysis of the specimens. This type of analysis is a quantitative one, differentially calculating the percentages of the total area occupied by bone, residual graft material, connective tissue and bone marrow. The main advantages of this technique are its relative simplicity and clinical relevance.

The easiest and most commonly used technique to quantify the tissue composition of histological specimens is by light-point counting. A matrix with 100 light points is superimposed over the region of interest (ROI). By counting the total number of points that fall on a specific type of tissue is the percentage of the ROI occupied by this tissue. Originally this technique was described by Schroeder and Münzel-Pedrazzoli in 1973, but has since been further modified, allowing the measurments to be done both manually or with the use of imaging analysis software (Schroeder and Munzel-Pedrazzoli, 1973).

Selection of the ROI for performing the measurements or calculations is a debatable subject, and no consensus was reached between the research groups. Cardaropoli *et al*. (2003) did their measurements in three zones (coronal, central and apical) of the

extraction sockets. Others (Hong *et al*., 2014) have performed histomorphometrical analysis on both the whole socket area and its vertical thirds (using image analysis software). The reproducibility and the significance in choosing ROI for a certain study is questionable. However, from the perspective of clinical significance and bearing in mind that: a) ARP is mainly performed for subsequent implant placement; b) the extraction socket's cross-section narrows as we advance apically; and c) peri-implant disease advances from the coronal portion apically – we may assume that the most apical content of the preserved extraction socket will be drilled out during the implant bed preparation, and therefore the most important area to evaluate the differential grafted socket composition would be the coronal one.

1.6 Aim of study

The aim of this study was to compare four novel and commercially available equine collagen based products against a commonly used xenograft control for ARP in a novel sheep mandibular extraction socket model with standardised buccal defect.

As there was little information about the behavior of the tested products and also the modified animal model, this study did not employ hypothesis testing. Instead, two research questions were considered:

1. How do the four novel BRG products behave in a tooth extraction model, when compared with a commonly-used xenograft or non-grafted tooth sockets?
2. What occurs in the modified tooth socket model at a single time point after grafting with bone replacement grafts.

Chapter 2 - Materials and methods

In this section we will describe the materials used for grafting the tooth extraction sockets, the surgical procedures undertaken, and the methods used to prepare and analyse the histological specimen. The Otago Animal Ethics Committee approved the study under protocol number AEC 78-14.

2.1 Experimental animals

Eleven crossbred adult ewes 3-4 years of age were used for this study, provided by the AgResearch Invermay Breeding Station. The selected sheep were required to have a weight above 70kg, as underweight animals have a slower recovery after surgical procedures. The animals were screened to exclude footrot, and their dentition was verified to be intact, suggesting a healthy periodontium. The animals selected for the study were individually tagged, and after treatment to control parasites and necessary immunisations, were released to a secure pasture for a few days before the surgery.

The surgical procedures for this study were performed in the Invermay AgResearch facilities, Mosgiel, New Zealand. The study animals were held in a separate paddock 48-72 hours prior to the surgery and were not allowed oral intake of food and fluids for 24 hours prior to their being scheduled for general anaesthesia. Some of the grafting materials that were tested in our study were also used by another surgical team in the same group of experimental animals for sinus augmentation procedures. The Otago Animal Ethics Committee approved multiple sites surgery. The results from the study of sinus floor elevation will be reported elsewhere.

An earlier published study (Liu *et al*., 2015) used a similar experimental model, but was unable to reach statistical significance of the results using groups of eight sheep. In the current study 10 sheep were included in the study group in order to increase its statistical power. Each animal received six treatment modalities: a negative control site with a non-grafted defect (CON); a positive control site grafted with bovine-derived xenograft and porcine collagen membrane (BX); and four test sites grafted with combinations of equine collagen products: membrane (CM), cone with/without 60%

hydroxyapatite/40% tricalcium phosphate (CO and CC respectively), and cone with integrated membrane ("Sombrero" concept, CS). For each experimental animal, the treatment modalities were decided using Latin-square allocation: CON, BX, CC, CS, CO, CO+CM.

The study design is summarised in Figure 2.1 and detailed further in Section 2.3. Details of the distribution of the experimental treatments are shown in Figure 2.2 and Table 2.1.

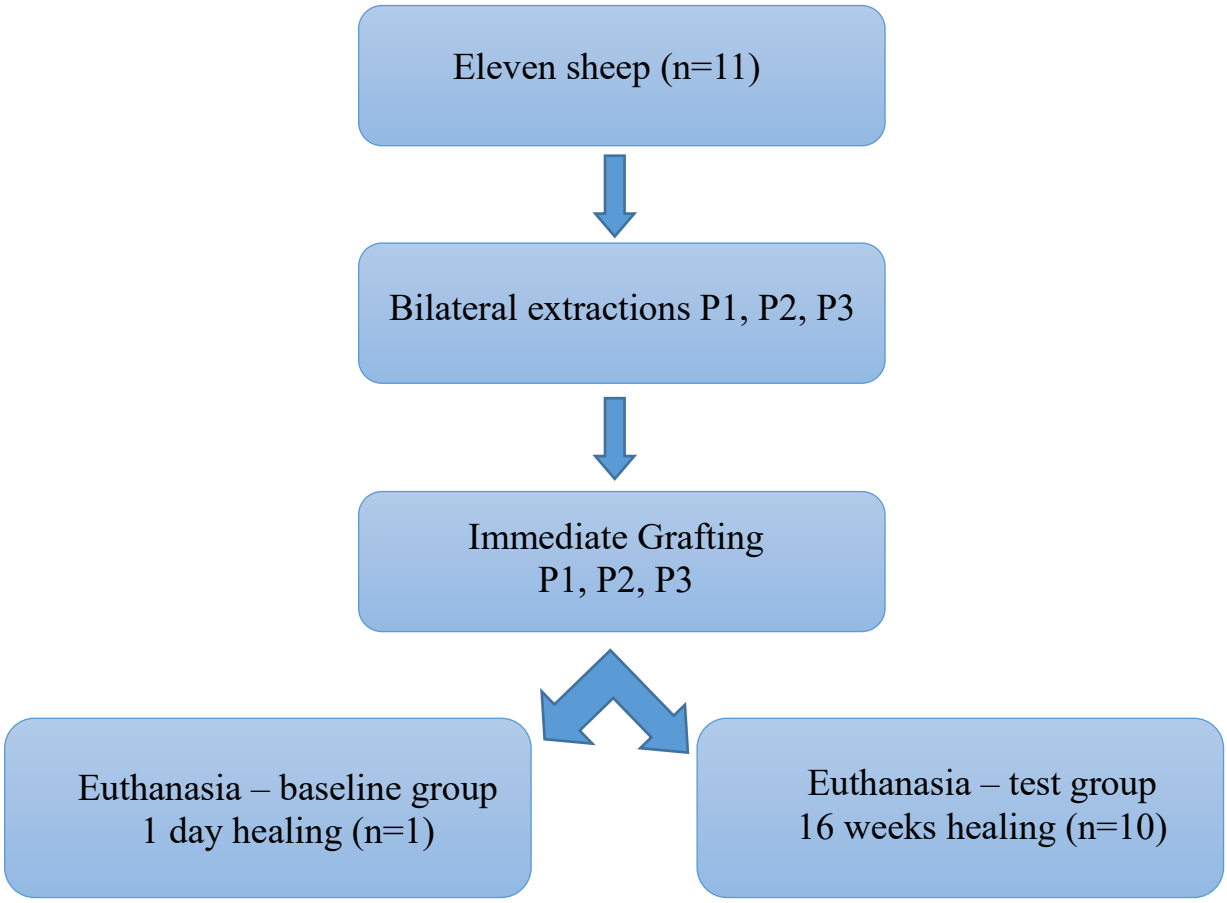

Figure 2.1. Flowchart of study design.

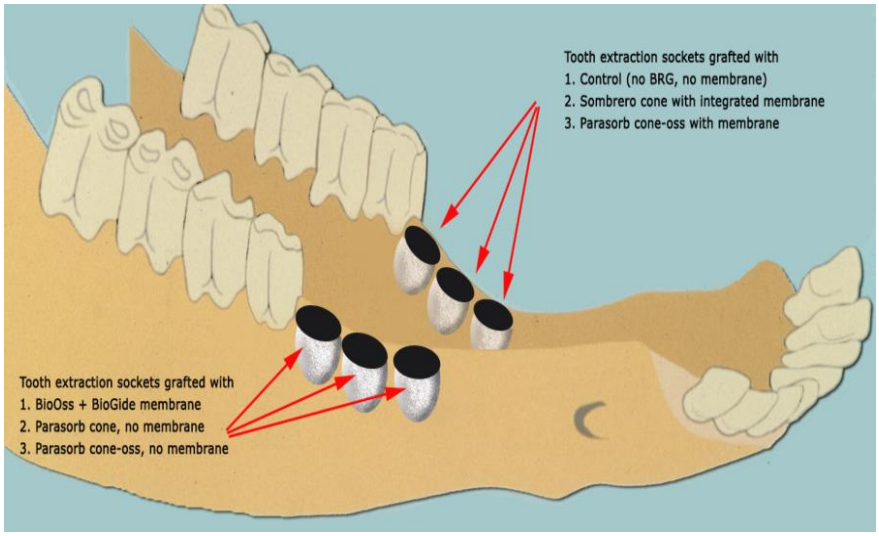

Figure 2.2. Graphic representation of a sheep mandible, with allocation of experimental sites.

Table 2.1 Latin square allocation of experimental sites.

No.	sheep	weight	L P1	L P2	L P3	R P1	R P2	R P3
1	412	80	A	B	C	D	E	F
2	413	80.5	F	A	B	C	D	E
3	414	78	E	F	A	B	C	D
4	415	83	D	E	F	A	B	C
5	416	72	C	D	E	F	A	B
6	417	88	B	C	D	E	F	A
7	418	78	A	B	C	D	E	F
8	419	70	F	A	B	C	D	E
9	420	95.5	E	F	A	B	C	D
10	421	88	D	E	F	A	B	C
11	409	91	C	D	E	F	A	B

LP1 – First Left Premolar

LP2 – Second Left Premolar

LP3 – Third Left Premolar

RP1 – First Right Premolar

RP2 – Second Right Premolar

RP3 – Third Right Premolar

Key	Socket grafting	Graft	Membrane	Study group
A –	Control site - no graft & no membrane	CONTROL	NONE	CON
B –	PARASORB Sombrero (collagen cone, 16mm high x Ø12mm, with integrated collagen membrane)	SOMBRERO®	INTEGRATED	CS
C –	Cone-Oss (new material, 15mm x ,12mm collagen cone containing bone granules) and & Resodont Forte RD2502 equine collagen membrane 25 x 25mm	CONE-OSS®	RES FORTE®	CO
D –	Geistlich BioOss-Collagenl with Biogide 25/25mm porcine collage membrane	BIOOSS-COLLAGEN®	BIOGIDE®	BX
E –	PARASORB Cone (collagen cone, 16mm high x Ø12mm) (no membrane)	CONE®	NONE	CC
F –	Cone-Oss (no membrane)	CONE-OSS®	NONE	CO+CM

2.2 Surgical and grafting materials

2.2.1 **PARASORB Cone®** (Resorba Wundversorgung GmbH, Nürnberg, Germany) (Figure 2.3a)

PARASORB Cone® is a commercially available resorbable type 1 collagen cone. One cone, sized Ø 1,2cm, height 1,6cm, contains 22.4mg of native equine collagen fibrils. The labelled uses of the product are haemostasis after an extraction, and ARP. It is not clearly stated whether it is recommended to cover it with a membrane for the latter indication.

A new package containing one cone was used for each animal in the allocated grafted sites.

2.2.2 **PARASORB Resodont®** (Resorba Wundversorgung GmbH, Nürnberg, Germany) (Figure 2.3b)

PARASORB Resodont® is a commercially available resorbable type 1 collagen membrane. The collagen used is of equine origin, and thus does not give rise to any ethical/religious issues. Resodont collagen membrane contains 2.8mg native collagen fibrils per 1cm². This product is manufactured according to a unique protocol (complete reconstitution of collagen). This membrane has been compared *in vitro* against other membranes, both resorbable (including Bio-Gide) and non-resorbable, showing positive results (Kasaj *et al*., 2008; Naujoks *et al*., 2013). According to the manufacturer, unlike Bio-Gide, this membrane can be used on both sides.

A new package of 22x25mm of PARASORB Resodont® was opened for each animal and trimmed to the size of the defect prior to placement over sites grafted with PARASORB Cone-Oss®.

2.2.3 **PARASORB Sombrero®** (Resorba Wundversorgung GmbH, Nürnberg, Germany) (Figure 2.3c)

PARASORB Sombrero® is a novel and commercially available hybrid product that combines a collagen cone and a non-separable absorbable equine type 1 collagen membrane in a single product. This hybrid product allows easier and less demanding handling properties. The collagen used in this product is equine type 1 collagen (31.2mg) without chemical additives or cross-linking agents.

A new package of PARASORB Sombrero®, was used for each animal in the allocated grafted sites.

Figure 2.3. **Equine collagen cones and membranes.** (Courtesy of Resorba)

a. PARASORB Cone®

b. PARASORB Resodont®

c. PARASORB Sombrero®.

2.2.4 **PARASORB Cone-Oss®** (Resorba Wundversorgung GmbH, Nürnberg, Germany)

Cone-Oss is a novel and not yet commercially available product consisting of an equine collagen cone staggered with biphasic calcium phosphate. The biphasic calcium phosphate used for this product is a combination of 60% hydroxyapatite (HA) and 40%

beta tricalcium phosphate (ß-TCP). The ß-TCP portion is resorbable, while the HA is planned to remain embedded in bone as a stable scaffold for long-term volume preservation (by offering a “regenerative room”). The concept of the product resembles that of Bio-Oss Collagen, however, there are a few major differences.

In Cone-Oss, the main part is the collagen, which is expected to be absorbed within two months by cell absorption, while biphasic calcium phosphate will only be partly absorbed (its ß-TCP portion) within six months by hydrolysis.

The composition of Cone-Oss® is demonstrated in a cross-section in Figure 2.4. The collagen component can be seen in “white”, while the collagen-BCP phase is stained with methylene blue (for demonstration purposes only). It is evident that there is a concentration gradient between the two phases. It is also visible that the periphery and the apical portion of the Cone-Oss® are compressible to allow better adaptation to various extraction socket sizes. The collagen content also offers primary haemostasis properties that are required for the subsequent healing process.

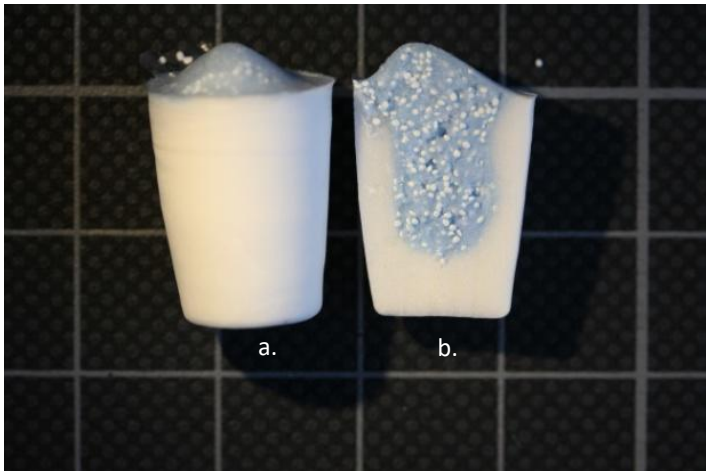

Figure 2.4. Composition of Cone-Oss®. (Courtesy of Resorba)

a. Whole cone

b. Cross-section of cone.

According to the manufacturer, in its next generation, Cone-Oss will be offered attached to a membrane, similar to PARASORB Sombrero. Hence, it could be used for both filling and covering of the defect, to provide true GBR.

A new package of PARASORB Cone-Oss® was used for each animal in the allocated grafted sites, covered with a single layer of PARASORB Resodont® membrane.

2.2.5 **BioGide®** (Geistlich Pharma AG, Wolhusen, Switzerland) (Figure 2.5a)

Geistlich Bio-Gide® is a widely used resorbable porcine collagen membrane. The membrane is produced from natural collagen without chemical additives or further cross-linking. Bio-Gide has a bilayer structure with one side being smooth and placed towards the flap, and the other being rough which faces the grafted site.

In our study, Bio-Gide® membranes were placed in a single layer over the extraction sockets grafted with Bio-Oss Collagen®. A new package of 25x25mm of BioGide® was opened for each animal and trimmed to the size of the defect prior to placement.

2.2.6 **Bio-Oss Collagen®** (Geistlich Pharma AG, Wolhusen, Switzerland) (Figure 2.5b)

Geistlich Bio-Oss® is a natural bovine bone mineral. This product has been intensely studied and clinically used. A simple search in PubMed for “Bio-Oss” yields almost 900 results, and this number will probably increase by the time our research is published. Therefore, some currently consider this material the gold standard of bone grafting (Schneider *et al*., 2009), and many other studies use it as a positive control while testing other bone grafting materials.

Bio-Oss Collagen is marketed by Geistlich as the next generation of Bio-Oss for various applications. It is comprised of 90% Bio-Oss granules (250-1000μm) embedded in 10% porcine collagen. This allows for better handling properties of the product. According to the manufacturer, the collagen content of the product is resorbed within a few weeks and does not have the barrier functions of a membrane, and therefore Bio-Gide membranes are recommended for use in ARP procedures.

Nevertheless, an off-label use of Bio-Oss Collagen without a membrane has shown positive results in a dog model (Araujo *et al*., 2008; Araujo and Lindhe, 2009b).

Commercial 500mg Bio-Oss Collagen® packages, one for each animal, were used in the allocated grafted sites.

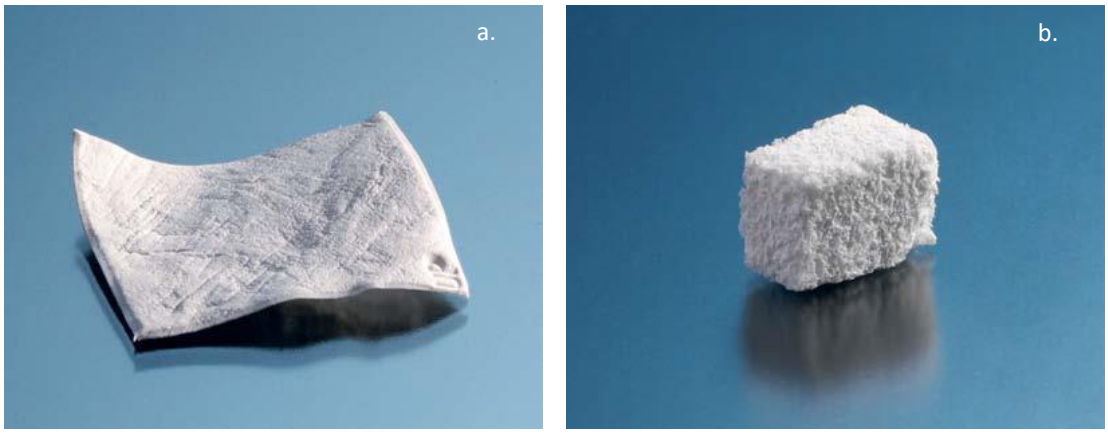

Figure 2.5. Grafting materials in control group. (Courtesy of Geistlich)

a. Bio-Gide®
b. Bio-Oss Collagen®.

2.3 Surgical protocols

All surgical procedures for this study were performed in the Invermay AgResearch facilities, Mosgiel, New Zealand. A designated operating theatre for large animal surgery was used. Sterile operating techniques for survival animal surgery were adopted as a standard for each surgical procedure.

2.3.1 General anaesthesia

Preoperatively, all experimental animals were pre-medicated with antibiotics (Trimethoprim, Amphoprim injection 1ml/15kg, Virbac New Zealand Ltd., East Tamaki, Auckland). General anaesthesia was induced by means of intravenous infusion of Thiopentone 20mg/kg (Bomac Laboratories Ltd., Manukau City, Auckland). After placement of the sheep on a mobile operating table, an endotracheal tube was inserted via oral route and secured to prevent displacement. Anaesthesia was maintained with 1-2% Halothane and Nitrous oxide/oxygen in a 1:2 ratio throughout the procedure. A gastric tube was placed to decompress the stomach, and the gastric content was left to drain freely into a container placed beneath the surgical table. A pulse oximeter was used to monitor the vital signs of the experimental animal during the surgical procedure.

2.3.2 Surgical sites preparation

After rotating the head of the sheep onto either the left or the right side, the mouth and the nose of the sheep were cleaned and disinfected with Betadine® solution (Alcon Laboratories, Inc. Rockville Pike, Bethesda, MD, USA). Sterile draping was used to cover the animal except for the oral cavity. The oral cavity was cleaned with sterile gauze soaked with 0.2% chlorhexidine gluconate solution (Savacol®; Colgate-Palmolive, New Zealand).

Mandibular premolars sites were selected for the grafting procedures, as their roots have a similar morphology compared to those of human teeth, and are the only teeth accessible for extraction without compromising the postoperative wellbeing of the experimental animal, since access to the molar area would require a cheek incision.

Local anaesthesia was administered by both buccal and lingual infiltrations around mandibular premolars using one cartridge of Mepivacaine HCl 2% with adrenaline

1:100,000 dental anaesthetic (Scandonest, Septodont, Ivoclar Vivadent Ltd., Auckland New Zealand). The additional desirable effect of the adrenaline contained in the local anaesthetic solution, is the relative control of hemorrhage from the surgical site due to its vasoconstrictive properties.

2.3.3 Tooth extraction protocol

A full thickness mucoperiosteal flap was elevated both bucally and lingually from the first mandibular molar extending anteriorly to approximately 2cm mesial to the first mandibular premolar using nos. 15 and 12 scalpel blades, respectively.

Marker notches were prepared in the cortical bone on the buccal aspect of the mandible mesial to the first premolar using a no. 8 round stainless steel bur at 1200rpm and saline coolant irrigation. The notches were filled with amalgam, to serve as a radio-opaque marker, and concavities were carved in the amalgam so that the notches would be identifiable on the impressions.

A minimally traumatic approach was adopted for the extraction of the mandibular premolars. Access to the contact area between the teeth was gained by means of dental elevators gently tapped on with a surgical mallet. Molar extraction forceps together with Coupland's and Cryer's elevators were used to mesially luxate and extract the premolars (P1-P3). Special care was taken not to damage the cortical plates, inter-radicular bone and to avoid root fractures (Figure 2.6).

Following the extractions, the sockets were curetted and baseline periapical radiographs were taken using a Rinn® holder in a paralleling technique to verify complete removal of the roots: non-standardised intra-oral radiographs were taken to facilitate descriptive analysis of the extractions sites prior to grafting and after healing.

Prior to surgery, a pre-existing sheep mandible was used to prefabricate custom trays suitable for this animal model. Impressions of the extraction sockets and the mandibular bone were taken with custom made trays and heavy and light bodied polyvinyl-siloxane impression material (Espress®, 3M ESPE, St. Paul MN, USA), to explore a possibility of tracking changes in bone volume (Figure 2.7).

2.3.4 Grafting sites preparation

Based on the availability of interdental bone, three root sockets on each side were selected for grafting.

The depth of the selected sockets was measured with a Biomet 3i Implant depth gauge (Biomet 3i, North Ryde NSW Australia; catalogue number DP020) to the closest 0.5mm. Whenever possible, the measurements were taken in the mesial root socket of each premolar.

The size of the sockets varied between the sites and between the animals, therefore the sockets were standardised using implant burs to at least 12mm deep x 5.2mm wide at their coronal portion. In cases where sockets were less than these dimensions, Southern Implants implant drills (Southern Implants, Irene, South Africa) were used to deepen and widen the site, successively twist drills 2mm (catalogue number D-20T-M15) and 3mm (catalogue number D-30T-M15) followed by a tapered dense bone drill measuring 5.2mm at the coronal portion and 13mm long (catalogue number D-52TP-13).

Dehiscence defects 5mm long and 2mm wide were created in the middle of the buccal wall of the selected sockets, using stainless steel burs at 1200rpm and saline as coolant. The defect walls were smoothed using a Mectron® Piezosurgery 2 Ultrasonic Unit (Henry Schein Shalfoon, Auckland NZ) with a diamond osteotomy insert (catalogue number OT1).

After creation of the defects, the width of the ridge at the crestal level was measured just mesially to each defect using a surgical Boley Guage calliper (Salvin Dental Specialties, Inc., Charlotte, NC U.S.A). The measurements were rounded to the closest 0.5mm (Figure 2.8).

2.3.5 Grafting the extractions sites

Each sheep received all six treatment modalities (one per site): a negative control site with a non-grafted defect (CON); a positive control site grafted with bovine-derived xenograft and porcine collagen membrane (BX); and four test sites grafted with combinations of equine collagen products: membrane (CM), cone with/without 60%

hydroxyapatite/40% tricalcium phosphate (CC, CO), and a cone with integrated membrane ("Sombrero" concept, CS).

The allocation of a treatment modality for each extraction socket with a standardised buccal dehiscence defect was done according to the randomisation table, using Latin-square allocation: CON, BX, CC, CS, CO, CO+CM.

Each experimental site was grafted with the appropriate grafting material to the level of the cortical bone, and when required, a membrane was placed over the graft.

Buccal and lingual mucoperiosteal flaps were sutured using resorbable 3/0 Polyglycolic Acid sutures (PGA Resorba®, catalogue number PA1117, Resorba Wundversorgung GmbH & Co., Nürnberg Germany) and the flaps elevated coronally to achieve primary closure (Figure 2.9).

A postoperative periapical radiograph was taken using a similar technique.

