A COMPARATIVE STUDY OF WOUND HEALING OF TWO COMMERCIAL

ACELLULAR DERMAL MATRICES: AN *IN VIVO* STUDY

A Thesis

by

SOPHIE ROSE COUTO

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Chair of Committee,	Kathy Svoboda
Committee Members,	Xianghong Luan
	Jeffrey Rossmann
	William Stenberg
Head of Department,	Larry Bellinger

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ABSTRACT

The overall objective of this study was to compare, *in vivo*, the wound healing and remodeling of two commercial acellular dermal matrices used for gingival augmentation in periodontal surgical procedures. The aims were to evaluate differences in the following at two time points: (1) gingival fibroblast density, (2) *de novo* collagen formation, (3) matrix degradation, (4) angiogenesis, and (5) males versus females.

This was a non-randomized controlled split-mouth study. Envelope flaps were surgically created in the maxillary quadrants of twenty-four Sprague Dawley rats. Each envelope flap was assigned to receive either (1) AlloDerm[TM] RTM or (2) OrACELL[TM]. Gingival tissue from one mandibular quadrant served as the untreated control. Six male and six female rats were treated for 7- or 21-days. Biopsies were obtained and processed for histologic analysis (H&E, Picro-sirius red, Verhoeff's solution) or RNA analysis (RT-PCR) to compare the expression of type I collagen (Col1a1), fibronectin (Fn-1) and VEGF-A (Vegf-A). A blinded evaluation of soft tissue thickness was performed based on clinical photographs taken before biopsy.

There was a greater density of fibroblasts for OrACELL[TM] compared to AlloDerm[TM] at both 7 and 21 days. There was a greater density of elastin present for AlloDerm[TM] compared to OrACELL[TM] at 7 days but no differences between groups at 21 days. There was a greater observed soft tissue thickness for OrACELL[TM] compared to AlloDerm[TM] at 7 days while the opposite was observed at 21 days. There were no differences between test groups in the percentage of birefringent collagen or in the expression of Col1a1, Vegf-A, or Fn-1. At 7 days, there was a significantly greater density of fibroblasts for males in the OrACELL[TM] group

compared to females. At 21 days, there was a significantly greater expression of Col1a1 for males in the OrACELL^{TM} group compared to females.

Based on the results of this study, early wound healing and remodeling of OrACELL^{TM} occurred more rapidly than that of AlloDerm^{TM}. Wound healing of OrACELL^{TM} was accelerated in male Sprague Dawley rats compared to females. Whether these results have positive or negative clinical implications for soft tissue grafting procedures in humans remains to be determined.

DEDICATION

I dedicate this thesis to my parents, Fernand and Irene, whose unfailing encouragement, patience, advice and support have made my academic pursuits possible everywhere they have led me.

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NOMENCLATURE

ADM	AlloDerm™
AL	Attachment loss
CAF	Coronally advanced flap
CEJ	Cemento-enamel junction
CON	Control
Col1a1	Alpha-1 type I collagen
CT	Connective tissue
CTG	Connective tissue graft
EMD	Enamel matrix derivative
FGF	Fibroblast growth factor
Fn-1	Fibronectin
GTR	Guided tissue regeneration
IGF-1	Insulin-like growth factor-1
IL-1	Interleukin-1
IL-8	Interleukin-8
KT	Keratinized tissue
MGJ	Mucogingival junction
ORA	OrACELL™
PDGF	Platelet-derived growth factor
PDM	Puros Dermis™
PEM	PerioDerm™

RT-PCR	Real-time polymerase chain reaction
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
VEGF-A	Vascular endothelial growth factor A
% BRC	Percentage of birefringent collagen

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Gingival Anatomy

The gingiva is one of the four main components of the periodontium, which also includes the cementum, periodontal ligament, and alveolar bone. The periodontium provides the fundamental support for teeth. The normal healthy gingiva consists of three distinct areas: the attached gingiva, the marginal or free gingiva, and the interdental gingiva (1).

The attached gingiva begins at the mucogingival junction (MGJ), the boundary between the alveolar mucosa and the gingiva. The MGJ exists on the mandibular facial and lingual surfaces and on the maxillary facial surfaces. The attached gingiva is composed of a stratified squamous epithelium that is keratinized or parakeratinized (2). The epithelial layers of the attached gingiva include the stratum basale, stratum spinosum, stratum granulosum and stratum corneum (3). Beneath the epithelium is a dense collagenous connective tissue (CT) layer (1). The attached gingiva is secured by collagen fibers to the underlying periosteum, which covers the bone and consists of two dense layers of connective tissue; an outer fibrous layer and an inner cellular layer (1), (4).

Although its thickness depends on the patient's periodontal biotype, the attached gingiva is on average 1.25 mm thick (5). In one-third of the population, the attached gingiva ends coronally at the free gingival groove, which marks the boundary between the attached gingiva and the free or marginal gingiva (6). Since the free gingival groove is not always present, the base of the gingival sulcus, or the shallow space between the tooth and the marginal gingiva, is used as the marker for the coronal termination of the attached gingiva. This vertical distance

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measured from the MGJ to the free gingival groove or base of the sulcus is referred to as the width of the attached gingiva. This is not to be mistaken with the width of the keratinized gingiva, which also includes the keratinized marginal gingiva.