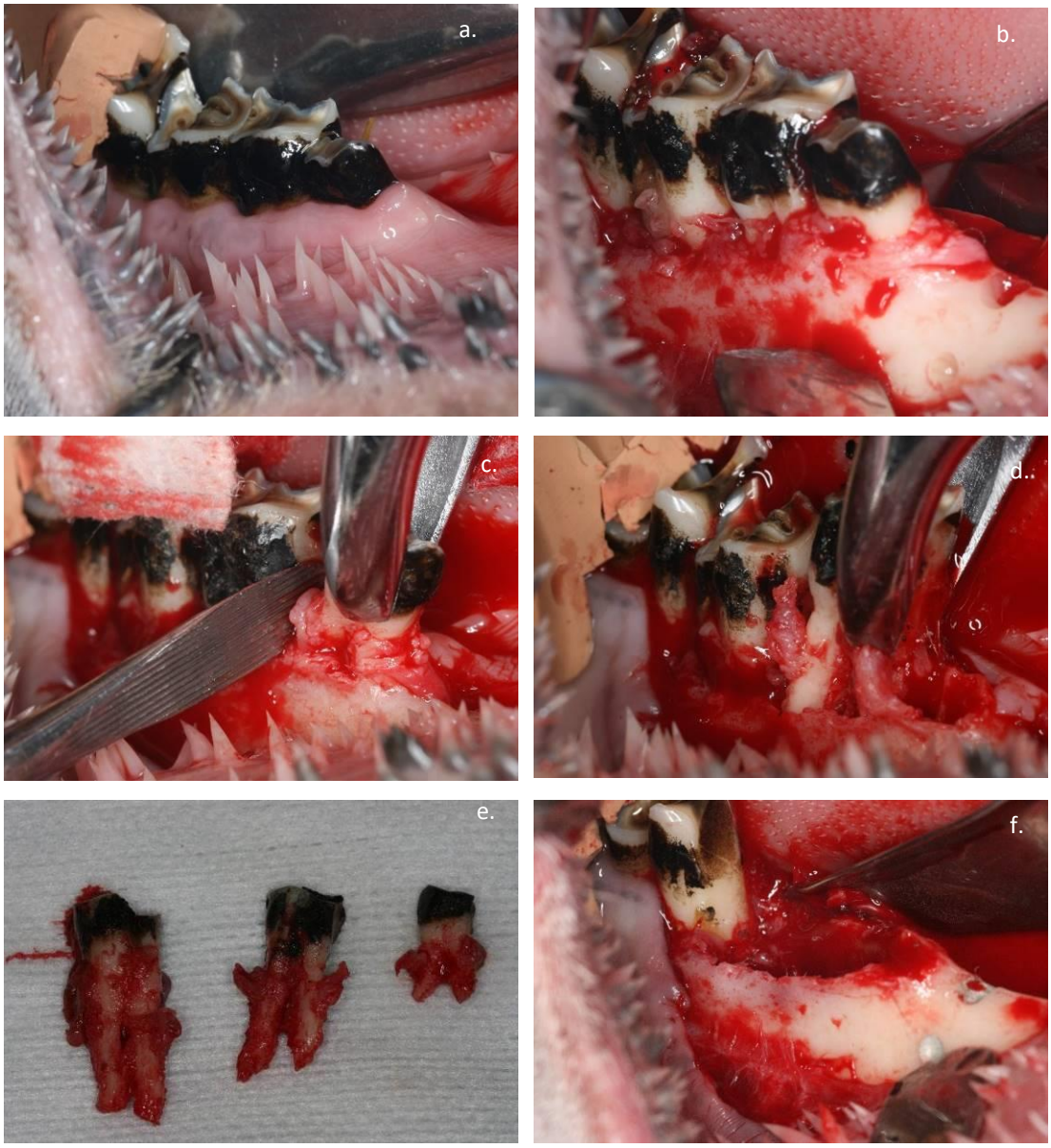

Figure 2.6. Extraction of mandibular premolars.

a. Preoperative photograph
b. Full thickness flap elevated bucally and lingually
c. Elevation of first mandibular premolar
d. Extraction of second mandibular premolar
e. Intact extracted mandibular premolars
f. Exposed edentulous ridge with amalgam markers.

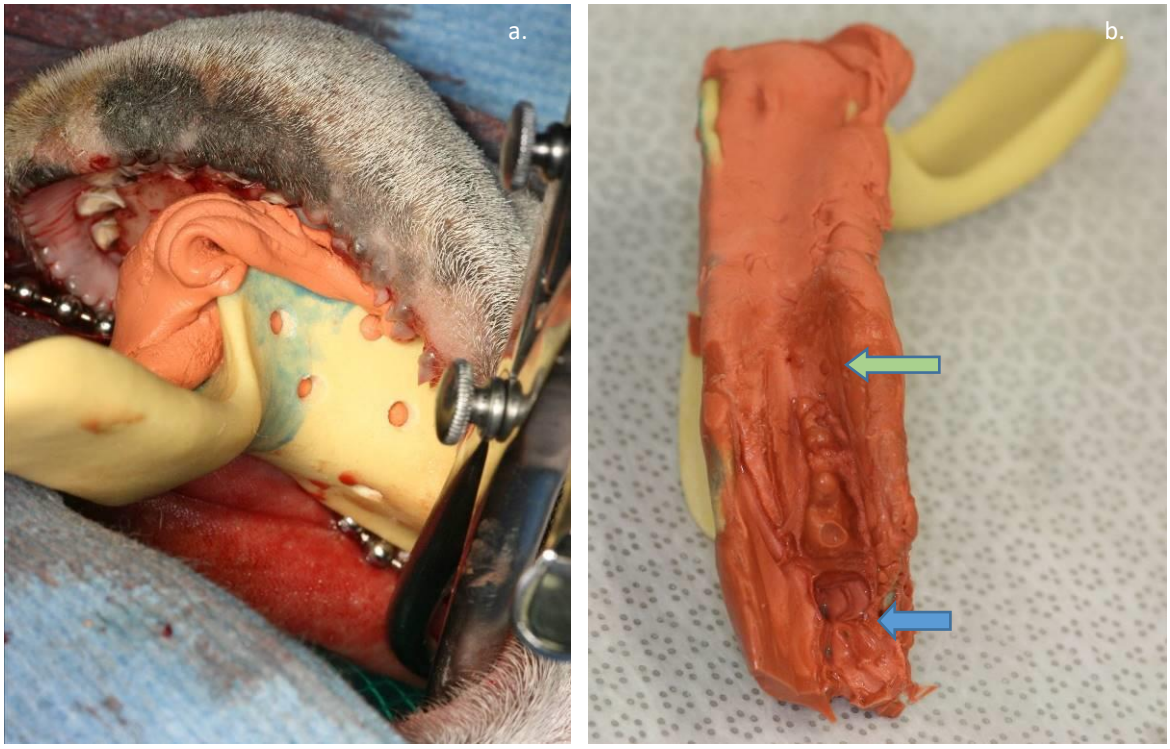

Figure 2.7. Impressions of the extraction sites.

a. Impression taking with custom tray and polyvinyl-siloxane

b. Impression, from bottom to top: 1st molar, extraction sockets, amalgam markers, edentulous diastema.

First molar

Amalgam markers

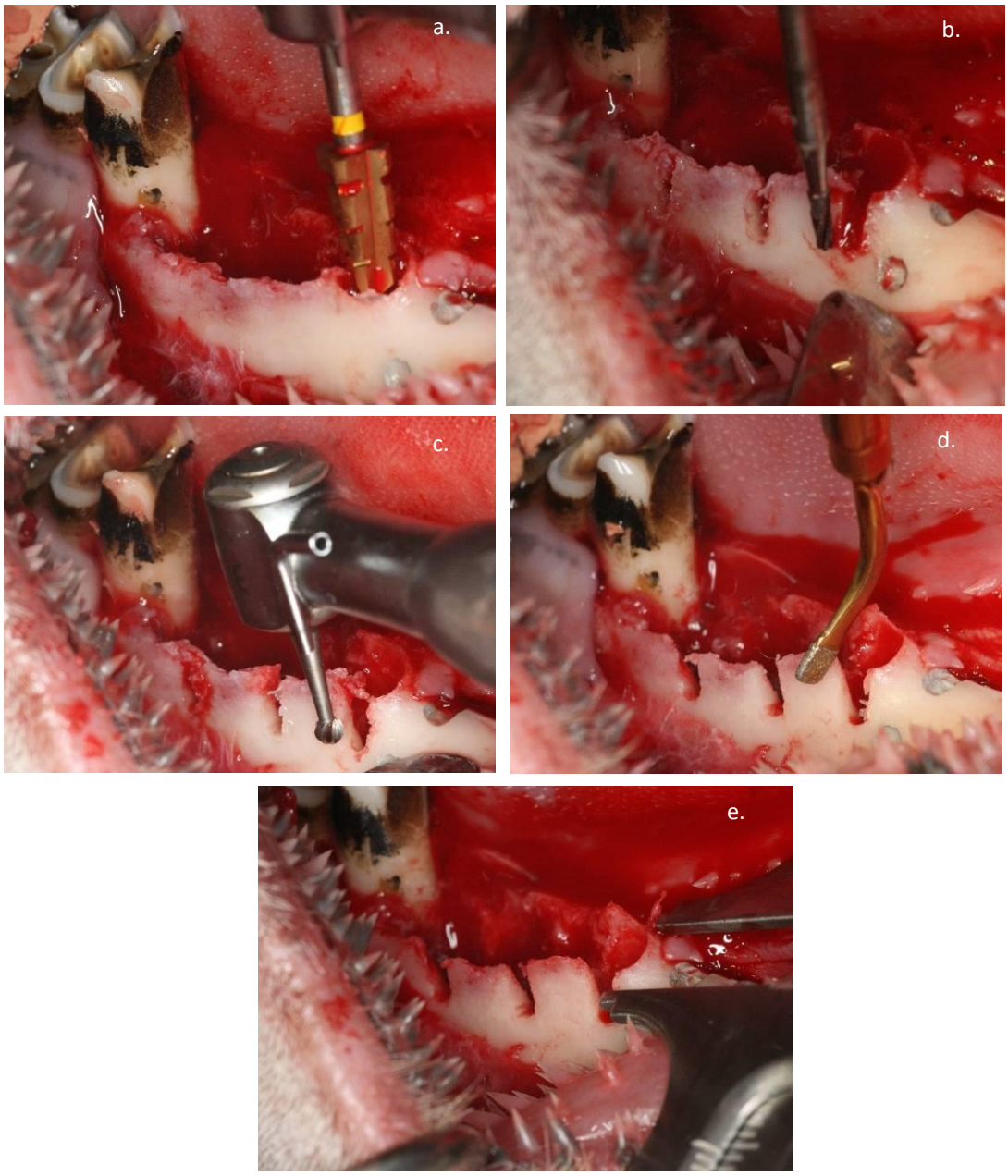

Figure 2.8. Site preparation.

a. Standardisation of grafting site depth
b. Preparation of buccal dehiscence defect – 5mm length
c. Preparation of buccal dehiscence defect – 2mm width
d. Smoothing of buccal defect walls with piezotome
e. Measuring the alveolar ridge width mesially to the created defect.

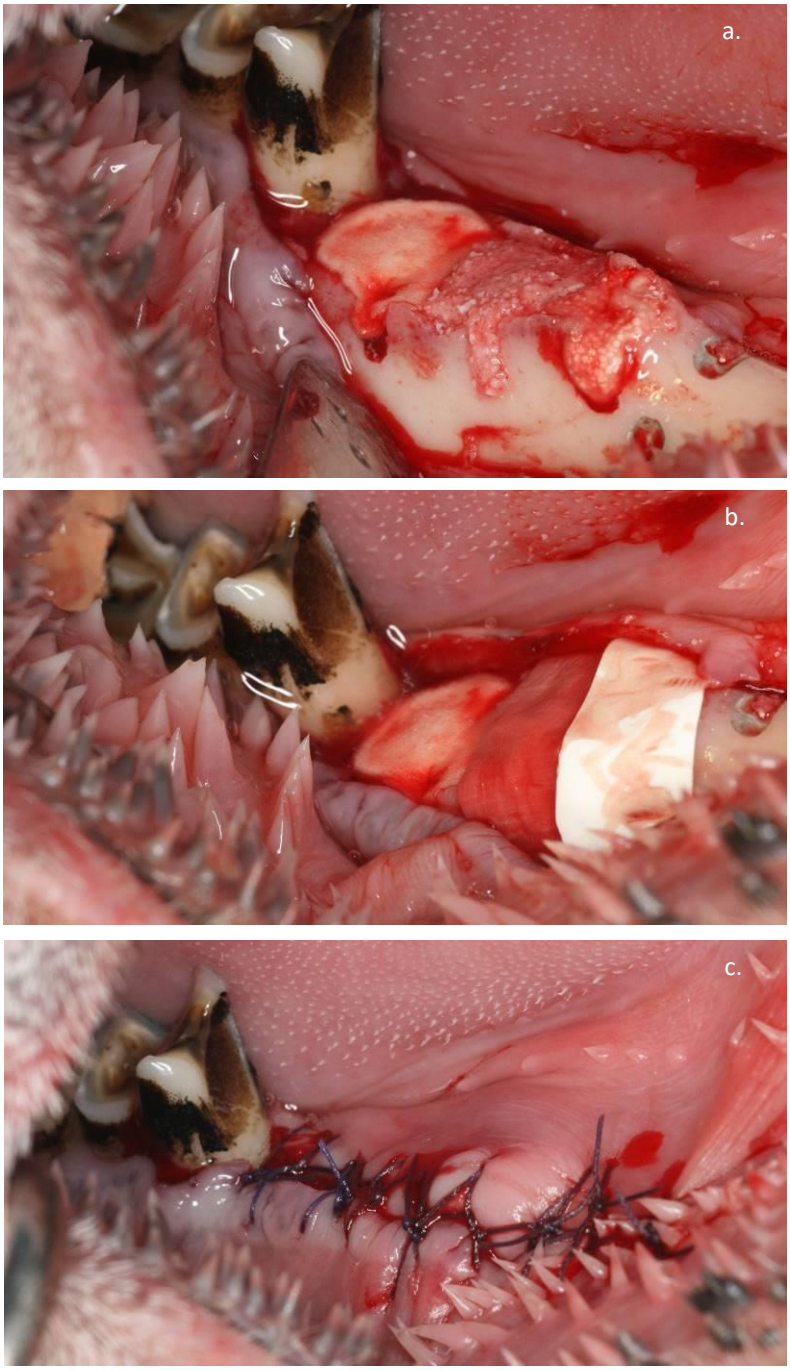

Figure 2.9. Site grafting.

a. From left to right – Sombrero®, Bio-Oss Collagen®, Cone-Oss®
b. Bio-Oss Collagen® covered with Bio-Gide®
   Cone-Oss® covered with Resodont®
c. Primary closure.

2.3.6 Postoperative management

Immediately after wound closure, 1ml of Bupivacaine hydrochloride 0.5% with adrenaline 1:200,000 long-acting local anaesthetic was administered by infiltration (Marcaine; AstraZeneca, North Ryde, Australia) for postoperative pain-relief.

Following extubation, the experimental animal was moved to a separate area for a few hours to allow for undisturbed recovery from anaesthesia, and was monitored by a veterinary technician.

For three days following the surgery, the sheep were kept in a designated paddock, where they were monitored and received postoperative regimen. Each animal had its mouth rinsed daily with 30ml of 0.2% w/v chlorhexidine gluconate solution (Savacol®; Colgate-Palmolive, NZ), as well as daily subcutaneous injections of anti-inflammatory medications (5ml Carprofen, Rimadyl® injection 50mg/ml, Zoetis, Mt Eden, New Zealand) and antibiotics (Trimethoprim 1ml/15kg). After three days of postoperative care, the experimental animals were returned to pasture, and were allowed to graze freely for the duration of the healing period.

2.3.7 Euthanasia and harvesting of mandibular blocks

A healing period of 16 weeks was chosen, based on a study published by our institution that used a similar animal model (Liu *et al*., 2015). This is equivalent to 21 weeks of healing in human subjects (Duncan 2005), an average period of healing following ARP prior to placement of a dental implant. Following this period, the experimental animals were again brought to the AgResearch Invermay facilities to be euthanised.

General anaesthesia was administered in a similar way to the initial surgical procedure. The delivery of anaethesia reduced the animal to a level of consciousness that was non-survivable. The sheep was placed on an operating table in a Trendelenburg position (supine position, with the legs being higher than the head) with the neck overextended. Using the sternocleidomastoid muscle as a guide on each side, the skin was incised, and the external carotid arteries were exposed by blunt dissection. Each artery was then cannulated with a 14G canula (Optiva TM, Smiths Medical, UK). The cannula was

stabilised with silk sutures both at the proximal and distal ends, to prevent its displacement and back-flow of the perfusing solution, respectively (Figure 2.10).

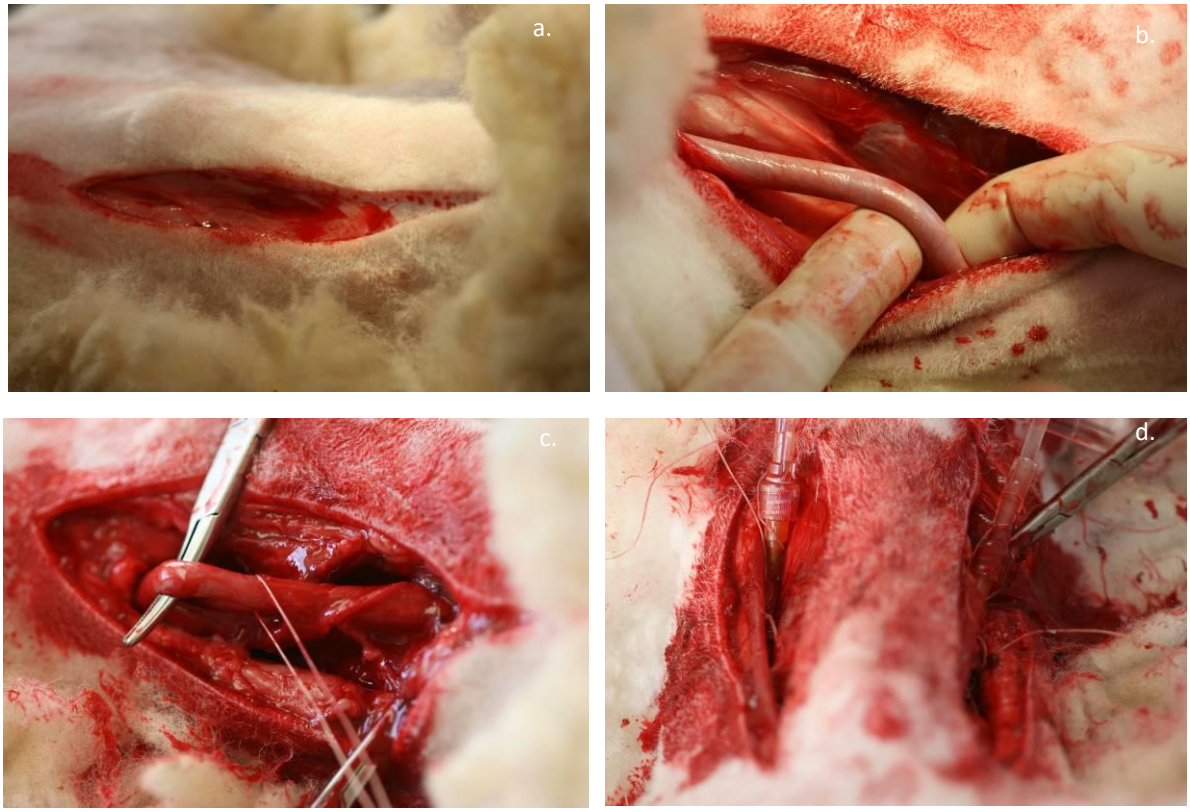

Figure 2.10. Perfusion protocol for euthanasia.

a. Neck dissection
b. Carotid artery identified
c. Preparation of ligatures prior to cannulation
d. Bilateral cannulation and ligation of the carotid arteries.

Each cannulated carotid artery was connected to bag containing 1L of normal saline (0.9% Sodium chloride, Baxter Healthcare Ply Ltd., NSW Australia) and 1.5ml of 5000IU heparin. The jugular veins were severed bilaterally with a scalpel blade, and the heparinised blood was allowed to drain into a container at floor level. Immediately after heparinisation, the carotid arteries were perfused with 1L chilled formaline fixative per side, using 10% neutral buffered formalin (NBF) (BioLab Ltd., New Zealand).

The mandibular specimens, containing the operated sites and some molar teeth, were retrieved by en-bloc resections, rinsed with water, and individually placed into sealed and labelled plastic containers, filled with 10% NBF for further processing.

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2.4 Specimen preparation

2.4.1 Specimen sectioning

The harvested mandibular blocks were radiographed in order to identify the surgical sites. For comparison with radiographs taken during the grafting, each block was placed onto an E-speed intra-oral Kodak dental film (Carestream Health, NY, USA), and exposed at a focal length of 38cm for 0.16 seconds using a Gendex Dental Systems machine (Monza, Italy). The films were later developed in an automatic processing machine.

The obtained radiographs were superimposed with postoperative radiographs on the same side, using molar teeth and amalgam pins (where available) as location markers. The borders between adjacent grafted sites were identified, and marked on the post-mortem radiographs. These markings were transferred onto the mandibular blocks to guide the sectioning (Figure 2.11a). The blocks were then sectioned in a bucco-lingual direction along the markings into individual grafted sites using a manual coping saw (Spear and Jackson, England). Each specimen was placed in a double labelled histology cassette.

2.4.2 Resin-embedded specimens

In order to perform non-demineralised histology, the tissues must be resin-embedded. The protocol for resin-embedding was initially described in 1982 in a published study by Donath and Breuner. It was further modified and refined by Duncan in 2005. Later publications from our institution (Liu *et al*., 2015) followed the modified version by Duncan, which was fully adopted for our study, and is attached in Appendix II.

The cassettes, each containing one specimen were placed in a covered glass container. The tissue samples were dehydrated in ascending grades of ethanol (20%, 40%, 70%, 95% and 100% respectively). The ethanol was then replaced with xylene (Ajax Finechem Pty Ltd, New Zealand) for clearing the tissues. The glass container with the specimen immersed in xylene was transferred to a rotating surface in a fume hood for four days, with two changes of xylene during that period. The specimens were then placed in pure methyl methacrylate (MMA) (Sigma Aldrich, USA) for one day with one change.

Mixtures of MMA-I, MMA-II and MMA-III were prepared following the attached protocol. Individual small glass jars were filled with MMA-III to a depth of 8-10mm one week prior to the embedding process to allow the MMA-III base to set.

Following the wash with pure MMA, specimens were immersed in MMA-I and MMA-II for two days each, and then the specimens were removed from the cassettes and each placed into an individual glass jar with a pre-set MMA-III base. A small paper sheet bearing the specimen's identification was placed inside each jar. The jars were filled to the top with MMA-III, sealed with screw top lids and were then left to set for approximately three weeks in a water bath in order to dissipate the heat produced by an exothermic setting reaction.

When the setting process was completed, the glass jars were broken to retrieve the acrylic resin blocks containing the tissue specimens (Figure 2.11b-c). These blocks were trimmed down and polished to an approximate rectangular shape with at least 2-3mm of acrylic resin surrounding the embedded specimen (Figure 2.11d). This was in order to simplify the subsequent slicing of the specimen. The trimmed blocks were marked with the specimen's identification code, using a permanent marker.

2.4.3 Sectioning of resin-embedded tissue blocks

The sectioning of the acrylic resin blocks was done by a Struers Accutom precision table-top microtome (Ballerup, Denmark) fitted with a diamond cut-off wheel (MOD 13 127x0.4x12.7mm). The resin blocks were firmly attached, using cyanoacrylate glue (MDS Adhesive QX-4, MDS Products Inc, Laguna Hills, CA, USA), to a larger acrylic block that was pre-mounted onto the cut-off machine in order to streamline the sectioning process, as well as to prevent any damage to the sectioning disc against the metal mounting arm. Sequential sections 650μm thick were cut and press mounted onto an opaque acrylic slide using cyanoacrylate glue. Prior to mounting, the identification code of the original specimen along with the sequential section number were transferred to the "tissue side" of the slides with permanent marker and were also engraved on the reverse side using a round drill with a straight dental hand piece.

Between four and 10 slides were prepared from each specimen, dependent on the original thickness (mesio-distal width) of the tissue specimen. For each specimen, two bucco-lingual sections representing the central area of the grafted extraction socket

were selected for further processing. The rest of the slides were kept in reserve. The selected slides with originally 650μm thick sections were further reduced to the final thickness of 80-100μm, verified with a digital micrometer (Digital Indicator, Mitutoyo, Japan), and polished using a Tegra-Pol rotating grinding machine (Struers, Ballerup, Denmark) and Silicon Carbide Paper (grit size #180 to #4000).

2.4.4 Staining

The slides were immersed in 20% ethanol in an ultrasonic bath for five minutes for superficial etching, followed by another five minutes in 1% formic acid for surface decalcification of the mounted sections. The sections were then stained with a mixture of one part MacNeal's tetrachrome (methylene blue, azur II and methyl violet) and two parts toluidine blue. The staining protocol is described in further detail in Appendix II-4. The stained slides were then rinsed with distilled water and left to be air-dried overnight on a benchtop.

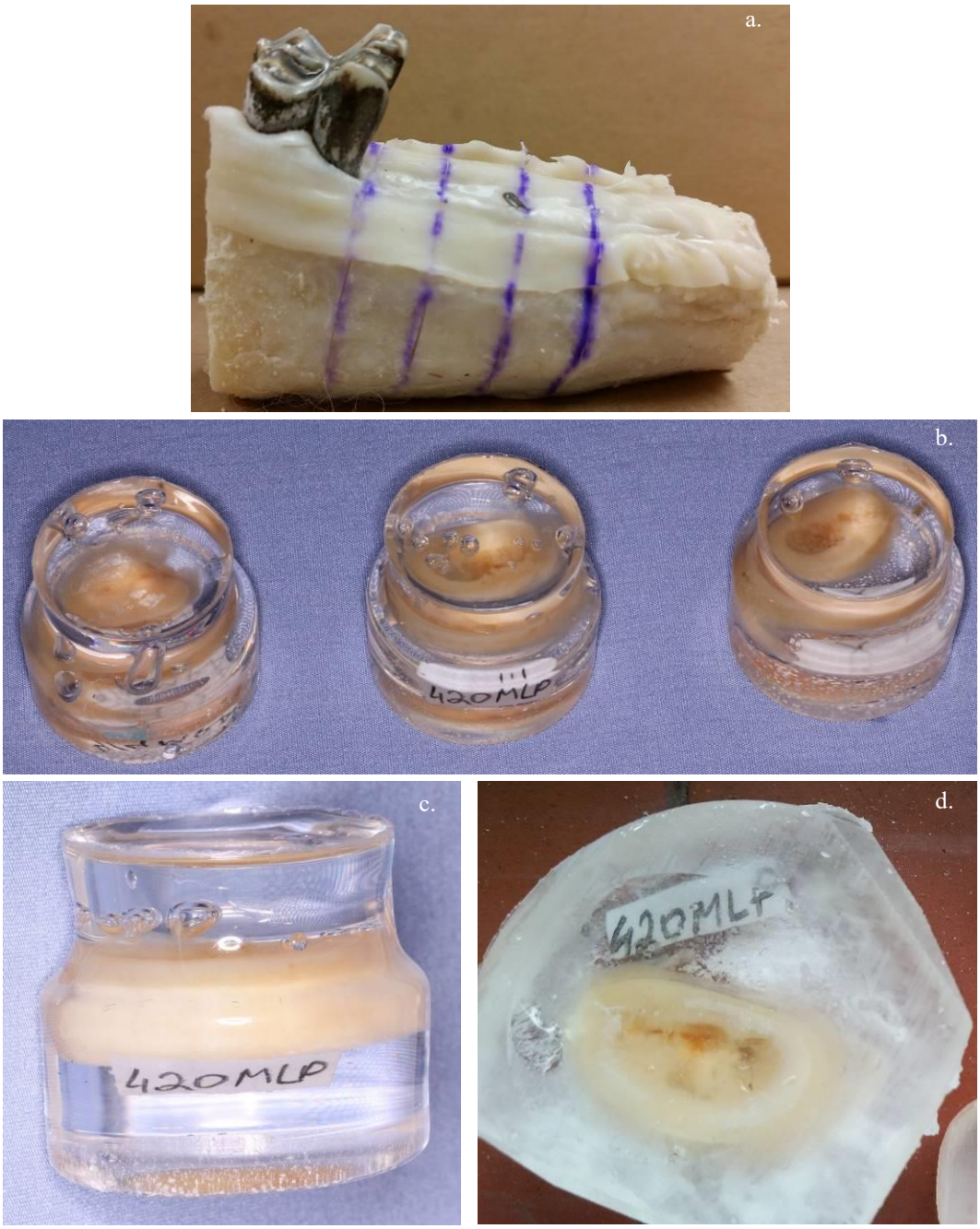


Figure 2.11. Preparation of mandibular specimen.

a. Mandibular block with marked grafted sites/segments
b. Each grafted site individually resin-embedded
c. Individual segment embedded in resin with identification marking
d. Individual segment with excess resin trimmed prior to histological sectioning.

2.5 Histological analysis

2.5.1 Imaging of histological sections

For histological analysis high resolution images were obtained using a confocal light microscope (Olympus AX70, Olympus Optical Co. Ltd, Japan) and an imaging system (Micropublisher 5.0 RTV, Qimaging) at 10x magnification. Using the Volocity 5.2.0 (Improvision, MA, USA) montaging software, each stained section was mapped into 200-400 (depending on the size of the entire scanned tissue) individual areas at 10x magnification with 10% overlap between the adjacent areas. A full series of individual images was automatically obtained and digitised for each slide. All the digital images related to a single slide were processed using Autopano Pro 2.5.2 (Kolor, USA) stitching software to produce a single panoramic image of the entire section, by automatically detecting the matching points in the overlapping areas of the individual images.

2.5.2 Histomorphometric analysis

2.5.2.1 Region of interest (ROI)

The newly formed bone was easily distinguished from the pre-existing bone by both the level of maturation and the intensity of the staining, with mature and well organised pre-existing bone stained at a significantly lighter level.

A 4x6mm rectangular ROI (Liu *et al*., 2015) was individually defined for each section using the following guidelines. Each scanned image was aligned so that the lingual cortex of the mandible would be vertical. The most coronal point of the pre-existing bone in the lingual cortical plate was identified. Since the lingual wall of the extraction socket was not altered during the surgical procedure, this was the most reproducible point for the histological measurements related to ARP. From that point, a horizontal line was drawn buccally, until it reached the most exterior part of the buccal wall along this plane. That point on the buccal cortex served as the bucco-coronal corner of the ROI. This definition of ROI served multiple purposes, the most important being reproducibility, balanced by minimal inclusion of pre-existing cortical bone in the ROI.

Furthermore, this ROI is highly representative of the area most likely to be chosen for installation of a dental implant. The coronal margin of the selected ROI was horizontally aligned with the most coronal point of the buccal wall. Effort was made to avoid the inclusion of 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 are most likely to be utilised for implant placement. The sheep mandible contains large areas of bone marrow space. The dimensions of the ROI were chosen in accordance with previous publications on a similar model, and are limited by the anatomy of the sheep mandible. A representative slide with ROI is shown in Figure 2.12.

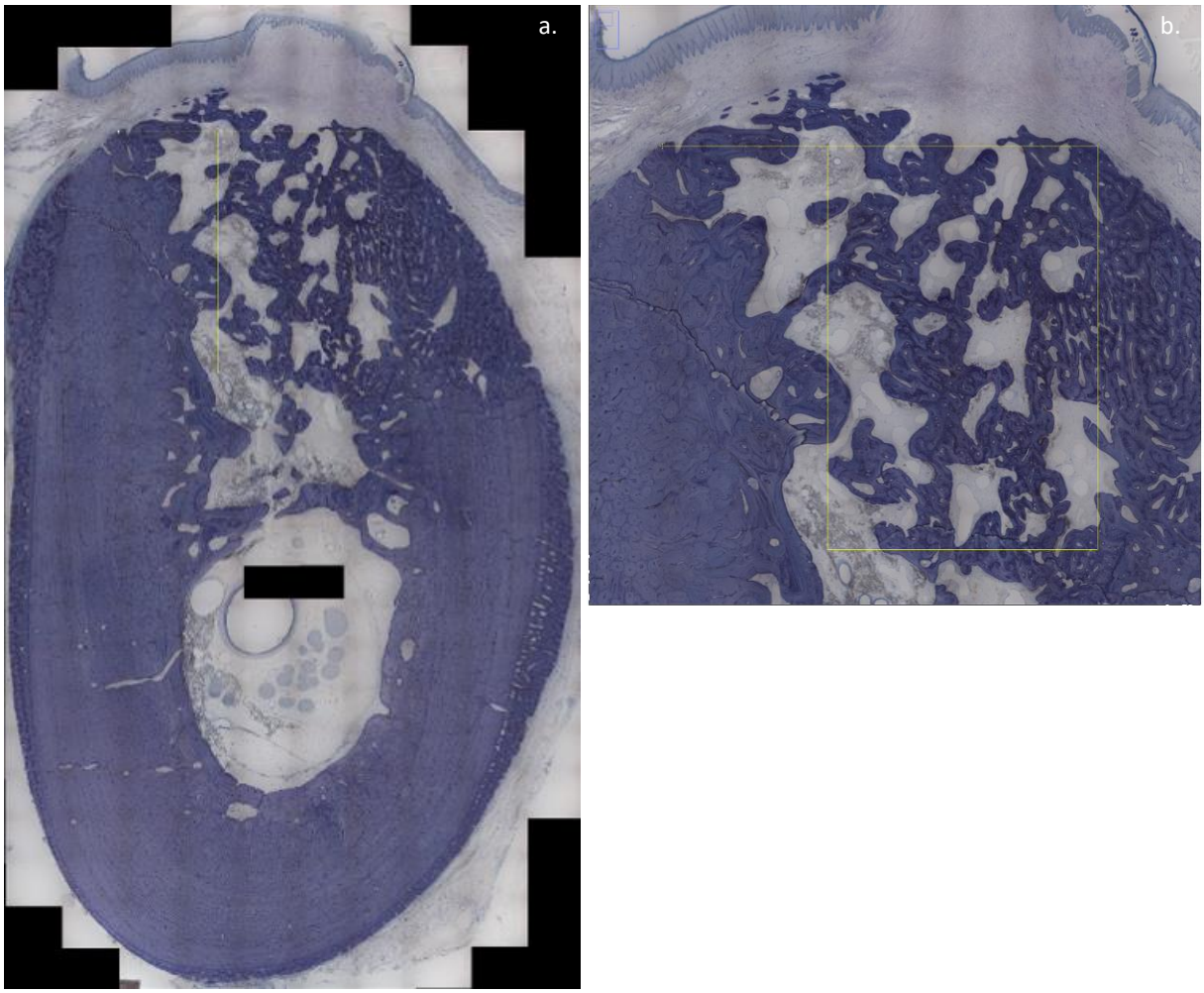


Figure 2.12. Representative slide with ROI 4mm wide and 6mm high (yellow).

a. Full-sized single section

b. Enlarged coronal area.

2.5.2.2 Histomorphometry

The percentage of area within the ROI occupied by hard tissue was determined for each slide. The measurements were done using a colour threshold plugin of image analysis computer software, ImageJ (version 1.50g, National Institute of Health, USA). By manually adjusting the colour thresholds, based on staining intensity of different tissues, area percentages of hard tissues within the ROI were calculated (Figure 2.13).

Each experimental site was represented by two bucco-lingual sections acquired from the centre of the extraction socket. Therefore, the average of the two measurements from each section was displayed as the mean for each treatment modality.

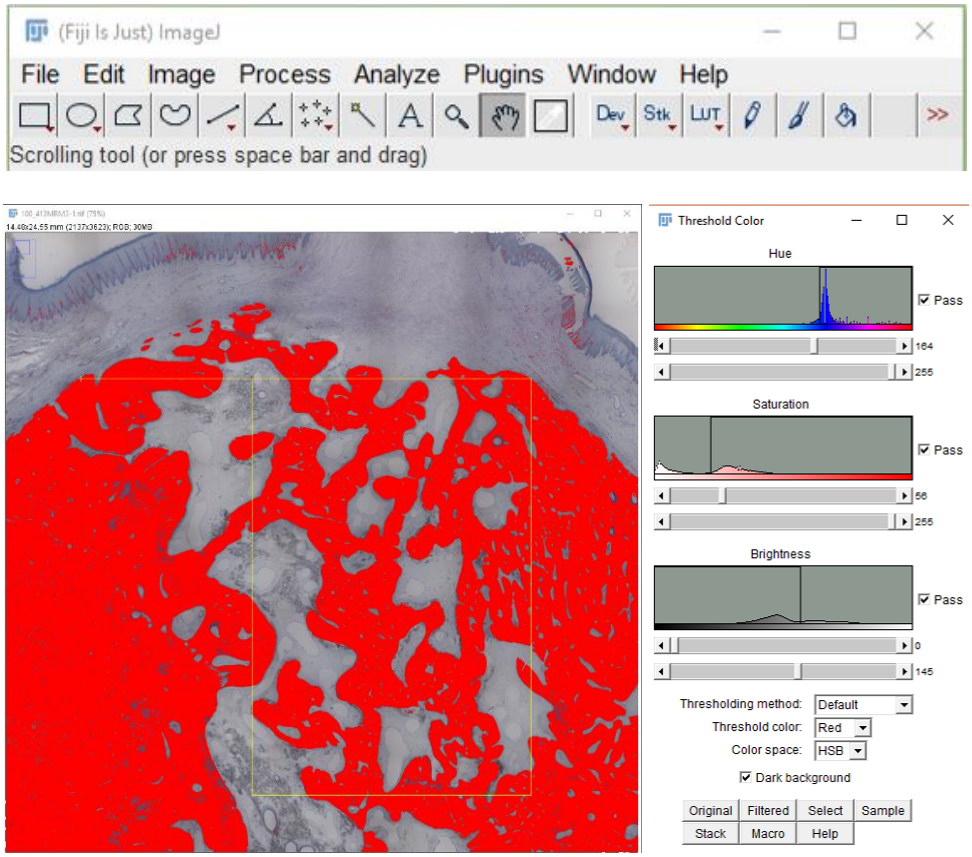


Figure 2.13. Manual selection of threshold for hard tissue (red) within the ROI.

2.5.2.3 Intra-examiner reliability test

Inter- and intra-examiner reproducibility of histomorphometric measurements was assessed. Using a random number generator, 10 specimens were selected for repeated measurements two months after the initial analysis was performed. The selected samples were re-measured by the primary investigator and another member of the team who was unaware of the results of the previous measurements. 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 pair of measurements. The intra-class correlation coefficients are generally high.

2.5.3 Histometry

Histometric measurements were done for each specimen using ImageJ software (version 1.50g, NIH, USA).

2.5.3.1 Bucco-lingual width of the alveolar ridge

The bucco-lingual width of the alveolar ridge was measured using the same reproducible strategy that was utilised to define the ROI. The length of the horizontal line, connecting the most coronal point of the pre-existing bone on the lingual cortex to the external aspect of the buccal wall was chosen to represent the width of the ridge.

2.5.3.2 Hard tissue "bridging"

The degree of hard tissue healing at the coronal aspect of the grafted extraction sockets was evaluated. The outline of the newly formed bone connecting the buccal and lingual cortices of the extraction socket was described as "coronal bridging" and was valued for each specimen as non-existent, partial or complete (Figure 2.14).

A band of hard tissue creating a distinct separation between the healed extraction socket and the marrow space was termed "marrow bridging" and was evaluated and described in a similar way, using the same values of complete, partial or non-existent.

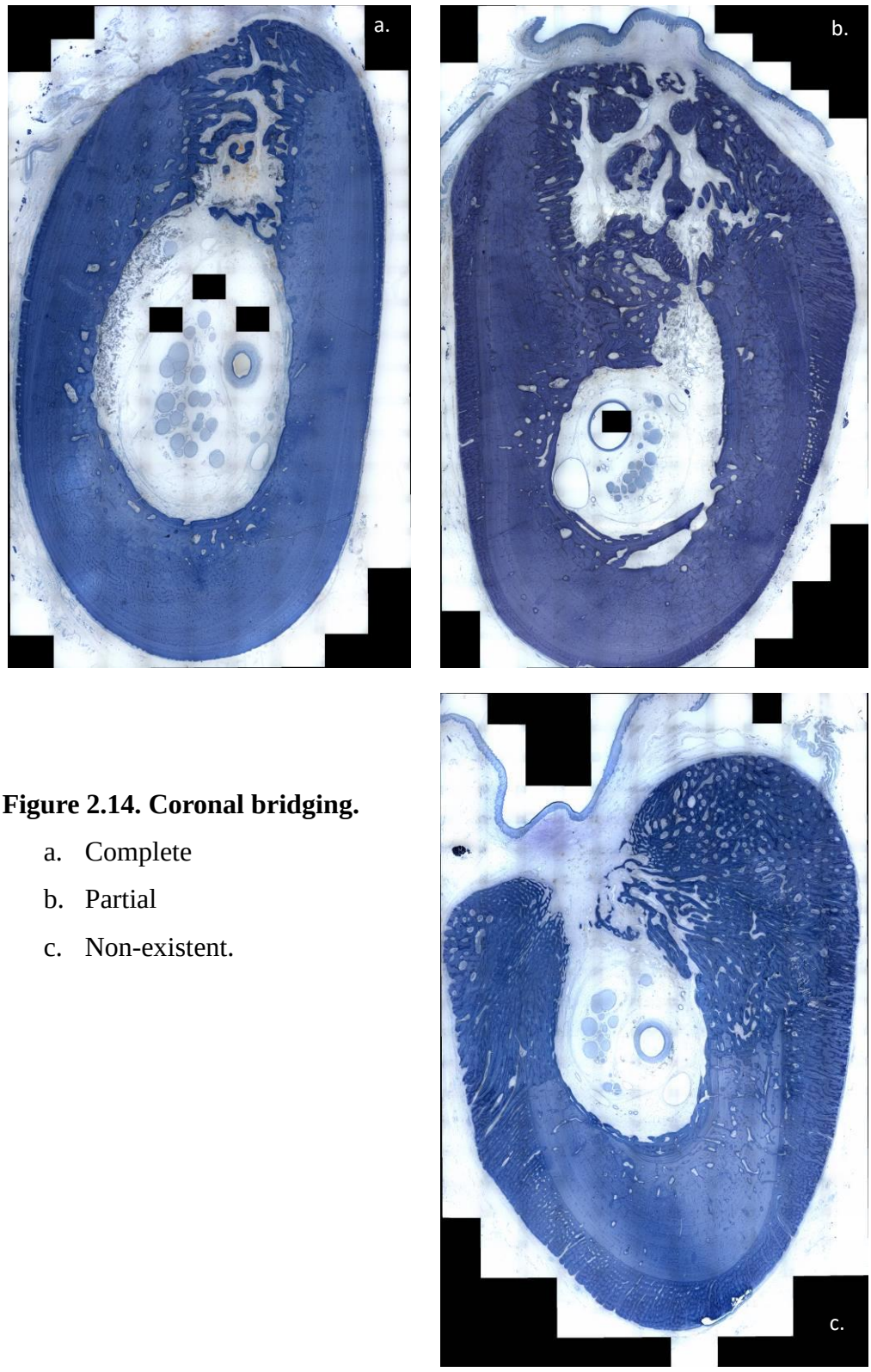


Figure 2.14. Coronal bridging.

a. Complete
b. Partial
c. Non-existent.

2.6 Statistical analysis

Mean values and standard deviations were calculated for all outcome variables. Where multiple sites were used (ROI, histological width), the mean of the two sites was used. For continuous outcomes, the difference between CON and the intervention groups was analysed by using a mixed model analysis with repeated measures for the treatment factor accommodated through a random effect for sheep and including the tooth position as a fixed effect. Following this, similar analyses were performed using BX as a reference against the other interventions. Estimated means and 95% confidence intervals (CI) for each treatment were estimated using marginal means. Differences were considered statistically significant when two-sided p was <0.05 with unadjusted post-hoc comparisons. For categorical analyses, pairwise exact McNemar's tests were planned as long as the number of discordant pairs was six or more (the smallest number needed for a statistically significant result without any adjustment for multiple comparisons, with eight discordant pairs needed for statistical significance with a Bonferroni adjustment). The statistical analysis was performed with Stata 14.1 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

Chapter 3 - Results

3.1 Handling properties of the grafting materials

3.1.1 PARASORB Cone® (CC) and PARASORB Sombrero® (CS)

The only difference between CC and CS is the presence of the membrane attached to the collagen cone in CS. Both materials could be easily removed from their packaging by holding their coronal parts with tweezers (Figure 3.1a). The collagen cone is a spongiform material, and therefore has to be handled with dry gloves and instruments. If the cone contacts saliva or blood prior to placement into the extraction socket, it loses its shape and texture, thus making the insertion into the socket very challenging.

In CS product, the attached membrane is very user-friendly, and once the collagen cone is inserted into the extraction socket, it holds the membrane in place and significantly reduces its tendency to migrate. However, the attached membrane needs to be trimmed to size, taking into account the diameter of the socket and the available interdental space.

3.1.2 Bio-Oss Collagen® (BX) vs Cone-Oss® (CO)

Both BX and CO grafting materials have particulate filler embedded in a collagen carrier matrix. We found that transferring the grafting material into the extraction socket was easier for CO, mostly due to its conical shape, compared to the brick-like shape of the BX (Figure 3.1b). Both products require condensation into the extraction socket. Due to the composition of the grafting materials, the filler particles held in place by the surrounding collagen matrix, results in minimal spillover during placement.

With both BX and CO, it is impossible to control the fill of the apical portion of the extraction socket, compared to other commercially available particulate grafting materials that fill the sockets in a bottom-up manner.

3.1.3 PARASORB Resodont® (CM) membrane compared to Bio-Gide®

The PARASORB Resodont® is an equine type 1 collagen membrane, whereas Bio-Gide® is composed of porcine type 1 collagen. Bio-Gide® membranes have two distinct surfaces, which dictate the correct placement of the rough surface towards the defect and the smooth surface facing the soft tissues. Resodont®, however, has two identical surfaces, and applying a specific surface towards the bone is irrelevant. Due to its relative rigidity, it is also easily handled in wet conditions.

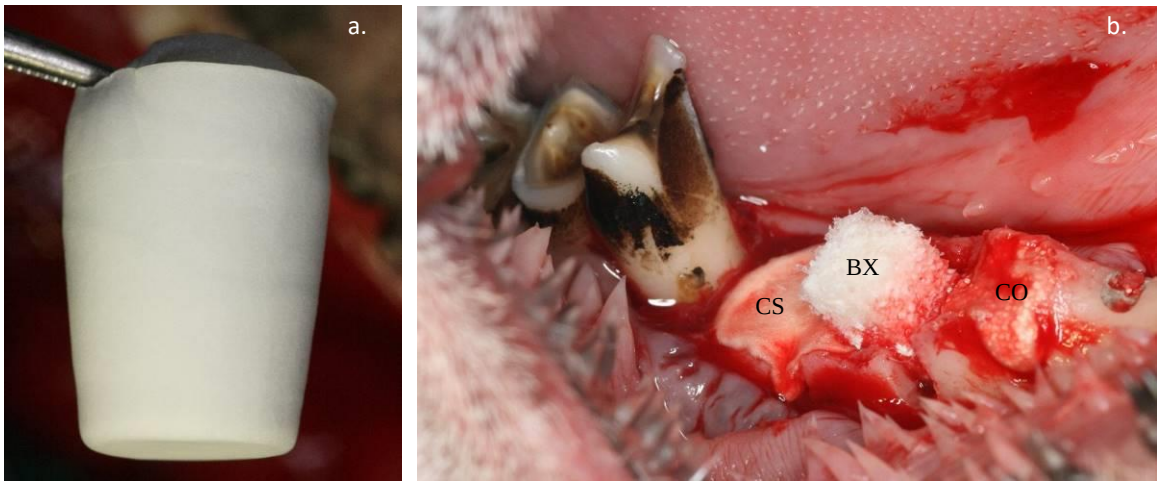


Figure 3.1. Grafting materials.

a. CC – Collagen Cone®
b. Grafted experimental sites:
    i. Anterior extraction socket grafted with Cone-Oss® (CO);
    ii. Middle extraction socket grafted with Bio-Oss Collagen® (BX), prior to condensing;
    iii. Posterior extraction socket grafted with PARASORB Sombrero® (CS).

3.2 Postoperative healing

No complications were observed during the immediate post-surgical period, when the animals were medicated and closely monitored before release to pasture. All the extraction sockets healed uneventfully at 16 weeks following the grafting procedure. There was complete bilateral soft tissue coverage of the surgical areas in all the study animals.

Post-mortem radiography revealed that one root tip was left behind in one of the experimental animals, but no complications were noted. The same root was present on the radiographs that were taken during the surgical procedure, however it was partially superimposed by the inter-radicular septum. This will be further discussed in the next section. This mandibular segment was not excluded from further histological analysis.

3.3 Radiographic examination of mandibular segments

Periapical radiographs were taken for each side of the mandible at three time-points: after tooth extractions and standardised defect preparation, after placement of graft materials, and post-mortem prior to sectioning the harvested tissue blocks and embedding the individual surgical sites in resin. Due to the nature of grafting materials used, BX, CO and CO+CM grafted sites exhibited levels of opacity comparable to the surrounding bone on post-grafting radiographs. CON, CC and CS sites, due to lack of filler particles, had a more radiolucent appearance (Figure 3.2a-d).

During 16 weeks of healing the grafted areas had undergone some remodelling. The standardised dehiscence defects could not be detected on post-mortem radiographs. Neither could we detect the grafted sites or the inter-radicular bone (Figure 3.2e-f).

We attempted to compare the radiographical vertical height at the grafted areas immediately after grafting and following euthanasia, however due to the high variation in the radiographs, such as angulation and lack of sufficient reproducible reference points, e.g. first molars and lower border of the mandible, this quantitative comparison could not be performed.

Nevertheless, qualitative observation of the radiographs was supportive of the assumption that the edentulous segments had undergone some vertical resorption during the healing period. On the intra-surgical radiographs that included a significant portion of the first mandibular molar, the horizontal level of the alveolar crest was mostly present at the level of or coronal to the furcation of the first molar (Figure 3.2a,c). On the other hand, on the post-mortem peri-apical radiographs, where the first molar was identifiable, in many cases the cortical bone of the edentulous area was located apical to the furcation of the first molar (Figure 3.2e).

Another noticeable finding was, that the amalgam pins that were created during the surgery as planned reference points for consecutive imaging analysis (Figure 3.2a-d), had disappeared on most of the post-mortem radiographs, and could only be located, fully or partially, in two out of 10 experimental animals (Figure 3.2e-f).

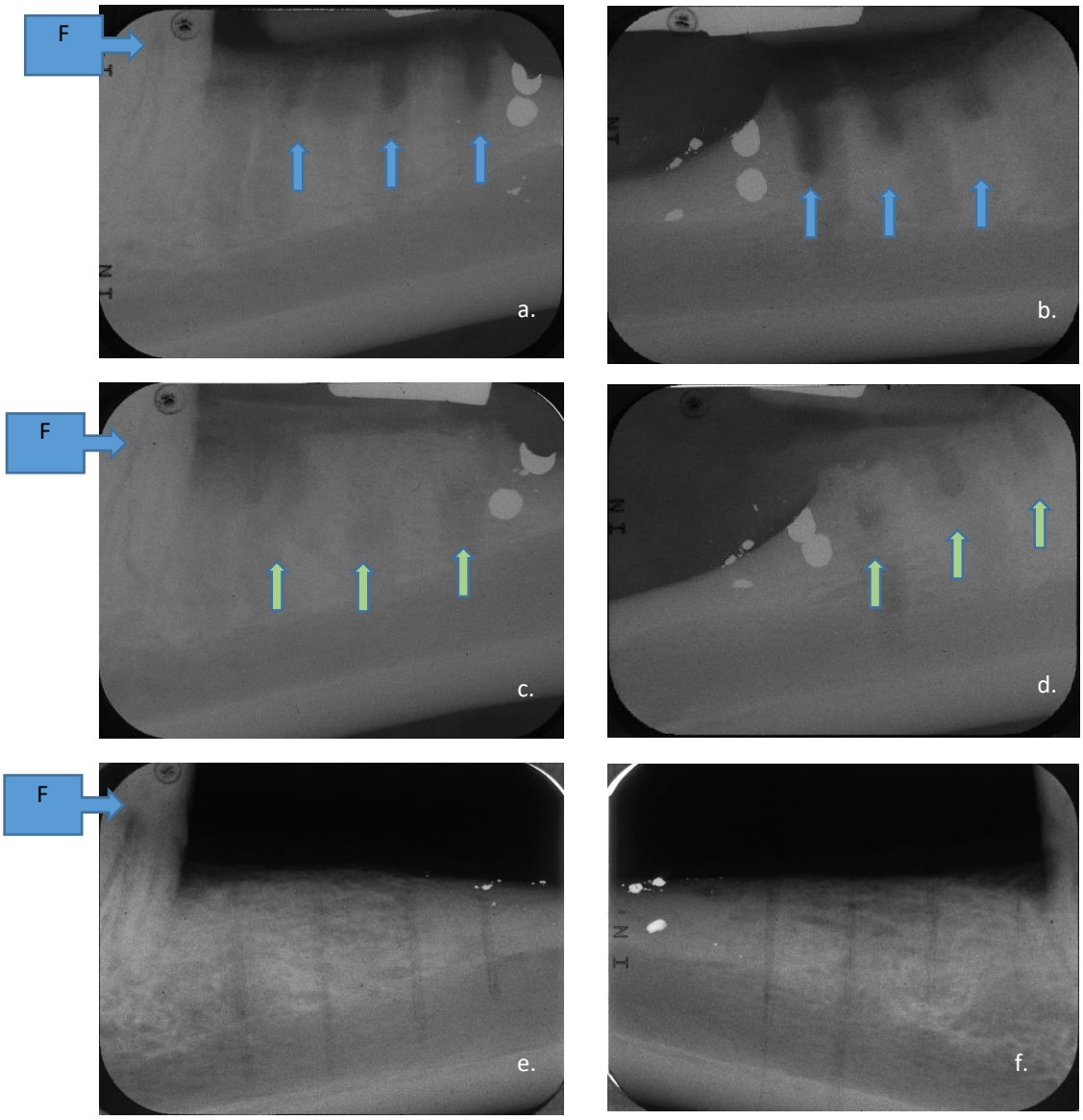
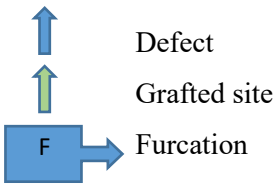


Figure 3.2. Radiographs of sheep 413.

a. Right – defects prepared
b. Left – defects prepared
c. Right – grafted (from left to right): CC, BX, CO+CM
d. Left – grafted (from left to right): CO. CON, CS
e. Right – post-mortem
f. Left – post-mortem.



Of special interest were the radiographs of sheep number 418. As previously noted, it had a residual root present near the grafted sites, and this could be used as a relatively good reference point.

On the intra-surgical radiograph, the most coronal point of the residual root is clearly seen apically in relation to the bone crest (Figure 3.3a) and the coronal border of the standardised defects that were 5mm in height. However, on the post-mortem radiograph the most coronal portion of the residual root is seen to be located coronally to the alveolar crest (Figure 3.3b).

When we superimposed both radiographs using the mesial aspect of the first mandibular molar, the residual root and the lower border of the marrow space as anatomical references, the vertical resorption of the alveolar ridge was better visualised (Figure 3.3c).

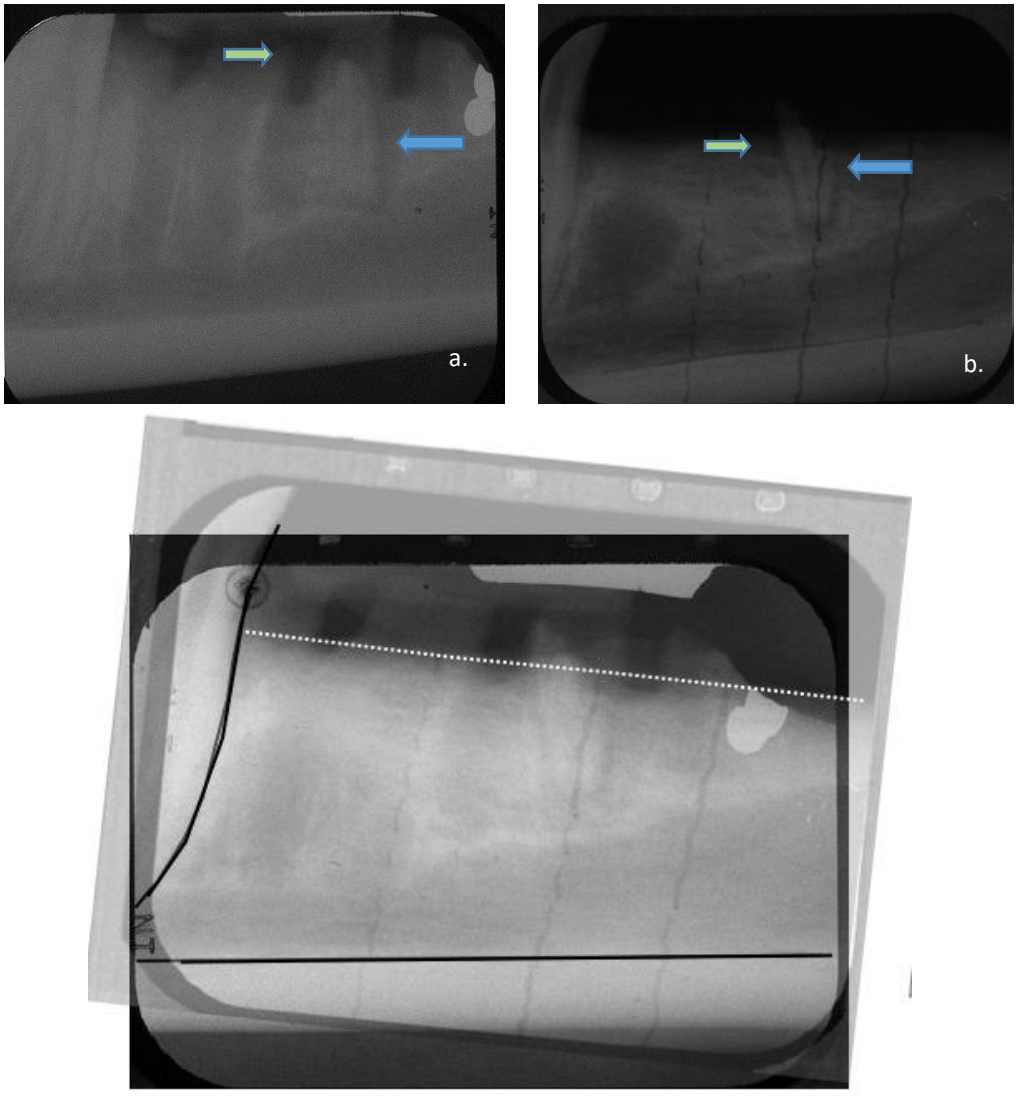


Figure 3.3. Radiographs of sheep 418.

a. Right – intra-surgical radiograph
b. Right – post-mortem radiograph
c. Superimposition using the mesial surface of the first molar and the lower border or marrow space as reference. White dashed line marks the post-mortem crest of the alveolar ridge.

Bone crest

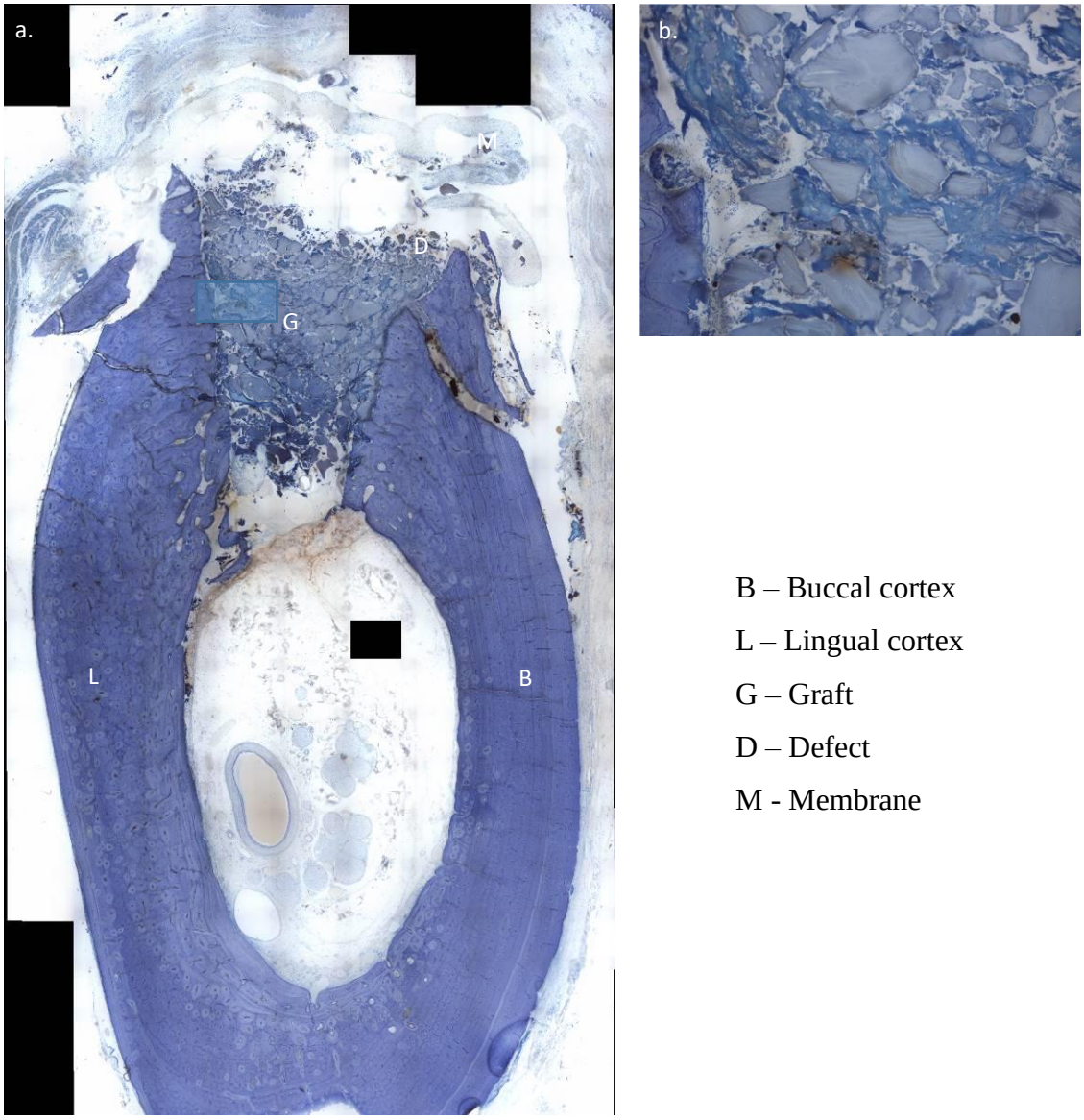
Residual root

3.4 Descriptive histology

The digitised images were grouped according to the grafting materials used (Appendix III) and were screened to detect any differences in healing patterns, such as size of bony trabeculae and completion of hard tissue healing at the alveolar crest. There were no different healing patterns found in either the grafted groups or by location of the site within the mandibular arch.

3.4.1 Baseline appearance of grafting materials

Sheep 412 was euthanised one day following the grafting surgery. The histological slides obtained from this animal provided us with baseline information about the appearance of the different grafted materials and native bone in our experimental model. All baseline specimens showed thick cortical bone, and large marrow space with scarce bony trabeculae. In the baseline sections, the buccal dehiscence defect was easily identified. Bony separation between the grafting materials and the apically located marrow space was rarely existent. The collagen component of grafted materials was stained with a light blue colour and had a wavy appearance. The xenograft particles were stained lighter than the existing bone due to the heating process used in preparation of this material, thus making the granules more dense and less stain absorbent (Figure 3.4). The β-TCP and HA particles of Cone-Oss® had a distinct and easily identifiable appearance of blue-to-grey round particles resembling cotton-wool (Figure 3.5).



B – Buccal cortex

L – Lingual cortex

G – Graft

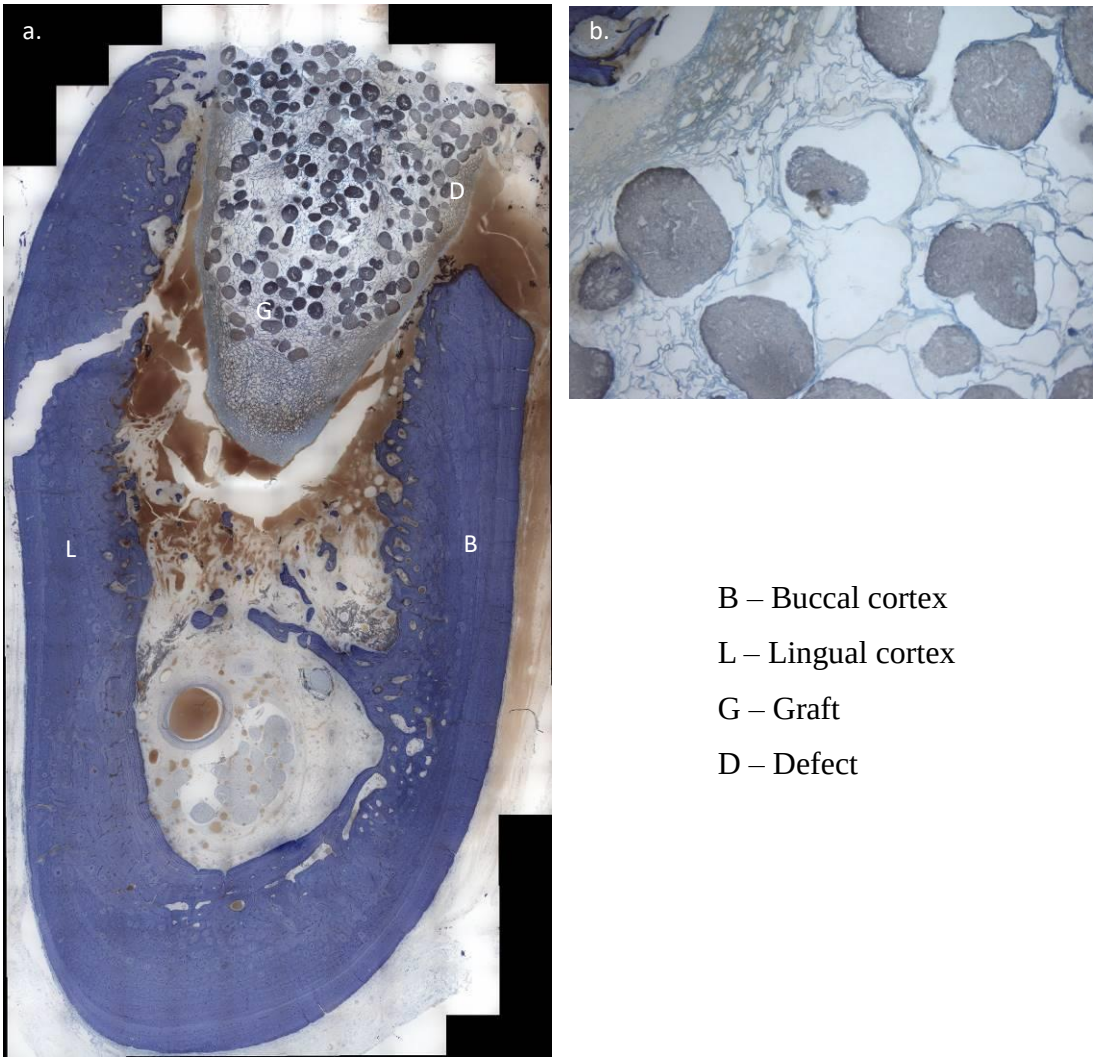
D – Defect

M - Membrane

Figure 3.4. Bio-Oss® and Bio-Gide® one day postoperatively.

a. Single whole section with grafted socket

b. x10 magnification of lingual part of the grafted socket.



B – Buccal cortex

L – Lingual cortex

G – Graft

D – Defect

Figure 3.5. Cone-Oss® one day post-operatively.

a. Single whole section with grafted socket

b. x10 magnification of the bi-phasic phosphate particles.

3.4.2 Sixteen weeks of healing

3.4.2.1 Soft tissue healing

In both the grafted and non-grafted extraction sites, the healed sockets were covered by thick, keratinised 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. Towards the centre of the alveolar crest, more collagen apposition was noted, suggestive of scar tissue formation at the site of the original incision.

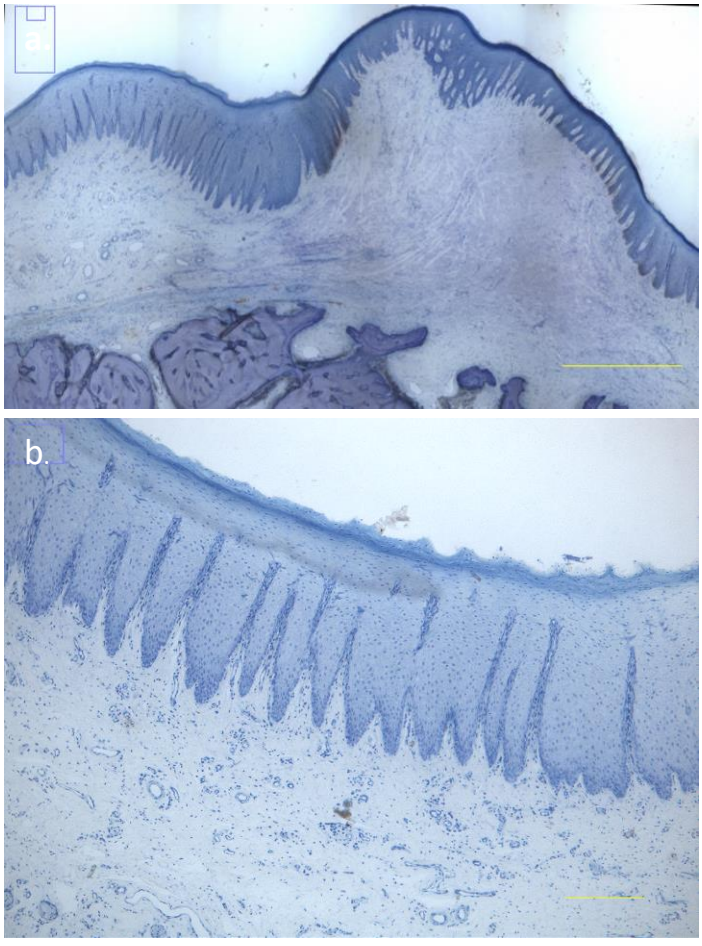


Figure 3.6. Mucosa healing.

a. Mucosa covering the coronal crest of the alveolar ridge
b. x10 magnification of epithelial lining and underlying connective tissue.

3.4.2.2 Bone healing in the area of buccal dehiscence defect

The experimental dehiscence defects that were created in the buccal wall of the extraction sockets were completely healed in all animals in all surgical sites with newly formed bone (Figure 3.7 orange dash line).

3.4.2.3 Bone healing inside the grafted sockets

For all treatment modalities, the newly-formed bone within the healed extraction sockets was predominantly woven bone, characterised by an irregular pattern and loose finger-shaped trabeculae (Figure 3.7 green arrow) surrounded by immature fibrovascular connective tissue. The majority of the trabeculae originated from the buccal and lingual walls of the extraction sockets, whilst the appearance of non-connected trabeculae can be explained by orientation of the sectioning relative to the long axis of these trabeculae.

3.4.2.4 Buccal and lingual crests

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 blue arrows). The newly formed bone was easily identified by its dense staining and lack of regularity. Mature pre-existing bone had a more organised appearance and stained in light blue. This newly formed bone within the bony defect was in direct continuity with the buccal and lingual socket walls.

3.4.2.5 Cortical bone overgrowth

Newly formed bone that was detected lateral to the original buccal and lingual cortical plates was a common observation in the histological slides. In 73 out of 120 specimens (60.8%), the newly formed bone could be seen at the lower border of the mandible, away from the original surgical exposure (Figure 3.8). For experimental animal 409, the healing process was accompanied with exuberant bone remodelling and overgrowth to the level that the anatomical reference points were no longer recognisable (Figure 3.9), and specimens from this animal had to be excluded from all further analyses.

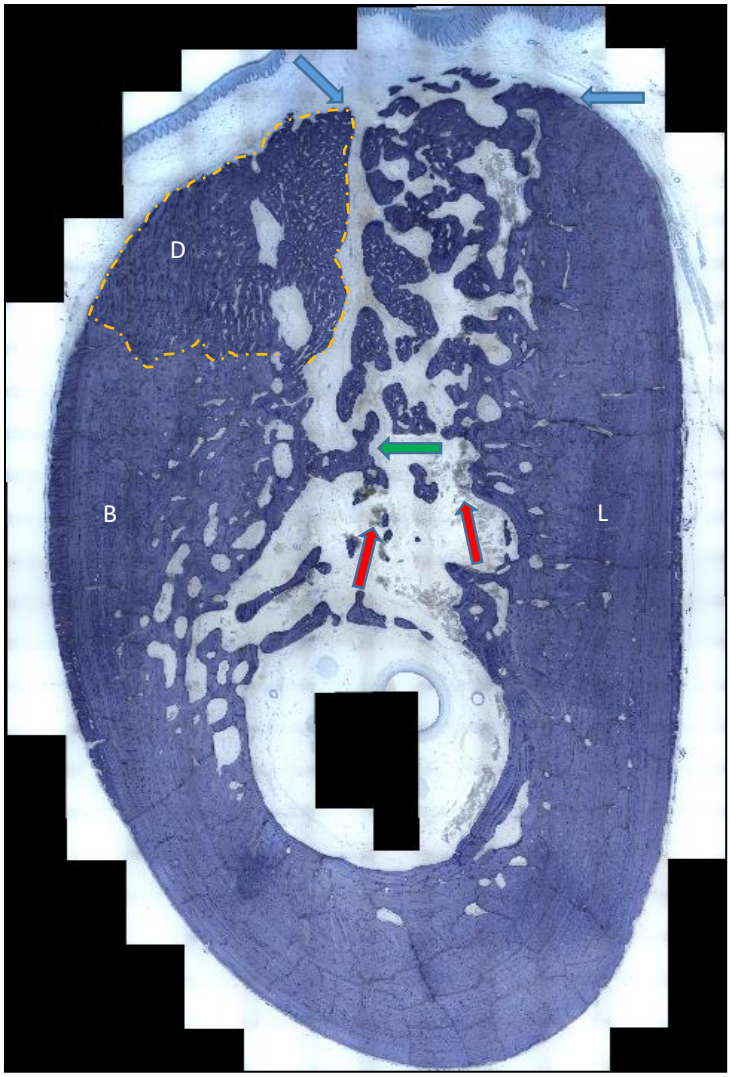


Figure 3.7. Full size view of a typical histological specimen.

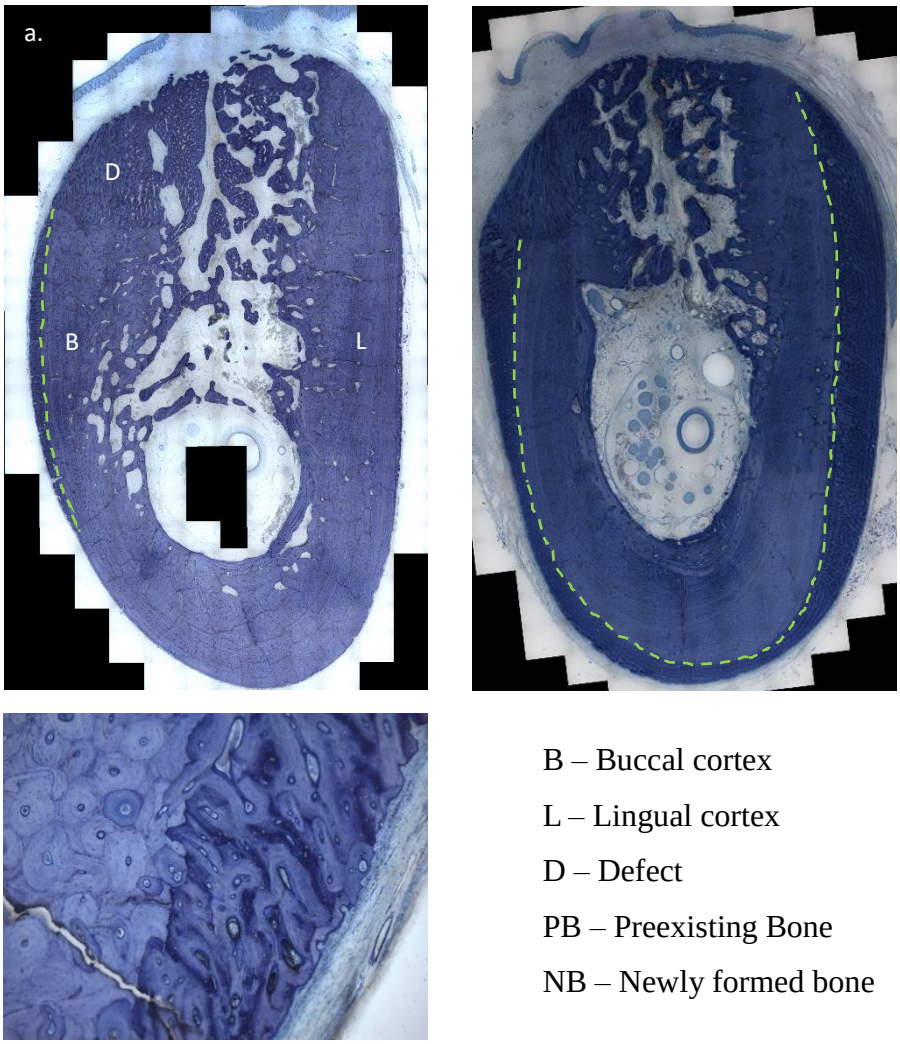


Figure 3.8. Bone overgrowth (demarcated with green dash line).

a. Socket with buccal defect healed with new bone, minimal buccal bone overgrowth
b. Socket with buccal defect healed with new bone, bone overgrowth on buccal and lingual crests, including the lower border of the mandible
c. X10 magnification demonstrating the border between the more structured preexisting bone and more irregular newly formed bone.

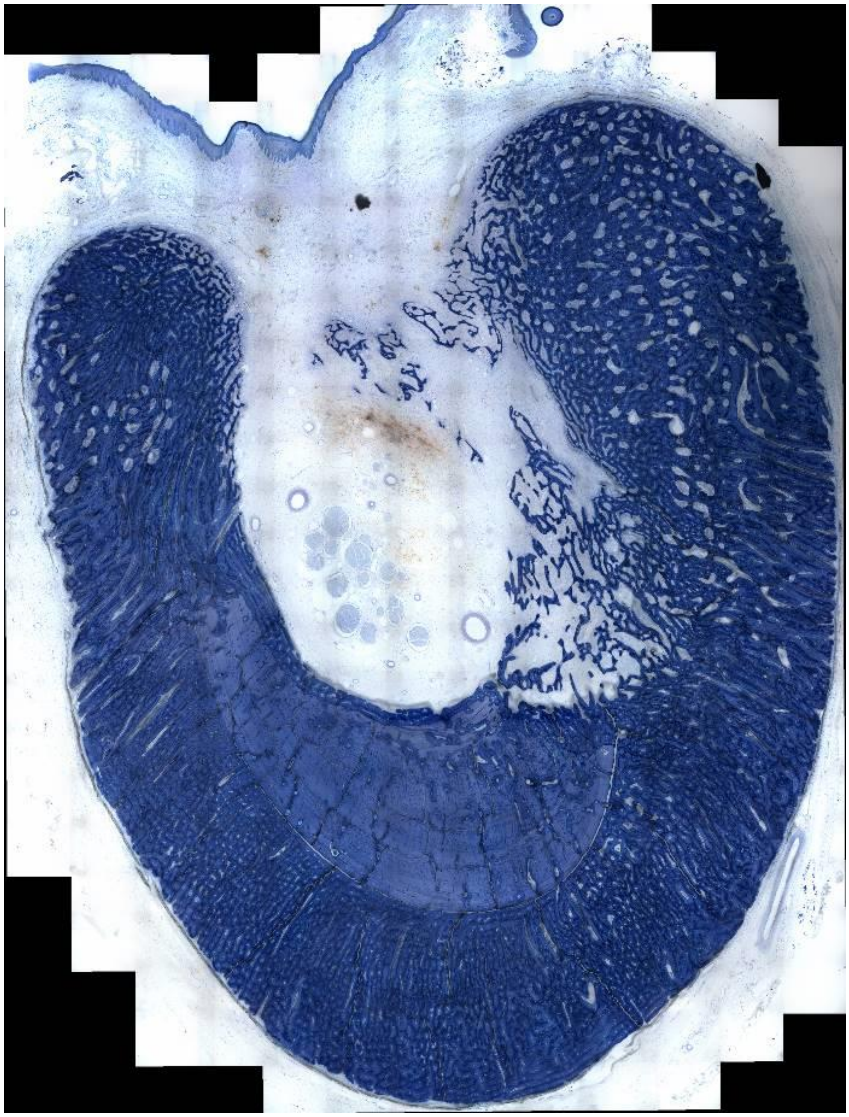


Figure 3.9. Exuberant remodelling and bone overgrowth.

3.4.2.6 Coronal and marrow bridging

The extent of coronal and marrow bridging was categorised as complete, partial or non-existent. For statistical calculations the specimens with partial and complete bridging were combined, as partial bridging may have appeared complete if the specimens were sliced at a different angle, and vice versa.

The results of these observations were summarised in Tables 3.1 and 3.2

Table 3.1. Crestal bone bridging.

	Crestal bone bridging (Number of sites)			
Treatment modality	**None**	**Partial**	**Complete**	**Partial + Complete**
CON	1	4	6	10
BX	1	2	8	10
CC	1	1	9	10
CS	1	2	8	10
CO	2	3	6	9
CO+CM	2	2	7	9

Due to the size of our sample there were too few events for analysis using mixed logistic regression and too few discordant pairs (max n=1) for McNemar's test on pairs of groups to achieve statistical significance.

Table 3.2. Marrow bone bridging.

	Marrow bone bridging (Number of sites)			
Treatment modality	**None**	**Partial**	**Complete**	**Partial + Complete**
CON	3	5	3	8
BX	2	6	3	9
CC	1	5	5	10
CS	2	5	4	9
CO	1	7	3	10
CO+CM	1	5	5	10

Similarly, in the analysis of marrow bridging there were too few events for analysis using mixed logistic regression and too few discordant pairs (max n=3) for McNemar's test on pairs of groups to achieve statistical significance. Therefore, for both crestal and marrow bone bridging, all comparisons with CON or BX were automatically deemed non-significant due to the sample size.

3.4.2.7 Histological artifacts

The methodology of preparation of slides for histological staining involved grinding down the initially 650μm thick slides to the thickness of 80-100μm, and then polishing prior to staining. This process caused some debris to accumulate next to the bone within the histological sections (Figure 3.7 red arrows) that had to be digitally adjusted in an image editor prior to performing histomorphometric calculations.

3.4.2.8 Residual graft material

Any remnants of the original collagen present in all of the tested grafted materials could not be visually detected. The β-TCP and HA particles of Cone-Oss® were also not seen in the healed grafted sockets. Bio-Oss® particles were found in half of the healed sites grafted with xenograft. In most cases, the residual Bio-Oss® particles were integrated within the newly formed bone. No foreign body reaction or encapsulation of these particles was documented (Figure 3.10). When several of the particles were examined under x10 magnification, some multi-nuclear osteoclast-like cells were noted at the periphery and directly adjacent to the particles, suggesting that the xenograft particles were being actively resorbed and replaced by the newly formed bone (Figure 3.11).

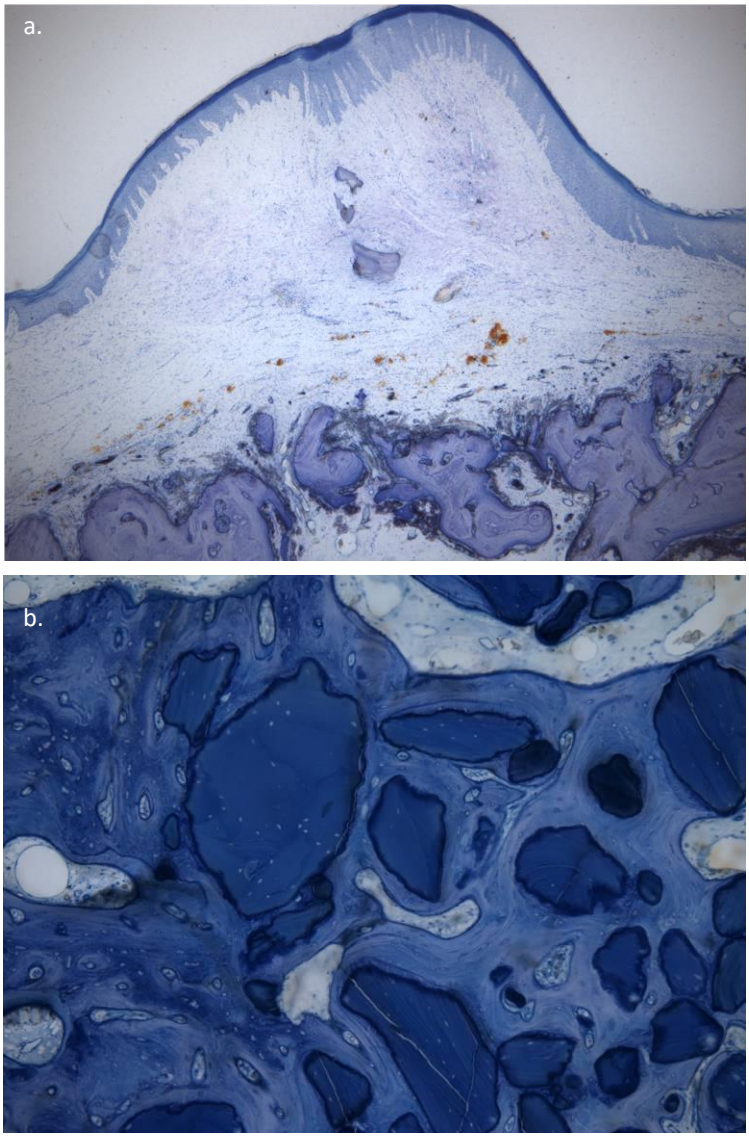


Figure 3.10. Residual graft in BX group.

a. Migrated Bio-Oss particles in soft tissue (x4 magnification)
b. Bio-Oss particles completely embedded in newly formed bone (x10 magnification).

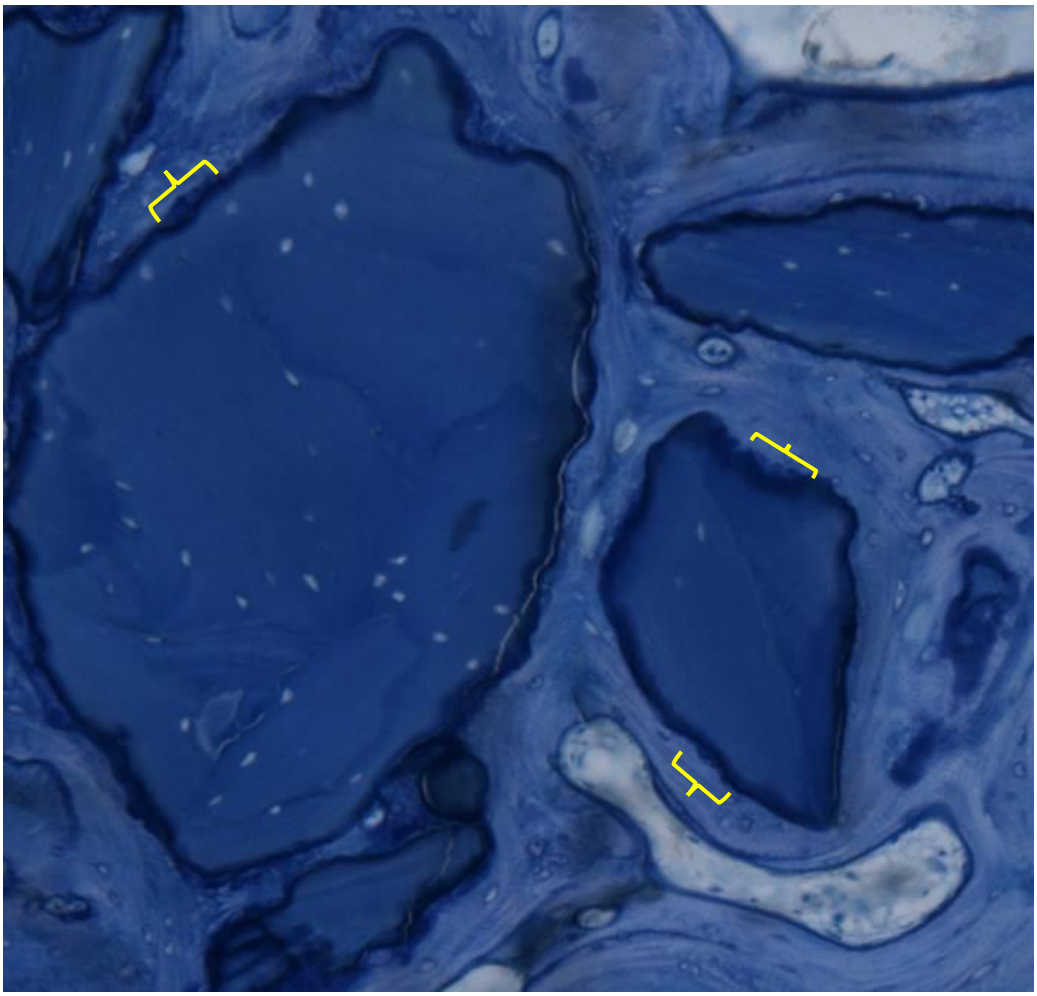


Figure 3.11. Resorption of xenograft particles.

Yellow brackets outline multinuclear, osteoclast-like cells at the periphery of xenograft particles embedded in newly formed bone.

3.4.3 Summary of findings in descriptive histology

Histological specimens obtained from an animal euthanised one day following surgery provided us with the baseline microscopic appearance of the grafted materials. This was useful in detection of the grafted materials within the healed sockets.

The healing pattern of the extraction sockets at 16 weeks was similar across the six different treatment modalities. The newly formed trabecular bone had formed in all the extraction sockets.

A bridge of hard tissue was detected at the coronal portion of the alveolar ridge in both grafted and non-grafted sockets. Similarly, a bony bridge was found to separate the healed socket from the marrow space. The small number of samples in our study was insufficient to conclude whether any of the treatment modalities had more of a tendency towards the formation of these bone bridges.

At 16 weeks, no equine collagen materials could be detected in the histological specimens. However, half of the processed slides had shown residual xenograft particles in the healed extraction sockets. When found, these BX particles were surrounded by newly formed bone, suggesting active resorption of the particles and replacement by bone.

3.5 Histomorphometric analysis

Two bucco-lingual sections representing the centre of each experimental site were chosen to measure the fraction of hard tissues within the ROI. The mean of two values was used as representative of the value for each socket. The representative values of the treated sites were clustered according to the underlying treatment modality.

3.5.1 Hard tissue fraction within ROI

Mean results for the calculation of hard tissue fraction for each treatment modality following 16 weeks of healing are presented in Table 3.3. The graphic representation of these results is illustrated in Figure 3.12. Individual results obtained for each histological section are listed in Appendix IV, Section 4.

Table 3.3. Fraction of hard tissues (%) within the ROI in surgical sites after 16 weeks of healing.

Treatment modality	Number of sites	Hard tissue (%) Mean ± SD	Hard tissue (%) (95% CI)	P value Compared to CON	P value Compared to BX
CON	9	49.6 ± 6.0	50.0 (43.3, 56.8)		
BX	8	54.5 ± 7.1	54.6 (47.4, 61.9)	0.305	
CC	9	57.0 ± 10.1	56.7 (50.0, 63.5)	0.113	0.630
CS	8	54.2 ± 9.9	54.0 (46.9, 61.1)	0.368	0.882
CO	9	54.5 ± 15.0	54.6 (47.8, 61.3)	0.282	0.986
CO+CM	7	57.3 ± 16.4	56.3 (48.7, 63.8)	0.167	0.733

*Significantly different from CON / BX ($p<0.05$)

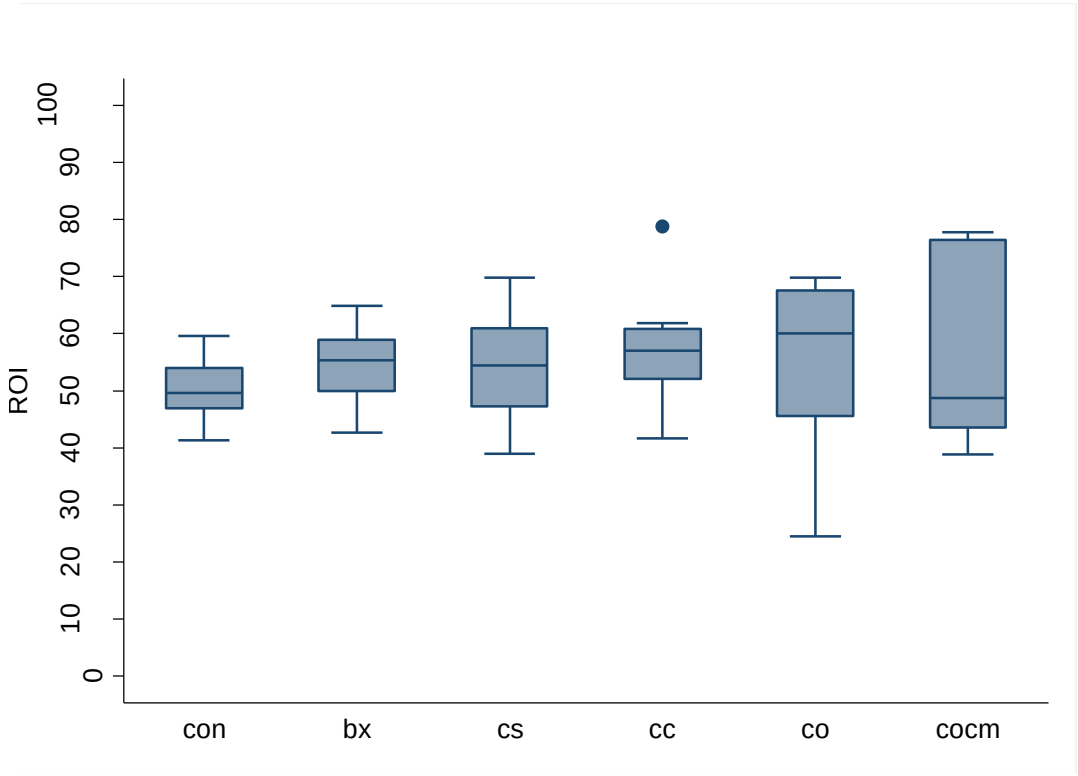


Figure 3.12. Fraction (%) of hard tissues within the ROI in surgical sites after 16 weeks of healing.

The amount of hard tissues formed in naturally healed non-grafted sockets was 49.6 ± 6.0%. The highest percentage of hard tissue after 16 weeks of healing was found in CO+CM group (57.3 ± 16.4%). This treatment modality also had the highest variability of the results, as can be seen from the standard deviation. The BX grafted sockets showed the least variability (54.5 ± 7.1%) amongst the active treatment groups. An overall comparison of hard tissue in extraction sockets subjected to all six treatment modalities did not detect statistically significant differences between the groups (p=0.687).

A series of individual t-tests were performed between the four treatment modalities based on equine collagen (Table 3.4). There were no statistically significant differences between the groups.

Table 3.4. Comparison of differences in hard tissue fractions between equine collagen based products (P values).

Treatment modality	CC	CS	CO	CO+CM
CC	---	---	---	---
CS	0.526	---	---	---
CO	0.602	0.892	---	---
CO+CM	0.918	0.620	0.706	---

*Significantly different ($p<0.05$)

3.5.2 Summary of findings in histomorphometric analysis

After 16 weeks of healing, formation of new bone was evident in all grafted and non-grafted extraction sockets. Residual graft materials were not detected in all experimental sites that were grafted with equine collagen based materials. Residual xenograft particles were found in half of the sites in the BX group. Therefore, in our analysis the fraction of hard tissue within the ROI was calculated, rather than the newly formed bone.

Our histomorphometric analysis failed to reveal statistically significant differences in the fraction of mineralised tissue within the ROI between the different treatment modalities, including the positive and negative control groups, CON and BX respectively, after 16 weeks of healing.

3.6 Histometric analysis

Two bucco-lingual sections representing the centre of each experimental site were chosen to measure the histological width of the alveolar ridge. The mean of two values was used as representative of the value for each socket. The representative values of the treated sites were clustered according to the underlying treatment modality.

These values, together with the baseline width recorded during surgery were used to calculate the width reduction after 16 weeks of healing.

3.6.1 Baseline width of the alveolar crest

As described in the methodology chapter, after the mandibular premolar teeth were extracted, the width of the alveolar ridge at each surgical site was measured at the mesial aspect of the created defect (Figure 2.8e). These values were grouped according to treatment modalities and the mean value and standard deviation for each group is presented in Table 3.5. Individual results obtained for each surgical site are listed in Appendix IV, Section 1.

It can be seen that the baseline values of the alveolar ridge width were similar across all the treatment modalities. Overall the test for baseline clinical widths suggests no difference between the groups (p=0.804).

Table 3.5. Baseline horizontal measurements of alveolar ridge width in millimeters.

Treatment modality	Baseline width (Surgical) Mean ± SD	P values Compared to CON	P values Compared to BX
CON	8.5 ± 1.5		
BX	8.5 ± 2.6	0.970	
CC	8.9 ± 1.9	0.950	0.920
CS	8.5 ± 2.2	0.263	0.247
CO	8.6 ± 2.6	0.706	0.678
CO+CM	8.5 ± 2.2	0.413	0.392

*Significantly different from CON / BX ($p<0.05$)

3.6.2 Histological width of the alveolar crest

At each histological section, the length of the horizontal line connecting the most coronal point of the pre-existing bone on the lingual crest with the external outline of the buccal crest was defined as the histological ridge width.

Mean results for calculation of histological width of the alveolar ridge for each treatment modality following 16 weeks of healing are presented in Table 3.6. The graphic representation of these results is illustrated in Figure 3.13. Individual results obtained for each histological section are listed in Appendix IV, Section 5.

Table 3.6. Final horizontal measurements of alveolar ridge width in millimeters.

Treatment modality	Final width (histological) Mean ± SD	P values Compared to CON	P values Compared to BX
CON	5.8 ± 1.6		
BX	7.4 ± 2.7	**0.007 ***	
CC	5.9 ± 1.8	0.963	**0.007 ***
CS	6.4 ± 1.8	0.228	0.156
CO	6.0 ± 2.2	0.846	**0.012 ***
CO+CM	6.3 ± 1.7	0.193	0.222

*Significantly different from CON / BX ($p<0.05$)

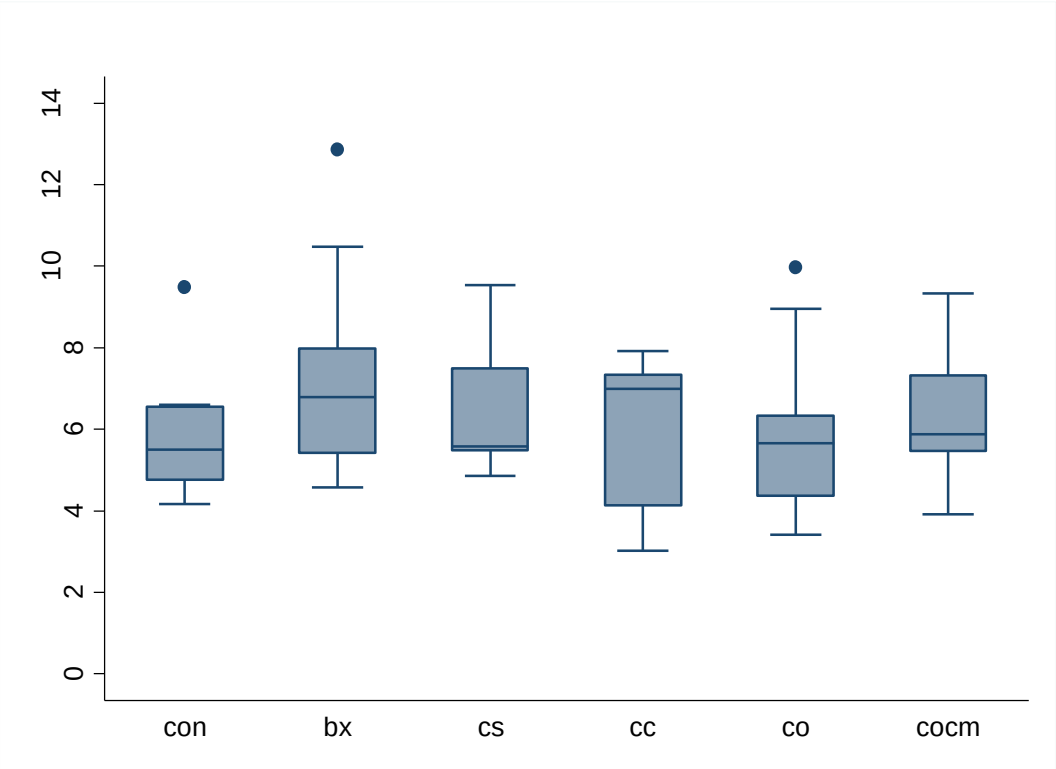


Figure 3.13. Horizontal measurements of the alveolar ridge width in millimeters.

An overall test for histological width measurements was performed following log transformation of the data to improve the normality of conditional residuals. The results detected statistically significant differences between the groups (p=0.048).

The mean histological width in naturally healed non-grafted sockets was 5.8 ± 1.6mm. BX sites had the widest healed alveolar ridges (7.4 ± 2.7mm), significantly different compared to the CON group (p=0.007). This group also had the highest variability of the results, as suggested by the standard deviation values.

The histological width values for CC and CO groups were very close to those of CON (5.9 ± 1.8 and 6.0 ± 2.2mm, p=0.963 and 0.846 respectively).

In a series of individual t-tests comparing the xenograft-grafted group against equine collagen products, we found that the mean histological width in the BX group was higher compared to each CC (p=0.007) and CO (p=0.012).

As with fraction of hard tissue within the ROI, the results of the four treatment modalities based on equine collagen were compared in a series of individual t-tests (Table 3.7).

Table 3.7. Comparison of differences in histological width of the alveolar ridge between equine collagen based products (P values).

Treatment modality	CC	CS	CO	CO+CM
CC	---	---	---	---
CS	0.248	---	---	---
CO	0.883	0.308	---	---
CO+CM	0.212	0.894	0.226	---

*Significantly different (p<0.05)

No statistically significant differences in histological width could be detected between these four treatment modalities.

3.6.3 Post-extraction alveolar ridge resorption

For experimental sites where both baseline and histological measurements of the alveolar ridge were available (n=51) changes were calculated in horizontal dimensions of the alveolar ridge, representing the post-extraction alveolar ridge resorption. The results were described in millimeters (Table 3.8).

Table 3.8. Post-extraction changes in horizontal dimensions of the alveolar ridge in millimeters.

Treatment modality	Δ-width Difference between baseline and final Mean ± SD	P values Compared to CON	P values Compared to BX
CON	-2.8 ± 2.1		
BX	-1.4 ± 4.1	**0.025 ***	
CC	-2.9 ± 1.5	0.955	**0.020 ***
CS	-2.3 ± 2.7	0.146	0.466
CO	-2.6 ± 1.2	0.634	0.075
CO+CM	-1.9 ± 2.1	0.184	0.442

*Significantly different from CON / BX ($p<0.05$)

Overall, there was no statistically significant evidence of difference between the groups (p=0.132) and this test was used as a gatekeeper test for post-hoc comparisons. However, in an exploratory examination of the post-hoc tests, the results would suggest a difference between BX and CON groups (p=0.025), although this would be rendered non-significant with a Bonferroni adjustment for multiplicity, as would the difference between BX and CC groups (p=0.020). The difference between BX and CO groups in these exploratory analyses did not reach statistical significance even without such adjustment, being only a tendency (p=0.075).

The same batch of results was also described as a percentage of the original width (Table 3.9).

Table 3.9. Loss in width of the alveolar ridge as a percentage from baseline.

Treatment modality	Width reduction as percentage of baseline Mean ± SD	P values Compared to CON	P values Compared to BX
CON	30.5 ± 23.2		
BX	9.7 ± 41.3	**0.002 ***	
CC	32.7 ± 14.7	0.885	**0.001 ***
CS	22.6 ± 23.9	0.097	0.183
CO	30.3 ± 12.3	0.827	**0.004 ***
CO+CM	20.6 ± 20.3	0.170	0.134

*Significantly different from CON / BX ($p<0.05$)

Overall, there was evidence of a difference between the groups (p=0.008) and so comparisons between the groups proceeded to post-hoc tests, which suggested that the BX group had less reduction in ridge width than CON (p=0.002), CO (p=0.004), and CC (p=0.001).

Non-grafted control (CON) sites had a 30% loss in alveolar ridge width (30.5 ± 23.2%). When the extraction sockets were grafted with bovine xenograft (BX), the reduction in the alveolar ridge width reduced three-fold (9.7 ± 41.3%). This difference was statistically significant (p=0.002).

The values for width reduction in the groups CC (32.7 ± 14.7%) and CO (30.3 ± 12.3%) were very close to those of CON (30.5 ± 23.2%). The means for CS (22.6 ± 23.9%) and CO+CM groups (20.6 ± 20.3%) were located approximately halfway between the results of the CON (30.5 ± 23.2%) and BX (9.7 ± 41.3%) groups, however no statistically significant differences could be detected.

The equine collagen based products were, once again, compared to identify any significant differences between the groups. The results are summarised in Table 3.10.

Table 3.10. Comparison of differences in width reduction of the alveolar ridge between equine collagen based products (P values).

Treatment modality	CC	CS	CO	CO+CM
CC	---	---	---	---
CS	0.074	---	---	---
CO	0.716	0.147	---	---
CO+CM	0.135	0.824	0.246	---

*Significantly different (p<0.05)

None of the groups were found to be different from others with statistical significance, however a non-significant tendency (p=0.074) could be observed between the groups CS and CC.

The changes in the alveolar ridge width are graphically represented in Figure 3.14.

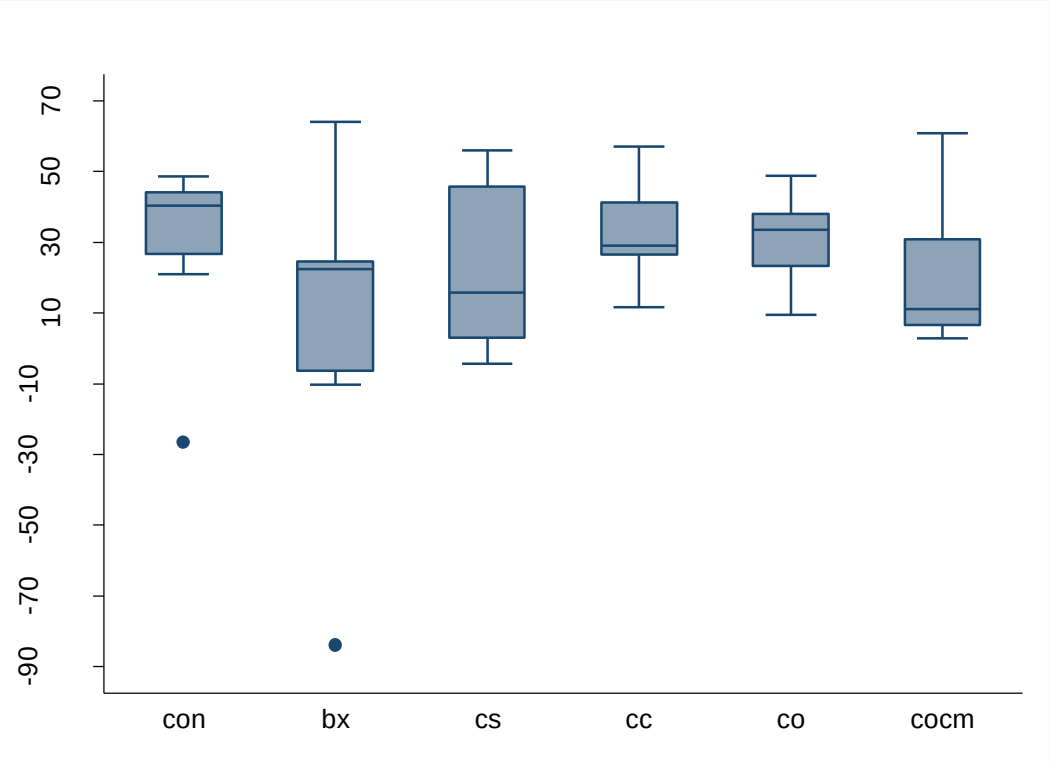


Figure 3.14. Loss in width of the alveolar ridge as a percentage from baseline.

From the Figure 3.13 two outliers are readily detectable. These suggest that in some of the specimens the alveolar ridge has actually expanded, rather than resorbed following the surgical procedure. These correspond to the specimens from sheep number 418 that belong to the CON and BX groups.

While some of the differences between the baseline and histological values may be explained by measurement errors and potential flaws in the model, as will be discussed in the Discussion chapter, we have recalculated the statistical data with these specimens excluded.

3.6.4 Summary of findings in histometric analysis

Baseline horizontal dimensions of the experimental sites were similar between the six treatment modalities, thus allowing for comparison of the effect of the treatment on the postoperative remodelling.

After 16 weeks of healing, a statistically significant difference was noted in the mean alveolar ridge width reduction when the BX group was compared with the non-grafted control, CON ($p=0.002$). BX showed better alveolar width preservation compared to the CC and CO groups, with statistical significance ($p=0.001$ and 0.004, respectively).

No statistically significant differences were found between the equine collagen based grafting products, however, a tendency to improved preservation of the horizontal dimensions of the post-extraction sites was noted in treatment modalities CS and CO+CM, where the intra-socket grafting material was covered by a barrier membrane, whether incorporated or as a separate product.

Chapter 4 - Discussion

4.1 Introduction

The current study was designed to serve two objectives. The first objective was to refine a previously developed tooth extraction socket model (Liu *et al*., 2015) in sheep for research of ARP. The second objective was to compare the healing of the extraction sockets with a standardised buccal dehiscence defect grafted with a range of novel equine collagen products against natural non-grafted healing and bovine xenograft-grafted controls. We improved the techniques for the extraction of mandibular premolars in sheep, and introduced a model of an extraction socket with a standardised buccal defect, which is more representative of the common clinical situations requiring grafting procedures for ARP. Following uneventful healing of the extraction sockets, we carried out histomorphometric and histometric analyses of the grafted sites.

4.2 Experimental results

In this chapter we will describe the results found in the current study, compare these to the evidence derived from previous studies in other animal and human models, and discuss the clinical relevance.

4.2.1 Summary of main results

After a healing period of 16 weeks, the tooth extraction sockets presented with hard tissue fractions ranging from 49.6% to 57.3% within the ROI. There were no statistically significant differences observed between any of the groups. Residual grafting material was detected histologically in half of the specimens grafted with the positive control xenograft (BX). No residual grafting material was detected in sites grafted with the equine collagen-based test materials, suggesting that these materials were completely resorbed within the defined healing period. In our study, bovine bone xenogaft was superior to the tested products in reducing post-extraction loss of the alveolar ridge width.

Non-grafted control sites showed a reduction in alveolar ridge width of approximately 30% during the 16 weeks healing period. A similar rate of post-extraction remodelling was recorded for the CC and CO groups. Positive control sites grafted with bovine bone

xenograft (BX) showed a three-fold decrease in horizontal changes (9.7%) and this finding was statistically significant (p=0.002).

Groups CS and CO+CM (incorporating an extra-socket membrane as well as the intra-socket graft) showed a 20% alveolar ridge reduction. Compared to the non-grafted controls (CON) this was not statistically significant, however the results suggested that barrier membranes play an important role in ARP procedures.

4.2.2 Volumetric fraction of hard tissues within ROI

4.2.2.1 Findings in the current study

In this study, the hard tissue fraction within the ROI in the non-grafted extraction sockets was 49.6 ± 6.0%. In the grafted sockets, mean values varied between 54.2% and 57.3%. No statistically significant differences could be identified between any of the experimental groups (p=0.687).

One explanation for the lack of differences between the groups may be the size of the study group, which may have lacked statistical power to detect true differences between the groups. Another possible explanation is that after 16 weeks of healing, the experimental sites reached a plateau in terms of remodelling. It would be beneficial to perform a similar analysis at various time-points to evaluate whether the differences between the groups would be more statistically pronounced during earlier stages of healing.

A recently published study from our institution examined extraction sockets grafting in a similar sheep model using two time-points, eight and 16 weeks of healing (Liu *et al*., 2015) with eight sheep euthanised at each time-point. The authors did not create a buccal dehiscence defect in their model and used slightly different reference points to determine their ROI for histomorphometric analysis. In the control, non-grafted group, the volume of mineralised tissue in the ROI was 45.7 ± 10.7%, which is comparable to our findings. On the other hand, a mean of 45.5% hard tissue was found in the BX group, whereas in this study the mean value for the BX group was 54.5%. The differences in the results between these studies can be explained by minor differences in surgical protocols, differences in the identification of ROI, and the use of different

commercially available bovine xenograft products and collagen membranes as positive controls.

Liu and co-authors (2015) did not observe statistically significant differences in their histomorphometric results between the experimental groups at both time-points. Additionally, the authors did not find a difference in the fraction of newly formed bone between eight and 16 weeks of healing, suggesting that the healing of the sockets may be complete at eight weeks in the sheep extraction model. Thus, observations made at earlier time-points may be advantageous in future studies.

In the discussion of the limited sample size, Liu *et al*. (2015) performed a post-hoc power analysis, and concluded that to achieve a 70% power for their study design, a significantly larger number of study animals would be required, 56 sheep for the eight week analysis and 18 sheep for the 16 week analysis. This suggests that the sheep tooth extraction model lacks the ability to discriminate between different treatment protocols and materials unless large numbers of animals are employed, which may limit the utility of this model.

4.2.2.2 Hard tissue fraction versus new bone and residual graft fraction

In our study we chose to measure the fraction of hard tissues within the ROI, and did not discriminate between new bone and the residual graft material fraction. The main reason for that was that no residual grafting materials were found in the groups treated with equine collagen-based products. Therefore, in five out of six groups, the hard tissue fraction was the same as the new bone fraction. The only group that had residual graft particles in the healed sockets was BX. In a study using a similar methodology and animal model, the fraction of residual graft in the BX group was 5.4 ± 5.6% after 16 weeks of healing (Liu *et al*., 2015). If we accept this value as representative of a healed sheep extraction socket grafted with bovine xenograft, compared to the overall mean values of the hard tissue fraction, the residual bovine xenograft would account for approximately 10% of the hard tissue fraction in this model, without changing the statistical outcomes.

This study was the first animal study to investigate equine collagen-based products from Resorba GmbH when used for ARP. Therefore, no direct comparison with previous studies was possible. We can however compare our data to that obtained in humans and in other animal models for non-grafted and xenograft grafted sites.

Cardaropoli *et al*. (2012) evaluated ARP in a human model, randomising 48 extraction sites in 41 patients into two treatment arms, a non-grafted control and a bovine xenograft covered by a collagen membrane. For histomorphometric analysis the authors used trephined sample from the implant osteotomy site. Similar to our results, they did not find statistically significant differences between the mineralised fractions in the BX test (44.80 ± 11.45%) and the non-grafted control (43.82 ± 12.23%) groups. In the test group, the authors found 18.46 ± 11.18% of residual xenograft particles, which did not impede successful placement of the dental implants.

In a recently published systematic review De Risi *et al*. (2015) performed a meta-analysis of histomorphometric data from 38 human studies of ARP procedures that described the relative fractions of new bone, residual graft and connective tissue. The authors found that both xenografts and alloplasts showed a volume of residual graft particles of around 20% after six months of healing. The percentage of connective tissue in xenograft and alloplast groups, as well as control sites varied between 45-55% at six months. Thus, the meta-analysis shows that the new bone fraction for these grafting materials in humans was between 35-45% after six months of healing. The authors concluded, that the lack of statistical differences between the different ARP procedures in terms of bone and connective tissue fractions, even at different follow up times, suggest that there may be no need to wait for as long as six months prior to the placement of a dental implant.

4.2.2.3 Clinical significance in implant dentistry

The importance of bone composition arises from the possibility of the grafted site being subsequently used for implant placement. For clinical purposes, the quality of bone is not described in histological terms. The surgeon is not usually aware of the volumetric percentage of mineralised or connective tissue within the potential implant site. Instead, many clinicians categorise the intended implant site based on the radiographic classification system developed by Lekholm and Zarb in 1985. According to this radiographic classification, Type I represents dense bone, characterised by a thick cortical plate and a dense appearance of trabeculation. Type IV is soft bone, and typically has a thin cortical plate and scarce radiographic trabeculation. In between these extremes lie Types II and III bone.

Histomorphometric analysis of edentulous ridge samples is believed to be correlated with the clinical classification of bone quality. Trisi and Rao (1999) attempted to quantify this in a study involving trephined bone specimens during implant surgery in 56 patients. The authors established the following ranges: samples with dense bone had a mean histomorphometric trabecular density of 76.54 ± 16.19%, whereas soft bone specimens scored 28.28 ± 12.02%. Interestingly, the operators' perception allowed them to distinguish with statistically significant confidence between dense and soft bone, but not between the intermediate gradients, even though they accounted for 48.3% of the specimens.

Type IV bone, which is mainly present in the posterior maxillary area in human patients, has always been a topic of concern in implant dentistry, as it is related to significantly lower implant survival rates (Bryant, 1998; Jaffin and Berman, 1991). Various methods have been suggested to increase the primary stability of implants in soft bone, such as under-preparation of the osteotomy (Degidi *et al*., 2015), development of implants with a more aggressive thread pattern (Valen and Locante, 2000) or drilling systems for densifying of osteotomy sites (Trisi *et al*., 2015). However, during healing, implant stability decreases for approximately two weeks after placement due to remodelling resorption (Branemark *et al*., 1997) and then increases due to gradual osseointegration.

During the past two decades there have been improvements in implant survival rates, especially in compromised areas such as the posterior maxilla; these improvements have been attributed to the surface modifications of newer generations of dental implants (Ivanovski, 2010). A meta-analysis of eight prospective multicentre studies has compared cumulative survival rates of 2614 machined-surfaced and 2288 Osseotite implants, stratified by bone density, as recorded by surgeons during osteotomy preparation (Stach and Kohles, 2003). The authors found that while in the machined surface group, the 4-years cumulative survival rates were significantly lower for soft bone (88.2%), compared to normal and dense bone (93.6%), in the Osseotite surface group the survival rates were similar (98.1% and 98.4% respectively). The conclusion was that bone quality has a definite impact on implant survival in machined surface implants, but not for surface modified implants.

Applying the cumulative volume of evidence, we can conclude that the histomorphometric differences reported by different studies, including our results,

between the percentage of bone or mineralised tissue of the potential implant site, may be evidence of the high variability of bone density between Type II and Type III bone, which is not clinically detectable by implant surgeons. These differences may be of limited clinical significance with respect to the newer implant surfaces available on the market.

In our study we could not detect any residual equine collagen-based material. This suggests that the collagen and the incorporated HA-TCP particles were completely resorbed in the sheep extraction socket model. While the complete resorption of the grafting materials is considered a favourable outcome by some clinicians, there is little research-based evidence that the small fraction of residual grafting material found with bovine bone xenograft has a negative effect on the long-term survival of dental implants.

4.2.3 Degree of alveolar ridge post-extraction remodelling

4.2.3.1 Non-grafted sites and BX positive controls

In our study, the alveolar ridge adjacent to the non-grafted extraction sockets became 30% narrower, whereas the BX group only reduced by 9.7% (p=0.002). Despite the high variability within the BX group, the results suggest that grafting extraction sockets with xenograft covered by a barrier membrane reduces alveolar ridge resorption by three-fold within the healing period of 16 weeks, in this sheep model.

There was one site where the residual ridge width (measured histologically) was significantly higher than the baseline intra-surgical width (specimen number 418 BX). The histological width of the healed site was actually 5mm wider than the baseline measurement. Careful re-evaluation of the histological slides suggests that the most coronal point used as the reference point for this measurement, shifted apically due to bone remodelling and loss of alveolar ridge height; the histological width was measured at a more apical point towards the middle of the alveolar ridge.

Exclusion of this specimen from the dataset had no impact on the calculations of the mineralised tissues within the ROI. However, there was a notable change in comparisons of alveolar ridge reduction. The difference between the CON and BX group became a trend rather than a statistically significant result, as with the new calculations, the p-value rose from p=0.002 to p=0.052. These results have to be viewed with caution, given the cut-off value of $p \leq 0.05$ to determine statistical significance.

Our findings are in agreement with previous research. In a pioneering study on human plaster models more than half a century ago, it was determined that both in the maxilla and the mandible, the buccal bony plate resorbs more that the lingual one (Pietrokovski and Massler, 1967). In a later study the authors confirmed their conclusions following a histological study in a rhesus monkey model (Pietrokovski and Massler, 1971).

A mean reduction in the alveolar ridge width of 35% was found in the non-grafted control sites (versus 12% for sites grafted with Bio-Oss Collagen®) in a dog model randomised controlled study of six months duration (Araujo *et al*., 2009; Araujo and Lindhe, 2009b). It is important to mention that in this study, only one root of the premolar was extracted, therefore the reduction measured was relative to the bone

around the root that was left in situ, and under the assumption of similarity of the ridge dimensions between the two roots.

In a prospective randomised human trial it was shown that untreated tooth sockets resulted in a 50% ridge width reduction within 12 months (Schropp *et al*., 2003). Two thirds of that resorption occurred in the first three months after the extractions. Systematic reviews and meta analyses have confirmed these findings, suggesting that a 32% reduction in the horizontal ridge dimension can be expected after three months of undisturbed healing, increasing to between 29% and 63% at six to seven months (Tan *et al*., 2012). Other reviews of the literature have pointed out that ARP procedures are effective in limiting the post-extraction ridge resorption compared with non-grafted healing (Avila-Ortiz *et al*., 2014). However, complete prevention of post-extraction dimensional changes is considered impossible (Ten Heggeler *et al*., 2011).

4.2.3.2 Sites grafted with test materials

In our study, similar levels of horizontal ridge width resorption were noted for the two test groups, CC and CO, 32.7 ± 14.7% and 30.3 ± 12.3% respectively. This was similar to the ungrafted control group (30.5 ± 23.2%) after 16 weeks of healing. Compared to the ConeOss CO group, the Collagen Cone CC group lacked biphasic calcium phosphate particles. However, this did not affect the amount of resorption in our model. In our study design, these sites had no collagen barrier membrane.

The differences between CO and CC groups compared to the control (BX) group were statistically significant. When the p-values were recalculated after removing the suspicious outlier in the BX group, compared to CC, the difference between the groups remained significant (an increase to p=0.018 from p=0.001), however the difference between BX and CO lost its statistical significance and became a noticeable trend (an increase to p=0.056 from p=0.004).

On the other hand, test sites with a collagen membrane, either embedded in the tested products (collagen cone + “Sombrero”, CS) or added as a separate product (collagen cone with particles + collagen membrane, CO+CM), showed less reduction in the horizontal dimension, 22.6 ± 23.9% and 20.6 ± 20.3% respectively. However, no statistically significant differences were found between these groups and either negative (CON) or positive (BX) control groups.

When the equine collagen-based products were compared with each other, no statistically significant difference was found between the groups. More differences were shown between the groups CC and CS, however, the results lacked statistical significance (p=0.074).

These results, although lacking statistical power, suggest that in our experimental model there was no benefit from the addition of biphasic calcium phosphate granules to the collagen cone in terms of reduction of post-extraction resorption of the alveolar ridge. The feature that made the difference, though not statistically significant, was the coverage of the grafted socket with a barrier membrane.

These findings are controversial when compared to the current literature. In a dog model similar to that described earlier, Araujo's group (2009) found that grafting the extraction sockets with a biphasic calcium phosphate graft was effective in ARP, without requiring a barrier membrane (Lindhe *et al*., 2013). In this study, however, the graft material comprised of α-TCP and biomimetic hydroxyapatite, the ratios of TCP/HA were not mentioned and no non-grafted control sites were used. Unlike our study, the authors reported that the graft material was not significantly resorbed inside the grafted sockets within three months of healing.

In another, slightly different dog model, Boix *et al*. (2006) extracted six maxillary and four mandibular premolars in three beagle dogs. The distal sockets were filled with injectable biphasic calcium phosphate, and the mesial sockets were left to heal as non-grafted controls for 13 weeks. No barrier membranes were used. The authors found that grafted sites had less vertical height loss, however they did not measure the width of the ridge.

In a recent human randomised controlled clinical trial on 36 patients, Mayer *et al*. (2016) used a 1:1 mixture of BCP (4BONE, Biomatlante ZA les Quatre Nations, France) and BCS (BOND BONE, MIS Implant Technologies Ltd., Israel) in grafted sockets versus non-grafted controls. The baseline measurements were performed with a calliper, and the final measurements were obtained in the same way at re-entry after four months of healing (Mayer *et al*., 2016). The flaps were coronally advanced to achieve primary closure without membrane barriers. At re-entry, no statistically significant changes were found in the treatment group compared to baseline measurements, whereas control sites showed significant width reduction.

Regarding the use of a barrier membrane, earlier literature suggests that a membrane alone can reduce the post-extraction reduction in the width of the alveolar ridge (Lekovic *et al*., 1997; Lekovic *et al*., 1998), However, in a recent review of literature, Horowitz and co-authors (2012) did not find significant evidence that placement of barrier membranes over the grafting material is necessary.

4.3 Discussion of the Model and the Method

In this section we will discuss in detail the elements of our experimental model that could have had an impact on the results of our study, compare the methods to those used by other groups in previous published work and suggest possible modifications to our method for future studies of ARP using the sheep animal model.

4.3.1 The sheep model

Sheep is a domestic animal that is a part of the human food chain in multiple cultures. The use of sheep as experimental animals is advocated over the use of companion animals such as dogs (An and Friedman, 1998) that have been widely used in periodontal research (Araujo and Lindhe, 2005; Berglundh and Lindhe, 1997; Cardaropoli *et al*., 2005; Fickl *et al*., 2008b; Rothamel *et al*., 2008). The sheep model appeared in the dental literature almost 20 years ago, in research of maxillary floor augmentation (Haas *et al*., 1998). Earlier publications using this experimental animal emerged from the University of Otago, as sheep are readily available for research in New Zealand. The sheep have been used to study regeneration of furcation defects (Danesh-Meyer *et al*., 1995), chronic inflammation models (Whelan *et al*., 1997), periodontitis and periodontal defects (Baharuddin, 2010), bone healing (Salmon and Duncan, 1997), dental implants (Duncan, 2005,2006) sinus grafting (Philipp *et al*., 2014) and recently for bone replacement materials grafting in extraction sockets (Liu *et al*., 2015).

4.3.1.1 Animal heterogeneity

For this study 11 sheep were purchased from a commercially available flock. The experimental animals were not inbred, and therefore were heterogeneous study subjects. This heterogeneity may account for differences in healing patterns between the animals, and differences in the obtained results, as can be seen from the standard deviation of the measured parameters. The advantage of this approach is that the results of the study can be used with more confidence to make possible conclusions about the effects of grafted materials in humans, which also present a highly heterogenous population. The disadvantage, however, is that where the sample size (N) is small,

heterogeneity may mask the effects of the treatment modalities, such that statistically significant comparisons are not revealed.

4.3.1.2 Protocols for tooth extraction in sheep

Bilateral mandibular premolars were chosen to be extracted for the current study. The number of grafting materials tested dictated the need for multiple extractions. Mandibular molar teeth were not included due to limited access and concerns about the postoperative residual grazing capacity of the animals.

A number of extraction techniques have been developed for extracting sheep premolar teeth. In the initial extraction protocol developed at the University of Otago (Duncan, 2005), a full thickness mucoperiosteal flap was raised and a shallow osteotomy, involving the coronal aspect of the periodontal ligament, was prepared around the teeth. The premolar teeth were then vertically sectioned to the furcation level, and separated roots were elevated. Sectioning of the teeth was performed due to the presence of dense and non-elastic mandibular cortical plates (May, 1970), that otherwise could result in root fractures. Following a healing period, the edentulous space was ready for experimental implant placement.

In a study of immediate dental implants in a sheep model, a Belgian group (Vlaminck *et al*., 2008) had also utilised vertical sectioning of mandibular premolars. The authors suggested the potential use of maxillary premolars in order to create a larger number of extraction sockets per animal. They have also proposed the use of periotomes for less traumatic extractions in future studies, as root fractures required more aggressive methods to remove the residual fragments.

In a recently published study Liu *et al*. (2015), did not remove any cortical bone on the coronal aspect, but rather severed the periodontal ligament around mandibular premolars using Piezosurgery® extraction tips. This assisted in mobilising the premolars, and the teeth were then extracted without longitudinal sectioning. However, the use of piezosurgery resulted in increased surgical time, and root fractures were still common.

In our study, the extraction technique was further modified. After raising full-thickness mucoperiosteal flaps both bucally and lingually, a chisel and a mallet were used to

wedge the interdental contact areas. Extraction forceps and elevators were used simultaneously to remove the teeth in the mesio-occlusal direction. This technique resulted in predictable extractions without the need for ostectomy or longitudinal sectioning of the teeth. Root fractures were uncommon in our study.

The current study was the first surgical experience with the sheep model for the primary investigator. The equine collagen-based tested materials had not been previously used by any of the clinical operators. A steep learning curve was observed in the surgical procedure, with one animal being operated during the first day of surgery, and then up to two animals per day on the consecutive days. Surgical experience of the operator had been identified as an important factor for success of surgical procedures in the oral cavity (Aghaloo and Moy, 2007; Cairo *et al*., 2012)

4.3.1.3 Healing of extraction socket in a sheep model

Duncan (2005) has comprehensively described healing in the sheep model. The healing times derived from his research are summarised in Table 1.3. In general, wound healing progresses faster in sheep than in human subjects. In the current study, the experimental animals were euthanised after 16 weeks of healing, which would correspond to 21 weeks of healing in humans. A similar time-point was chosen for a previous study of grafted extraction sockets in a sheep model (Liu *et al*., 2015). The choice of this healing period is not arbitrary. It is widely accepted in clinical practice that grafted extraction sockets are left to heal for a period of four to six months prior to re-entry and the placement of a dental implant (Branemark, 1985; Esposito *et al*., 2010; Quirynen *et al*., 2007). Therefore, the chosen time for euthanasia would coincide relatively with the timeframe appropriate for an implant placement in the clinical situation.

We examined histological specimens at one time-point of 16 weeks. Full regeneration of the buccal defect was seen in all specimens, both grafted and non-grafted. Furthermore, no evidence of residual test graft materials was found in our study, although residual xenograft was found in the positive control (BX) sites. In a recent study with a similar methodology (Liu *et al*., 2015), the mean fraction of residual xenograft, after 16 weeks in the sheep extraction model, was 5.4%. Therefore, the use of two time-points with an earlier one in a sheep model would be advisable, in order to more closely follow the healing process of a grafted extraction socket.

4.3.1.4 Standardised buccal dehiscence defect

Creation of the buccal defect was chosen for our model in order to closely simulate a common clinical condition, where the buccal bony plate is damaged either during the extraction process or as a result of the pre-existing inflammatory or infective condition (Leblebicioglu *et al*., 2015; Venkateshwar *et al*., 2011). We found that a buccal defect of this size healed uneventfully within 16 weeks, even in the CON group. We question the utility of creating such a defect in this animal model if future studies are undertaken. Alternatively, the healing pattern of the buccal defect in a sheep model can be followed up in more detail in future studies utilising multiple time-points over a shorter healing period.

Furthermore, the buccal defect caused issues during analysis. The buccal cortex of the study animals was traumatised in three ways: by raising a full thickness flap, by extraction of the teeth, and by creation of buccal defects. We suggest that the effect on post-extraction remodelling of the buccal cortex was cumulative. A significant portion of the buccal cortex in the healed sections was composed of newly formed bone, thus complicating the histometric measurements. While the elevation of the flap could not be spared, due to extreme difficulty of extracting sheep teeth, the creation of the defect has inserted another variable in this new model that still needs comprehensive validation.

4.3.1.5 Bone overgrowth outside the original envelope in a sheep model

Due to ethical considerations, all new implantable devices and biomaterials should be tested in an animal model, prior to trials in human subjects. When choosing between different available animal models, the key question is the degree of transferability of the results obtained in pre-clinical animal studies to human physiology (Pellegrini *et al*., 2009; Stadlinger *et al*., 2012). Despite the differences in some of the physiological aspects, large animals are more similar to humans in bone remodelling and regenerative capacity (Aerssens *et al*., 1998; Pearce *et al*., 2007).

One of the differences between sheep and human mandibular bone is the generation of new bone lateral to the original cortical bone, as was observed in our study. Unlike humans, sheep are ruminants, and due to the constant grazing, their mandibles are exposed to strong shearing and bending forces. This overgrowth might serve as a compensatory mechanism that allows the sheep mandible to withstand these forces

after the loss of three masticatory units on each side. This finding accords with the results of Duncan *et al*. (1998a,b) and Duncan (2005), who reported an increase in the thickness of the alveolar cortices due to external overgrowth following the creation of critical size defects and the installation of dental implants in the sheep mandible.

Similar periosteum-induced osteogenic activity is not evident in human subjects. In fact, it is found in pathological conditions, such as Garre's osteomyelitis (Kadom *et al*., 2011) or osteosarcoma arising in previous Paget disease (Rana *et al*., 2009)

While not being of extreme importance in studies of biocompatibility of biomaterials or osseointegration of dental implants, this phenomenon can negatively influence the use of a sheep model in studies of ARP.

4.3.1.6 Reference points for measurements

In our study we examined several ways to allow for comparison of post-extraction and post-euthanasia measurements. None of them proved to be effective, therefore another approach needs to be considered for future studies using this model.

During the surgery we created two defects on each side of the mandible using a round bur, and filled the defects with amalgam to serve as radiographic markers. Implantable radiographic markers have been widely used in studies of bone growth (Haas *et al*., 1998), and similarly can be used in studies monitoring bone resorption. If plain radiography is used to trace the changes, controlling the orientation of the markers in relation to the film is an obvious limitation. In contrast, the use of three-dimensional radiography, which became much more readily available, allows a more accurate comparison of the images taken at different time-points, provided we know the exact physical dimensions of the markers. The other problem with this technique that we encountered was that the implanted markers did not always remain in the place where they had been inserted. In fact, the amalgam markers were radiographically noticeable, fully or partially, only in two of the experimental animals.

Immediately post-extraction, we took impressions of the edentulous mandible using custom trays and polyvinyl-siloxane impression materials. The impressions were poured in plaster, and the models were scanned in order to obtain a three-dimensional image. After euthanasia, the resected mandible blocks were scanned with a dental CBCT (Sirona Galileos, Salzburg, Austria). Efforts to overlay and compare these two

three-dimensional images were made by Prof. Albert Mehl from the Department of Computerized Dentistry at the University of Zurich. However, he could not find an algorithm that could compare the objects. According to his professional opinion, two DICOM data sets would be required to do so. Having an option to perform computed tomography scans at different time-points with the same setting would be advantageous and could provide us with comparable information. This radiographic technique is used in both animal (Whelan *et al*., 1997) and human models (Mohammed *et al*., 1998) as a reproducible non-invasive measurement. However, in our animal research facility, this equipment is currently not available. But, even if three-dimensional radiographic evaluations were at our disposal, some implanted radiographic markers could be advantageous, as the cusps of the teeth in ruminants are subject to constant wear from grazing (Kaiser *et al*., 2009), and therefore might not be reliable reference points.

4.3.1.7 Multiple extraction sites

In our sheep experimental model, there is an edentulous diastema between the premolars and the incisor area and in our study we extracted three double rooted mandibular premolars on each side. This could be comparable to extracting three molar teeth in a human subject without premolars present prior to the extraction, thus creating a long edentulous span. This could impose certain limitations on our ability to generalise the results of the study, as follows.

Alveolar ridge remodelling after tooth extraction has been studied for the last 50 years (Pietrokovski and Massler, 1967). Human studies were limited to investigating the changes in single extraction sockets (Pietrokovski and Massler, 1967; Schropp *et al*., 2003). Landmark studies using dog models (Araujo and Lindhe, 2005; 2009b; Cardaropoli *et al*., 2003; Cardaropoli *et al*., 2005) were also limited to evaluating the dimensional changes in single tooth extraction sites with and without grafting. They used hemisection of the canine premolars with extraction of one of the roots and root canal treatment of the remaining root. This proposed technique allowed the measurements of horizontal and vertical changes of the extraction sites compared to the dentate adjacent sites that were assumed to have remained unchanged during the healing period. However, it also limited the applicability of gathered information to single tooth extraction sites surrounded by existing dentition.

There is a lack of information about the resorption rates of alveolar bone in adjacent multiple extraction sites. The literature in this field is scarce. Only recently has a study been published comparing the alveolar ridge remodelling after one, two and three consecutive teeth in a dog model (Al-Askar *et al*., 2013). In their model, the authors used the extraction classification system suggested earlier by one of the group members (Al-Hezaimi *et al*., 2011). Using five beagle dogs and recording measurements by micro-computerised tomography after four months of healing, the authors found that the residual bucco-lingual width of the alveolar ridge was significantly reduced at sites with multiple extractions, compared to single tooth extractions, in both anterior and posterior locations. It is important to note that the described study used only five experimental animals, with teeth remaining on both sides of the edentulous span, and no histological results were reported.

The same research group published another study evaluating alveolar ridge resorption using the same dog model (Al-Hamoudi *et al*., 2015). The extracted sites were grafted with particulate xenograft, covered by a collagen membrane, and primary closure was achieved by coronal advancement of the flaps. There were no ungrafted control sites. Micro-computerised tomography after four months of healing did not show significant differences in the bucco-lingual width of the alveolar ridge between different groups and different sites at various heights from the cemento-enamel junctions of the teeth adjacent to the edentulous gap. Comparing their results to their previous study with a similar model, the authors concluded that ARP procedures are more crucial in multiple extraction sites, as larger reduction of the ridge width can be prevented.

To the best of our knowledge, ours is the first description of multiple extraction sites adjacent to a wide edentulous diastema for the study of ARP. This warrants for further evaluation and validation of the protocol in future studies. Using different grafted products in such spatial proximity risks possible lateral spillover of the grafting materials. Incorporating negative non-grafted control sites in random locations in relation to the unilateral remaining dentition might further complicate the interpretation of the study results.

4.3.1.8 Sectioning of the mandibular block

After euthanasia, resected blocks of mandibular bone were sectioned into individual grafted sites prior to embedding in resin. Due to the lack of radiographic markers or the

lack of ability to identify the grafted sites on the radiographic images, double superimposition had to be used for sectioning. During the first stage of superimposition, an immediate post-surgical periapical radiograph, that visualised the grafted defects, was superimposed with the post-mortem periapical radiographs, using the first mandibular molar, inferior alveolar canal and, if present, the lower border of mandible as reference points. The markings for the proposed sections were drawn on the post-mortem periapical radiograph. These were transferred to the mandibular section during the second stage, in which the post-mortem radiograph was superimposed on the mandibular block using the mesial aspect of the first molar as reference.

This two-staged process of superimposition could increase the cumulative error into the final sectioning procedure. An additional source of error could be different angulation of radiographs, due to anatomical limitations, between the post-surgical and post-mortem radiographs. In our opinion, a simple measurement during the surgery could simplify future sectioning, and reduce the requirement for superimpositions. This could be achieved by measuring the linear distance in millimeters between the mesial aspect of the first mandibular molar to each of the grafted sites, and especially to the mesial and distal aspects of the surgically created defects, as these are the areas of interest for subsequent histological evaluations. This measurement could be utilised in future studies using the same experimental animal model.

4.3.2 Sources of bias - blinding of the examiners

In our study, the primary investigator who analysed the outcomes of the experiments was actively involved in animal surgery and preparation of the specimen. At the time of surgery, the blinding of the operators was impossible due to the distinct differences between the grafting materials. It was also difficult to achieve blinding at the preparatory stages following euthanasia, as the tissue blocks and surgical sites had to be labelled for identification. While no residual grafting materials were found in the four treatment modalities studied, the residual particles in the BX group had a distinct histological appearance and thus were easily identified.

Despite the lack of blinding, the measurement methods were found to be very reliable and reproducible when 10 randomly selected specimens were re-examined by the primary investigator two months after the initial analysis. A high level of agreement was also found after repeat analysis by a research team member who was blinded as to

which group the histological specimens belonged to, suggesting a low risk of bias during analysis.

4.3.3 The grafting materials

In this study we compared a variety of equine collagen based materials for ARP with and without barrier membrane coverage against non-grafted naturally healed sockets and sockets grafted with bovine xenograft particles and covered with porcine collagen membrane.

4.3.3.1 Bio-Oss Collagen®

Bovine xenografts are widely used for bone grafting. Bio-Oss® is the market leader both in clinical practice and in periodontal research. Some even describe it as the new "gold standard" for bone grafting (Schneider *et al*., 2009). A simple search in PubMed engine with "Bio Oss" as keyword yields 925 results. Bio-Oss Collagen® is the newer version of this popular grafting material; this contains 10% collagen which contributes to its handling properties and easier adaptation within grafted defects. Reported survival rates of dental implants placed into alveolar ridges and maxillary sinuses augmented with Bio-Oss are comparable to implants placed into natural bone (Aghaloo and Moy, 2007; Sanz-Sanchez *et al*., 2015).

Xenograft particles have slow rates of resorption, and residual grafting material can still be found in regenerated sites after a prolonged healing time (Artzi *et al*., 2000). Some controversy exists in the literature regarding the desirability of a full resorption of the grafting materials. While some studies reported delayed bone healing in extraction sockets grafted with this material (Araujo *et al*., 2008; Araujo and Lindhe, 2009b), especially in the early stages (Araujo *et al*., 2009), to date, there is no reported evidence of long-term negative effects. Furthermore, osseointegration of subsequently placed dental implants was not hindered in dog (Berglundh and Lindhe, 1997) or human models (Valentini *et al*., 1998).

There is sufficient cumulative scientific evidence for the use of Bio-Oss® and Bio-Oss Collagen® as positive controls in studies of ARP. In our experimental model, the Bio-Oss (BX) group had significantly less reduction in alveolar ridge width after 16 weeks

of healing. The mean width reduction in the BX group was only one third of the reduction observed in the non-grafted CON group.

On the histological level, the residual BX particles in our study were found well integrated and surrounded by the newly formed bone. The presence of multinuclear osteoclast-like cells suggested ongoing resorption of the particles and replacement with native bone.

4.3.3.2 Biphasic calcium phosphates (BCP)

Alloplasts are synthetic materials used for bone grafting. Their main advantages include a much more cost-effective production and batch homogeneity, compared to allografts and xenografts. Alloplasts might be considered a safer alternative to xenografts and alloplasts, and can be more culturally acceptable due to religious and animal rights concerns.

BCP, a mixture of HA and β-TCP, is being proposed as a potential material for bone regeneration (LeGeros *et al*., 2003). Its chemical composition shows much similarity to the inorganic component of the natural human bone (Nilsson *et al*., 2004). The HA component of the composite material serves as a slowly resorbable scaffold, while the more rapidly-resorbing β-TCP serves as a local reservoir of calcium and potassium ions for apposition of new bone. These materials have attracted interest in the research of ARP in animal and human models (Boix *et al*., 2006; Kesmas *et al*., 2010; Machtei *et al*., 2013; Mayer *et al*., 2016).

Different ratios of HA/β-TCP have been tested in different study models. In a study testing different compositions of BCP for treatment of periodontal osseous defects in a dog model, higher HA/β-TCP ratios gained more attachment with 85/15 showing superior results (Nery *et al*., 1992). Another study in mini pig mandibles, comparing different formulations of BCP against autogenous bone and bovine xenograft, found that while 20/80 BCP achieved similar results to autografts, 60/40 and 80/20 BCP grafts resembled the results of the xenograft group (Jensen *et al*., 2009). When the amount of bone formation and degradation of graft material was compared between the BCT-grafted groups, the authors noted it was inversely proportional to the HA/TCP ratio. However, a recently published study showed similar results between 70/30 and 30/70 BCP groups in a study investigating reconstruction of deficient mandibular ridge

in dogs (Nevins *et al*., 2013). It is important to mention that a barrier membrane in those studies covered all the grafting materials.

In our study, the BCP material used was Cone-Oss®, with a HA/β-TCP ratio of 60/40. No residue of this grafting material was found in our model after 16 weeks of healing. It could have been advantageous to have some additional earlier time-points along the healing process in order to track the resorption rate of the grafting material in a sheep model.

Our study showed that the mean reduction in the width of the alveolar ridge reached 30% when a collagen membrane did not cover CO, which was similar to the non-grafted CON group. In a CO+CM group, in which Cone-Oss® was covered by a collagen membrane, the horizontal resorption dropped to 20%. As previously noted, our data lacked the statistical power to detect significant differences between the groups.

4.3.4 Exclusion of experimental sites

In our study, one sheep was euthanised on the first postoperative day, to serve as a histological baseline. The remaining 10 sheep were allowed to heal for 16 weeks. One of them, sheep number 409, showed a complete lack of osseous healing in all six surgical sites, as well as exuberant bone remodelling suggestive of chronic inflammation. The specimens of this experimental animal were excluded from our analysis. The remaining nine animals provided us with 54 experimental sites, and thus 108 histological slides for evaluation. Ten out of 108 slides were also excluded from the analysis due to inability to detect the reproducible reference points necessary for measuremets. The excluded slides were labeled as "N/A" in sections 1 and 3 in Appendix IV. This reduction in the size of the sample could have decreased the power of our study.

4.3.5 Histomorphometric analysis of hard tissue fraction in ROI

In our study we chose to measure the hard tissue fraction in the ROI using a semi-automated segmentation technique. Hard tissue was delineated by manual selection of thresholds for each and every slide, filtering out the portions of images that lay outside

the selected values of hue, saturation and brightness (Figure 2.10). The identified remaining fraction within the selected area of interest was measured and calculated as a percentage.

In histological studies, there are two commonly used methods to calculate fractions of different tissues within a selected ROI. The earlier studies have predominantly used the technique of light-point counting. A matrix of standardised dimensions with 100 evenly dispersed points allowing light transition is superimposed on the histological slide. By counting the total number of light points coinciding with a certain tissue type, a relatively accurate estimation of the percentage of area occupied by this tissue type can be calculated. The technique is simple to use, easily reproducible, and does not require a learning curve or any computerised calculations. This technique was first described in the field of periodontology in 1973 (Schroeder and Munzel-Pedrazzoli, 1973) and was later modified and widely used in the studies of grafting materials for ARP (Araujo and Lindhe, 2009b; Artzi *et al*., 2000; Cardaropoli *et al*., 2005)

In our study we used computer-assisted image analysis software to measure the tissue fractions, similar to later studies in the same field of research (Heberer *et al*., 2008; Hong *et al*., 2014; Liu *et al*., 2015).

The results of the analysis may change depending on what technique is used to gather the data. Leichter and co-workers (1998) compared the results of both techniques in a study evaluating bone regeneration in furcation defects in a sheep model. The authors found that, despite the high reproducibility rates of each technique, the results obtained by either technique are not directly comparable with each other. Bone percentages within the defects measured by using stereology were consistently higher compared to those measures by computer-assisted analysis of the same histological slides. It was also noted that stereology was easier to learn, and that the total amount of time spent on analysis of each slide was three times greater in the computer-assisted analysis group. Similar results were obtained in a later study (Duncan, 2005) comparing utilisation of both techniques in analysis of the percentage of bone-to-implant contact in a sheep model. The author found the results obtained with the use of stereology were significantly higher compared to the results obtained by computer-assisted analysis of the images. Other studies, however, found that both techniques provided comparable results, without statistically significant differences (Amenabar *et al*., 2006; Montgomery *et al*., 2008).

It may be concluded, that while stereology remains the gold standard for such measurements due to the ease of use and no inherited bias that is introduced by the researcher when manually selecting the thresholds for computer-assisted segmentation, both techniques are valid; caution is advised when comparing the results of studies where the measurement techniques differed.

The semi-automated computer-assisted analysis technique chosen for our study is highly dependent on the process of histological preparation, contrast of histological staining, adequate illumination and focus of the microscope at the time of capturing the images. All these factors eventually affect the quality of the digital images available for the analysis, and therefore the difficulty of manually setting up the correct thresholds for segmentation.

For example, in non-demineralised sections, the initial 650µm thick sections were ground down to the final thickness of 80-100µm, and then polished prior to histological staining. The process of grinding and polishing created a smear layer of ground debris that concentrated adjacent to the bone in the slides. Despite the acid etching as part of the staining protocol, some of the debris remained on the slides and created an artifact, which required additional image processing before colour thresholding could be applied.

4.3.6 Histometric analysis methodology

The sheep animal model has been previously used for sinus floor augmentation (Haas *et al*., 2002a; Haas *et al*., 2002b), dental implant placement (Duncan *et al*., 2015; Vlaminck *et al*., 2008) and bone grafting materials (Liu *et al*., 2015). Our study was the first to measure the changes in the horizontal dimension of the alveolar ridge after teeth extractions in a sheep model.

In a landmark study evaluating post-extraction changes in man, it has been established that an average of 30% reduction in the alveolar ridge width can be expected within the first three months, increasing to 50% within a year (Schropp *et al*., 2003). This is in line with the results of our study, confirming mean alveolar width reduction of 30.5% in the non-grafted control group (CON). The decrease in vertical dimension of the buccal plate was reported to be 1.2mm following 12 months of healing (Schropp *et al*., 2003). Viewing these results from the perspective of the suitability of the edentulous site to

accept a dental implant, a loss of approximately 1-1.5mm in the vertical dimension can, in most cases, be managed during implant placement surgery. However, a loss of 30-50% of ridge width may require a separate grafting procedure, before implant placement can be attempted. Moreover, choosing a 10mm dental implant instead of an implant longer by 1mm will reduce the total surface area available for osseointegration by 9% (1/11), whereas decreasing the implant diameter by 1mm from 4.5mm to 3.5mm would result in a 22% (1/4.5) decrease in the implant surface area.

There are several methods to measure dimensional changes of the alveolar ridge. Bone sounding is the first method that has been long used in implant dentistry in order to determine the dimensions of the alveolar ridge without employing three-dimensional radiography (Quinlan *et al*., 1998; Stumpel, 2008). This method involves custom made occlusal stents, allowing measurements to be made in the same site at consecutive appointments. In human subjects the method requires local anaesthesia, as soft tissues must be punctured with a sharp instrument in order to reach the alveolar bone. In our experimental model this approach would have proved more complicated for several reasons. For each measurement the animal would have had to be put under general anaesthesia. Furthermore, the dental anatomy of the ruminants included an edentulous span between the premolars and the anterior teeth. Due to the lack of teeth mesial to the extracted premolars, the stability of such a stent would be questionable. Moreover, problems could have arisen with occlusal adaptation of the stent at consecutive time-points, since the occlusal morphology of the grazing animals could have undergone some changes due to functional tooth wear (Every *et al*., 1998; O'Brien *et al*., 2014). The removal of lower premolar teeth bilaterally could potentially increase the tooth wear of the remaining molars; this needs to be determined in future studies. Finally, cortical bone overgrowth cannot be identified with sounding, and thus would not allow us to accurately measure the post-extraction resorption.

Computed tomography is a commonly used method of non-invasive evaluation of alveolar ridge dimensions in implant dentistry (Benavides *et al*., 2012). In an animal model, the experimental animals have to be put under general anaesthesia to take the scans. During the planning phase of our study, we did not have the appropriate facilities available for animal computed tomography. Furthermore, the bone overgrowth outside the pre-existing bony envelope that was observed in histological specimens presented a further problem that could have impacted on the suitability of CT in our animal model.

When we used the dental CBCT to scan the harvested mandibular blocks, radiographically we could not differentiate between the pre-existing and the newly formed cortical bone. This could have been due to either insufficient resolution of the available scanner, or the similar radiographic appearance between the pre-existing and the newly formed cortical bone.

In our study we used two sets of measurements to calculate the changes in alveolar ridge width. The baseline ridge dimensions were registered during the surgical phase, using a direct measurement of the ridge width with a caliper at the crestal level of the ridge mesial to the buccal defect. The final measurements were obtained from the histological specimens, measuring the horizontal distance from the most coronal identifiable point of the pre-existing bone on the lingual wall to the outer surface of the buccal wall.

Our chosen methodology for this study clearly has several limitations that may make the interpretation of the results more difficult. These could not be foreseen in the planning of our study, but should be taken into account in future experiments.

First, the baseline measurements were taken clinically and the final measurements were done on histological slides. Each processing step (fixation, sectioning, embedding, grinding and staining) changed the dimensions of the alveolar ridge and potentially introduced an error. In our model we were not able to overcome this problem. At baseline, axial histological sections of the mandible could not be obtained from a living experimental animal for obvious reasons. Following euthanasia, direct clinical measurements of the alveolar bony ridge could not be performed, as the overlying soft tissues had to be preserved for histological processing. Even if that could have been done, we would not have been able to determine visually whether the visible cortical bone was pre-existing or a recently formed overgrowth.

Second, the baseline measurements were done mesial to the buccal defect, whereas the histological slides were shifted slightly distally, as they represented the centre of the grafted socket and defect. Moreover, the created buccal defects underwent complete clinical and radiographic healing, and could not be identified in the mandibular blocks. Therefore, in future studies shorter healing periods should be considered, and/or the creation of the buccal defect will have to be abandoned and measurements should be

performed and sections taken at places that could be repeatedly and reproducibly identified.

Third and lastly, given the impression that in our model notable vertical resorption might have occurred, it can be argued that the baseline and the final measurements of horizontal changes were not necessarily recorded at the same vertical height. It may be more relevant if the most coronal point of pre-existing cortical bone on the lingual wall that we used as a reference point had shifted more apically due to post-extraction bone remodelling. In future studies, consideration should be given to identifying constant and reproducible reference points for detecting changes in vertical dimensions of buccal and lingual cortices.

When planning a study in an animal model, it is important to review the techniques that have previously been used by others. Being the pioneers in developing the current protocol in sheep, we could only learn from other animal models. The most popular animal used for ARP studies is the dog.

As previously mentioned, Araújo and Lindhe (2005, 2009b) have carried out a series of *in vivo* studies exploring the alveolar ridge remodelling using the beagle dog model. In their protocol, canine premolars were hemisected, one of the roots was rootfilled and left in place, while the other was extracted and either grafted or left for spontaneous healing. For the histometric measurements, the authors made an assumption that the alveolar process is similar in shape and size for both mesial and distal roots of the canine premolars, and therefore the baseline measurements of dimensional changes were obtained after euthanasia from the sites with the remaining roots in situ.

Making a similar assumption in the sheep model would lead to additional errors, as the mesial and distal roots of sheep mandibular premolars were found to be different in shape and size (Duncan, 2005). Other problems would need to be overcome if repeating Araujo's model is attempted in sheep. Root canal treatment of sheep teeth has yet to be attempted. The other problem would be the extraction of only one of the roots in sheep premolars, as due to the thickness of the cortical plates the teeth can only be elevated mesially. Therefore, extraction of a single root would be impossible without significant osseous resection, which contradicts the objectives of this model.

4.4 Conclusions and recommendations for future research

4.4.1 Conclusions

The aim of our study was to evaluate novel equine collagen-based bone grafting materials for alveolar ridge reservation versus non-grafted control sites, with bovine xenograft as the positive control. We evaluated the histological specimens for healing patterns, carried out histomorphometric analysis of mineralised tissue development in a region most suitable for implant placement, and compared the treatment modalities with respect to the reduction of post-extraction loss of the alveolar ridge in the horizontal dimension.

All experimental sites healed uneventfully. The sheep showed exceptional healing capacity, with all the buccal dehiscence defects completely resolved with newly formed bone. During the healing process, the amalgam markers were lost in the majority of the specimens. Furthermore, as part of the remodelling process, newly formed bone was shown to appear outside the pre-existing bony envelope of the mandible to allow the bone to withstand the shearing forces during grazing, thus making the analysis of the results more challenging. These limitations will need to be addressed and further refined in future studies using the sheep model for ARP.

No distinctive patterns of healing were documented for any of the treatment modalities. No statistically significant differences were observed between the study groups in the percentage of coronal and marrow bone bridging, and the fraction of mineralised tissues within the redefined ROI. There were no statistically significant differences between the amounts of hard tissue formed in the un-grafted tooth socket when compared with any of the grafted sites.

Residual bovine xenograft particles remained embedded in newly developed bone, but underwent gradual resorption. Equine collagen-based grafting materials were fully resorbed within 16 weeks of healing. Further studies with multiple healing time-points are required to more closely establish their rate of resorption.

Bovine xenograft covered by a membrane barrier decreased the post-extraction loss of ridge width three-fold. Treatment protocols without a barrier membrane showed a level

of the reduction in alveolar ridge width similar to the non-grafted controls. The tested materials showed complete resorption during healing without preserving the bone dimensions.

This study reports a modified tooth extraction socket in a sheep model with clinical applications in the field of ARP. This is the first time that the equine collagen-based grafting materials have been tested in a large animal model.

While the sheep tooth extraction model may require additional refinements for evaluating different materials and treatment modalities for ARP, the results of our study have suggested that the experimental equine collagen-based materials are biocompatible, undergo full resorption within 16 weeks, do not prevent ingrowth of newly formed bone into the grafted sockets, and have a tendency to reduce the alveolar ridge resorption, when covered by a barrier membrane. With larger study samples, this trend may be proven statistically significant. The results obtained from the BX group in our study further support the use of xenograft as the benchmark for comparison of other grafting materials for ARP.

4.4.2 Clinical significance

The dimensions of the alveolar ridge following dental extractions are of major importance for subsequent prosthetic rehabilitation. Insufficient height and/or width of the edentulous ridge may preclude placement of dental implants without challenging vertical and/or horizontal augmentation of the residual ridge. Various ARP techniques have been shown to reduce, but not completely prevent, the post-extraction dimensional changes (Darby *et al*., 2009). Most of them require placement of grafting materials of various origin inside the fresh extraction socket (Wang *et al*., 2004). While the benefit for ARP procedures are recognised, no single technique or graft material was proven to provide superior results (Atieh *et al*., 2015; Horowitz *et al*., 2012).

While the extractions are basic procedures routinely performed by the dental professionals, ARP requires more surgical experience. Cost-efficient grafting materials and minimally invasive techniques that require very limited surgical experience could popularise ARP procedures amongst general dental practitioners.

PARASORB Sombrero® is novel hybrid product that is comprised of a collagen cone with a non-separable attached collagen membrane. This product, as per the manufacturer's recommendations, requires minimal marginal flap elevation to tuck the membrane between the alveolar bone and the periosteum, while the cone portion holds the membrane in place and prevents its displacement. PARASORB Cone-Oss®, an equine collagen cone staggered with biphasic calcium phosphate, is not yet commercially available. The fully resorbable particle content of this product makes the collagen cone easier to handle and less likely to lose its shape when it contacts the body fluids. The manufacturer plans to combine the two into a single product, in which a particle filled collagen cone will be attached to the barrier membrane.

Our study has demonstrated the biocompatibility of the equine collagen based materials, their osteoconducive potential, and the full resorbability of the biphasic calcium phosphate particles. We also noted a non-statistically significant trend for better outcomes for preservation of the alveolar ridge width, which occurs when the grafted material is covered by a barrier membrane. This study can serve as a base for testing the future generation of these grafting materials in *in vivo* animal studies.

The current study may also contribute to the popularisation of the sheep animal model in the study of ARP, GBR and implant dentistry.

4.4.3 Future research potential

4.4.3.1 Refinement of ARP model in sheep

This study was the first to describe the changes in horizontal dimensions of the sheep post-extraction alveolar ridge, whether grafted or not.

In our experimental model, we encountered many challenges, such as extraction surgery, insertion of anatomical markers, multiple adjacent bilateral extractions, cortical bone overgrowth, reference points for measurement, histological staining. These multiple experimental methods should be further evaluated in further studies in the sheep model in order to study the effects of the different parameters on the outcome of the grafting procedures. Multiple high resolution computed tomographic scans should be taken preoperatively, immediately postoperatively, and at a number of time-points prior to euthanasia. Short-term general anaesthetics should be trialed in sheep models for allowing multiple sedations for these scans. Extraction of maxillary

premolars could be attempted in order to decrease the number of extracted mandibular units, and thus the compensatory cortical bone overgrowth. Non resorbable surgical fixation plates or screws can be utilised instead of amalgam pins to serve as fixed anatomical markers, in relation to which clinical and radiographic linear and volumetric measurements can be made. There is however, a risk of these markers being exposed to the oral cavity, and subsequently infected. Multiple time-points can be used to study the dynamics of post-extraction alveolar ridge remodelling. Counter-staining techniques can provide more details about the different tissue types in the histological specimens. Further description and refinement of our model would allow for more reproducible results, and potentially lower numbers of experimental animals in order to achieve statistical significance.

4.4.3.2 Further development of equine collagen based grafting materials

The results of our study suggest that a barrier membrane is desirable for a decrease in reduction of the alveolar ridge width. The next generation of grafting materials for ARP by Resorba is an equine collagen cone with embedded biphasic phosphate particles firmly attached to a barrier membrane. The addition of particles will enhance the handling properties of the collagen cone in a liquid environment, and the attached membrane will be less prone to displacement. This new product would need to be tested against non-grafted natural healing and a xenograft control in a larger sample of experimental animals in order to increase the statistical power of the study.

Another potential use of biphasic phosphate particles may be to serve as a carrier for osteogenic bioactive substances, such as BMPs. Currently, the use of commercially available human recombinant BMPs is limited in oral surgery and periodontology, mainly due to the high costs of the material. However, with improved cost effectiveness, these should also be considered for intraoral bone grafting.

4.4.3.3 Insertion of dental implants following ARP

In modern dentistry the main reason for ARP procedures is the subsequent insertion of dental implants. Further studies are required to evaluate the placement of dental implants into extraction sockets grafted with various grafting materials in terms of primary stability, bone-to-implant contact, implant survival, and the potential effect of the development and the progression of peri-implant disease.

In our study we observed complete resorption of equine collagen based grafted products, compared to partial resorption of the xenograft particles. It would be of interest to see if this difference results in variations in the outcome of implant therapy.

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Appendices

Appendix I

1. Medications used on experimental animals

Medication name	Purpose	Admission Route	Dose
Thiopentone	General Anaesthetic	Intravenous	20mg/kg
Halothane	General Anaesthetic	Inhalation	1-2% (to effect)
Nitrous Oxide	General Anaesthetic	Inhalation	1:2 (to effect)
2% Mepivicaine HCL (with 1:20,000 adrenaline)	Local Anaesthetic	Local infiltration	2 x 2.2ml cartridges around surgical site at beginning of surgery
0.5% Bupivicaine HCL (with 1:200,000 adrenaline)	Long lasting Local Anaesthetic	Local infiltration	5ml around surgical site at completion of surgery
Trimethoprim	Antibiotic	Intramuscular	1ml/15kg for 3 days following surgery
Carprofen	Anti-inflammatory agent	Intramuscular	5ml once/day for 3 days following surgery

2. Chemical reagents used

Distilled Water (dH_20), (purified via reverse osmosis unit, RiOs™ unit, Millipore Intertech, USA)

Xylene, $C_6H_4(CH_3)_2$, (Ajax Finechem Pty Ltd, New Zealand)

Ethanol, C_2H_5OH, (High grade, Absolute Ethanol, Thermo Fisher Scientific, USA)

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

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

Xylene, $C_6H_4(CH_3)_2$, (Ajax Finechem Pty Ltd, New Zealand)

Concentrated Hydrochloric Acid (HCl), (100317.2500, Merck, Germany)

Di-Ammonium Oxalate Monohydrate, (1.01190.1000, Merck, Germany)

Phosphate Buffered Saline (PBS), (Gibco™, Invitrogen Corporation, NZ)

3, 3’ diaminobenzidine (DAB), (Sigma D3939, Sigma Aldrich, USA)

3. Equipment used

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 Carbide Paper, Grades 180-4000 (Struers, Ballerup, Denmark)

Accutom, cutting machine, (Struers, Ballerup Denmark)

Incubating/shaking machine (Multitron®, Infors HT, Switzerland0

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

Appendix II

1. Resin for embedding

Ingredients

Methyle methacrylate (Catalogue number M55909, Sigma Aldrich, USA)

Benzoyl peroxide (Catalogue number 517909, Sigma Aldrich, USA)

Dibutylphthalate (Catalogue number 524980, Sigma Aldrich, USA)

Xylene, (Ajax Finechem Pty Ltd, New Zealand)

Method for MMA I

4 parts Methyl methacrylate

1% Benzoyl peroxide

1 Part Dibutylphathalate

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

2. Embedding protocol

Immerse specimens, previously dehydrated in ethanol, in xylene for 4 days in fume cupboard on a rotating platform. Change to fresh xylene after 2 days.

Wash specimens in methyl methacrylate (MMA) monomer.

Transfer specimens to MMA I for 2 days in fume cupboard on a rotating platform.

Fill glass jars with MMA III to one third height, and place in a light-proof plastic container partially filled with water. Leave undisturbed at room temperature for 2-3 days, until set.

Immerse specimens in MMA II for 2 days in fume cupboard on a rotating platform.

Retrieve each specimen and its identification tag from the histological cassette. Place the specimen and the tag flat in an individual glass jar with a pre-set MMA III base. Fill the jar with fresh MMA III, and tightly close the lid.

Place glass jars in half-filled water bath in light-proof plastic container. Leave undisturbed at room temperature for at least 2 days, until fully set.

4. Staining with MacNeal's Tetrachrome / Toludine Blue solution

Solution A

0.5g Methylene blue (Catalogue number 15943 Merck, Germany)

0.8g Azur II (Catalogue number 9211 Merck, Germany)

0.1g Methyl violet 2B (Catalogue number M 0527Sigma Aldrich, USA)

250ml Methanol (Catalogue number 1.06009.6025, Merck, Germany)

250ml Glycerol

Stir with magnetic stirrer until no precipitate seen

Leave for 12 hours at 50°C then 3 days at 37°C

Solution B

Toluidine blue in 100ml distilled water + 1.0g borax

Solution A+B

10ml Solution A

5ml Solution B

85ml distilled water

Staining protocol

Place slide in 20% ethanol in Coplin jar

Place Coplin jar in ultrasonic bath for 5 minutes

Replace ethanol with 0.1% formic acid

Place Coplin jar in ultrasonic bath for 5 minutes

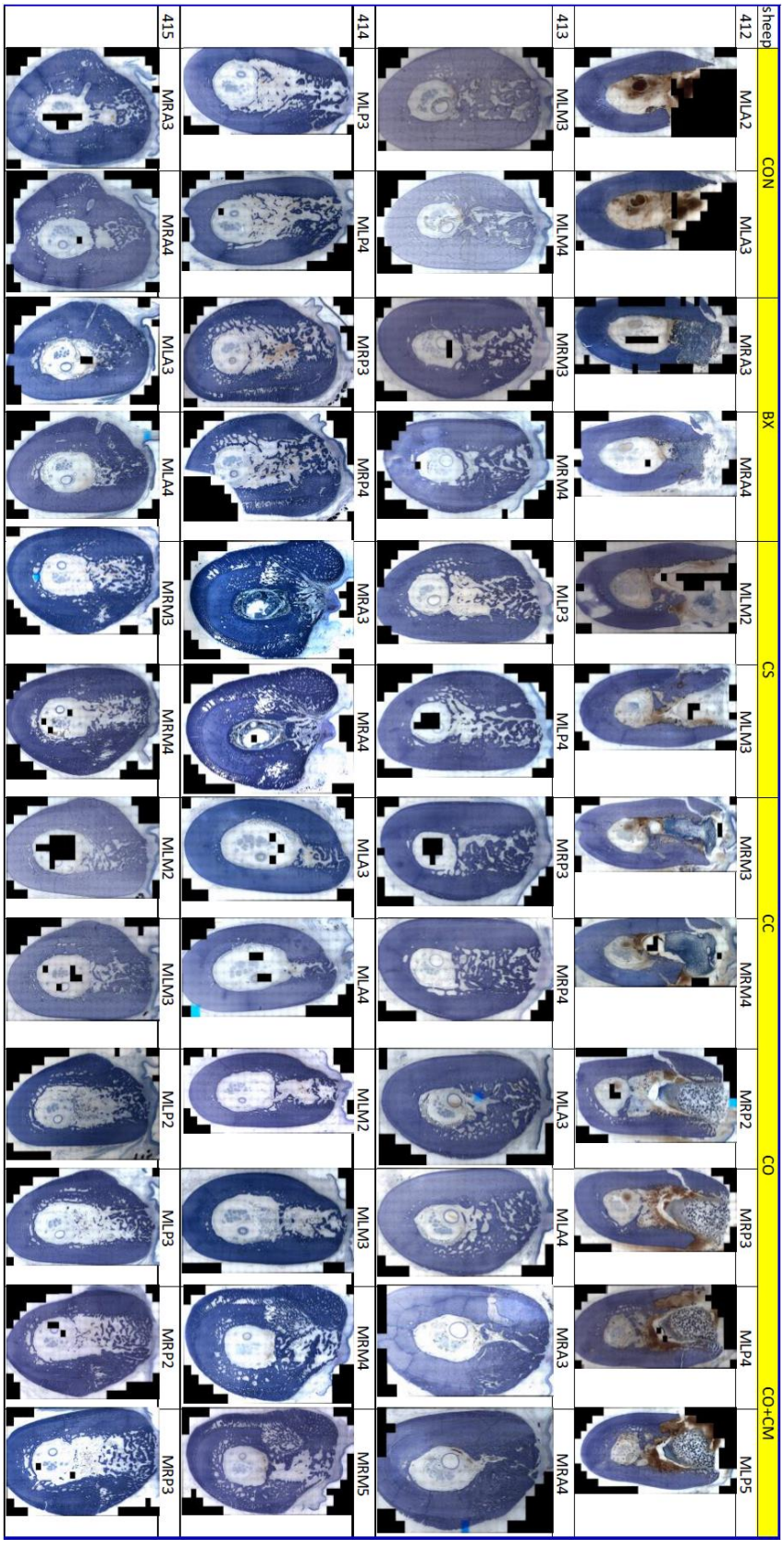
Wash slide with tap water

Cover section on slide with Solution A+B for 5 minutes

Rinse slide with distilled water for 5 minutes

Leave overnight to dry on a benchtop at room temperature

Appendix III Overview of histological slides



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	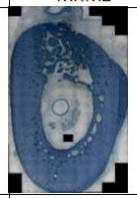		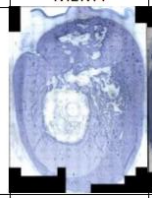	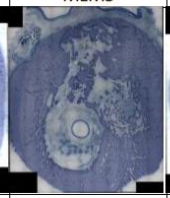	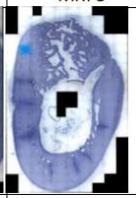	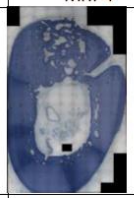	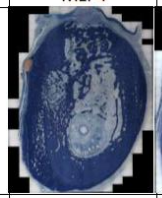	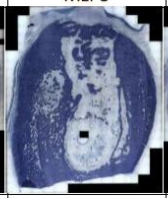	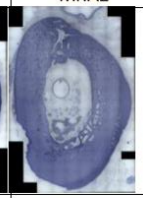	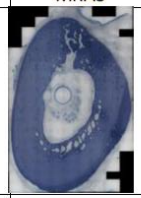	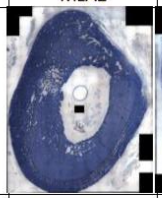	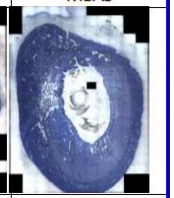
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		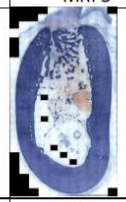	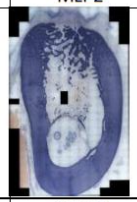			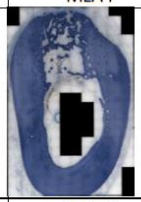		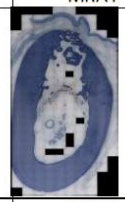		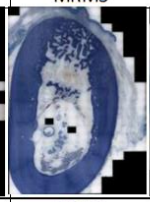		
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		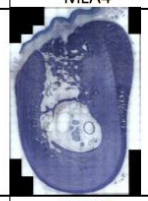	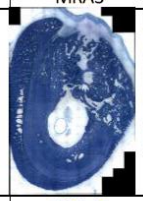	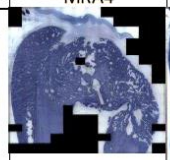	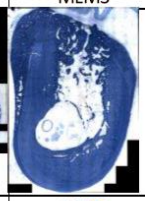	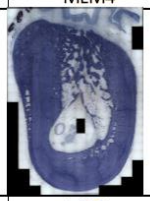		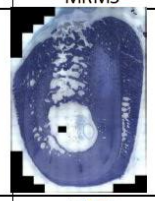	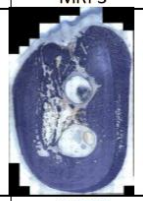		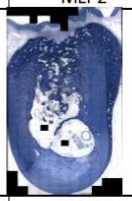	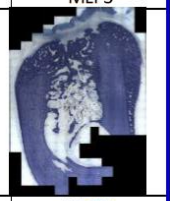
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		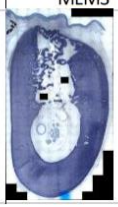	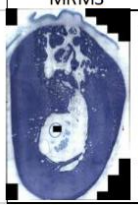									

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	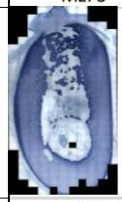											
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	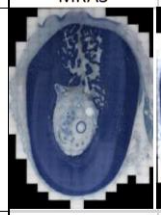		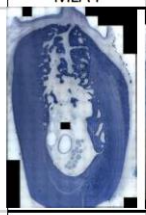								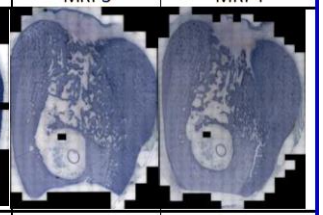	
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	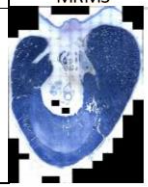	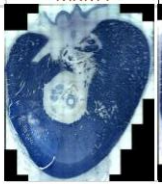	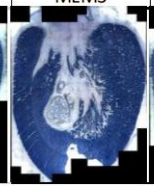	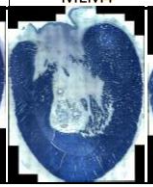	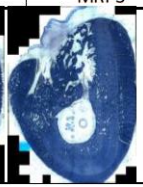			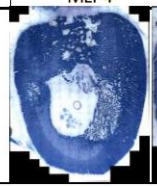	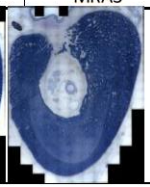		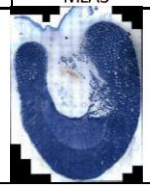	

Appendix IV Clinical and histological data

1. Clinical alveolar ridge width, measured at the mesial aspect of the defect

No.	sheep	weight (kg)	date	L P1 (mm)	L P2 (mm)	L P3 (mm)	R P1 (mm)	R P2 (mm)	R P3 (mm)
1	412	80	23-Jan-15	8.00	10.00	12.00	7.00	9.00	11.50
2	413	80.5	29-Jan-15	7.00	10.00	11.50	6.00	8.50	11.00
3	414	78	29-Jan-15	5.00	7.00	10.00	4.50	6.50	9.50
4	415	83	28-Jan-15	5.50	9.50	10.50	6.00	8.00	9.50
5	416	72	30-Jan-15	6.00	7.50	10.50	5.50	8.00	9.00
6	417	88	26-Jan-15	6.00	8.50	10.50	7.00	8.50	11.00
7	418	78	27-Jan-15	7.50	9.50	10.50	7.00	11.00	13.00
8	419	70	28-Jan-15	6.00	7.50	11.00	6.50	9.00	10.50
9	420	95.5	26-Jan-15	7.00	8.50	10.00	6.00	10.00	15.00
10	421	88	27-Jan-15	6.50	8.00	11.00	7.50	8.50	11.00
11	409	91	30-Jan-15	6.5	8	9.5	6	8	10

2. Crestal hard tissue bridging

No.	sheep	CON	BX	CS	CC	CO	CO+CM
1	412	0	0	0	0	0	0
2	413	2	2	2	2	2	1
3	414	1	1	1	2	2	2
4	415	2	2	2	2	2	2
5	416	2	2	2	2	2	2
6	417	1	2	2	2	2	2
7	418	2	2	2	1	1	2
8	419	2	2	2	2	2	2
9	420	1	2	2	2	1	2
10	421	2	2	2	2	1	1
11	409	1	1	1	2	0	0

“0” – none;

“1” – incomplete;

“2” – complete.

3. Mineralised tissue fraction (%) within ROI

sheep	CON		BX		CS		CC		CO		CO+CM	
413	MLM3	MLM4	MRM3	MRM4	MLP3	MLP4	MRP3	MRP4	MLA3	MLA4	MRA3	MRA4
	53.71	54.78	56.92	53.65	50.98	51.70	55.66	58.44	68.87	70.74	76.26	79.36
414	MLP3	MLP4	MRP3	MRP4	MRA3	MRA4	MLA3	MLA4	MLM2	MLM3	MRM4	MRM5
	39.38	44.05	55.72	55.00	N/A	N/A	57.08	66.60	26.29	22.70	N/A	N/A
415	MRA3	MRA4	MLA3	MLA4	MRM3	MRM4	MLM2	MLM3	MLP2	MLP3	MRP2	MRP3
	53.62	48.63	70.23	59.40	41.97	35.98	57.45	48.24	47.01	44.14	47.23	50.17
416	MRM2	MRM3	MLM4	MLM5	MRP3	MRP4	MLP4	MLP5	MRA2	MRA3	MLA2	MLA3
	57.16	62.09	59.20	53.23	50.16	70.10	56.51	47.41	74.01	62.78	52.23	42.76
417	MRP2	MRP3	MLP2	MLP3	MLA3	MLA4	MRA3	MRA4	MRM2	MRM3	MLM4	MLM5
	56.45	39.7	50.52	44.97	47.55	42.60	38.32	44.98	43.97	45.10	42.05	44.81
418	MLA3	MLA4	MRA3	MRA4	MLM3	MLM4	MRM4	MRM5	MRP3	MRP4	MLP2	MLP3
	44.37	38.31	N/A	N/A	69.91	69.61	61.42	60.23	62.11	57.88	78.45	74.43
419	MLM2	MLM3	MRM3	MRM4	MLP3	MLP4	MRP3	MRP4	MLA4	MLA5	MRA4	MRA5
	48.12	45.55	45.33	39.91	51.43	47.16	56.50	58.12	71.68	63.31	35.61	41.97
420	MLP3	MLP4	MRP2	MRP3	MRA3	MRA4	MLA2	MLA3	MLM3	MLM4	MRM3	MRM4
	49.04	59.02	N/A	61.68	60.07	54.82	84.02	73.40	62.65	61.73	64.13	72.22
421	MRA3	MRA4	MLA4	MLA5	MRM4	MRM5	MLM3	MLM4	MLP2	MLP3	MRP3	MRP4
	46.34	52.89	48.53	55.82	57.15	66.34	51.96	50.33	47.86	N/A	N/A	N/A
409	MRM3	MRM4	MLM3	MLM4	MRP3	MRP4	MLP3	MLP4	MRA3	MRA4	MLA3	MLA4
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

4. Re-measurement of fractions of hard tissue within ROI for reproducibility

	Primary investigator		Research member
Slide	Original	Repeat	Repeat
413MLP4	51.7	50.63	51.128
415MLM3	48.24	46.15	61.112
415MRM3	41.97	39.46	59.958
416MLA2	52.23	52.96	53.141
416MRA3	62.78	63.54	65.13
417MLP2	50.52	49.67	49.842
418MLP3	74.43	75.63	74.451
418MRM4	61.42	59.74	59.985
419MLP3	51.43	51.08	50.745
420MLM3	62.65	62.27	64.639

5. Histological alveolar ridge width (mm)

sheep	CON		BX		CS		CC		CO		CO+CM	
413	MLM3	MLM4	MRM3	MRM4	MLP3	MLP4	MRP3	MRP4	MLA3	MLA4	MRA3	MRA4
	4.86	6.7	6.5	6.32	5.57	5.48	5.52	5.09	5.12	5.53	5.95	5.27
414	MLP3	MLP4	MRP3	MRP4	MRA3	MRA4	MLA3	MLA4	MLM2	MLM3	MRM4	MRM5
	6.31	6.89	10.42	10.53	N/A	N/A	N/A	4.02	6.78	5.89	N/A	N/A
415	MRA3	MRA4	MLA3	MLA4	MRM3	MRM4	MLM2	MLM3	MLP2	MLP3	MRP2	MRP3
	4.47	5.01	5.11	4.05	6.39	5.85	7.17	6.82	6.81	5.60	6.62	8.04
416	MRM2	MRM3	MLM4	MLM5	MRP3	MRP4	MLP4	MLP5	MRA2	MRA3	MLA2	MLA3
	4.32	4.62	7.29	8.68	6.59	4.28	7.44	7.23	2.49	4.33	5.13	5.77
417	MRP2	MRP3	MLP2	MLP3	MLA3	MLA4	MRA3	MRA4	MRM2	MRM3	MLM4	MLM5
	7.22	5.9	7.05	6.53	6.20	5.05	4.24	4.00	5.76	5.55	6.01	5.75
418	MLA3	MLA4	MRA3	MRA4	MLM3	MLM4	MRM4	MRM5	MRP3	MRP4	MLP2	MLP3
	10.85	8.14	12.87	N/A	9.71	9.36	7.80	8.04	10.64	9.31	9.04	9.62
419	MLM2	MLM3	MRM3	MRM4	MLP3	MLP4	MRP3	MRP4	MLA4	MLA5	MRA4	MRA5
	3.78	4.56	6.61	6.97	4.01	5.70	7.30	7.62	4.00	3.62	5.39	7.25
420	MLP3	MLP4	MRP2	MRP3	MRA3	MRA4	MLA2	MLA3	MLM3	MLM4	MRM3	MRM4
	4.86	5.43		5.41	4.75	6.29	2.46	3.57	4.48	4.24	4.20	3.63
421	MRA3	MRA4	MLA4	MLA5	MRM4	MRM5	MLM3	MLM4	MLP2	MLP3	MRP3	MRP4
	5.56	5.45	5.14	4.95	8.09	9.65	6.41	7.73	7.25	10.66	N/A	N/A
409	MRM3	MRM4	MLM3	MLM4	MRP3	MRP4	MLP3	MLP4	MRA3	MRA4	MLA3	MLA4
	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A