In his classic study, Bowers showed that there is great variation in the width of the attached gingiva, ranging from 1 to 9 millimeters (7). Not only does it vary depending on the patient's biotype; it differs in different areas of the mouth (7). In general, Bowers noted that the attached gingival width is greater in the maxilla compared to the mandible with the greatest width over the incisors. This zone of attached gingiva becomes narrower at the canine and first premolar area and then increases slightly over the second premolars and molars (7). Voigt evaluated the mandibular lingual attached gingiva and found the greatest amount at the first and second mandibular molars and the least amount at the incisors and canines (8). The width of the attached gingiva has also been shown to increase with age (9) and tends to be narrower in locations with high frenum attachments (7). The malposition of a tooth in the dental arch also affects its attached gingival width, with facially-positioned teeth having greater and lingually-positioned teeth having lesser attached gingiva (7).

The marginal gingiva is not attached. It forms the gingival collar surrounding the teeth and the soft tissue wall of the gingival sulcus (1). The marginal gingiva begins at the free gingival groove or base of the sulcus and extends slightly coronal to the cemento-enamel junction (CEJ), the boundary between the enamel of the anatomic crown and the cementum of the anatomic root. The coronal border of the marginal gingiva is termed the free gingival margin. The normal marginal gingiva is on average 1 mm wide (1) and 1.56 mm thick (5). It increases in thickness from anterior to posterior and is directly proportional to the depth of the gingival

sulcus (5). The junctional epithelium is a band of stratified squamous epithelial cells that firmly attaches to the tooth surface by means of a basal lamina (1).

The interdental or interproximal gingiva occupies the space under the contact point between the teeth. The shape of the interdental gingiva may vary but it is typically concave and composed of stratified squamous non-keratinized epithelium (10). When the teeth are in normal contact, the interdental gingiva is bordered facially and lingually by keratinized papillae which fill the gingival embrasure spaces. The amount of papilla fill is proportional to the height of the underlying interproximal alveolar bone (11). When there is a diastema or space between the teeth, there are no interdental papillae (1).

The alveolar mucosa differs from the attached gingiva in several important ways. Unlike the robust, coral pink and stippled attached gingiva, the alveolar mucosa is red, smooth and shiny because its epithelium is thin and not keratinized (1). The connective tissue is also loosely arranged and contains a greater quantity of blood vessels, which can be visualized beneath the surface (1). It is more delicate and less resistant to the insults of mastication and inflammation (12).

Gingival Recession

Gingival recession is the exposure of the root surface of a tooth via the apical migration of the free gingival margin away from the CEJ (13). Along with a lack of keratinized tissue (KT), gingival recession is the most common type of mucogingival deformity (14). Others include decreased vestibular depth, aberrant frenum position, gingival excess and abnormal color (15). Gingival recession can lead to dentin hypersensitivity, poor esthetics and carious or non-carious cervical lesions such as abrasions or erosions (14). Risk factors for the development of gingival recession include a thin periodontal biotype, a reduced alveolar bone thickness due to

tooth malposition, and the absence of attached gingiva (16). Other contributing factors include traumatic tooth brushing habits (17), trauma from lip or tongue piercings (18), aberrant frenum or muscle attachment (19), intrasulcular restorative margins (20), orthodontic therapy (21), and periodontal inflammation (22). Gingival recession also increases with age (23).

A controversy in the periodontal field is whether a minimum width of attached or keratinized gingiva is necessary for the prevention of gingival recession and inflammation. In a 1972 study of dental students with good oral hygiene, Lang and Loe found that most tooth surfaces with at least 2 mm of keratinized gingiva were clinically healthy (24) while tooth surfaces with less than 2 mm had inflammation. They concluded that at least 2 mm of keratinized gingiva, or 1 mm of attached gingiva, was necessary to maintain gingival health (24).

In contrast, Miyasato *et al* reported that areas with less than 1 mm of keratinized gingiva were not more prone to acute inflammatory changes than areas with greater than 2 mm of keratinized gingiva (25). In 1987, Wennström found that with good oral hygiene, minimal or no attached gingiva does not necessarily result in recession (26). Kennedy *et al* also reported that with good oral hygiene it is still possible to maintain periodontal health despite an absence of attached gingiva (27).

A recent systematic review concluded that in patients with suboptimal plaque control, attached gingiva is important to maintain gingival health (20). The current consensus on this topic is that even though a minimum amount is not necessary to prevent attachment loss when optimal plaque control is present, approximately 2 mm of keratinized tissue and 1 mm of attached gingiva is desirable (20).

The long-term consequence of untreated gingival recession has been studied. Serino *et al* reported that 3 mm and 4 mm recession sites in patients with excellent oral hygiene deteriorated

in 67% and 98% of cases, respectively (28). When Agudio *et al* compared treated recession sites to untreated contralateral sites, they reported that recession was reduced in 83% of treated sites but increased in 48% of untreated sites over an average 23.6-year follow-up period (29). A recent systematic review and meta-analysis has reported that even in patients with good oral hygiene, untreated facial gingival recession defects were more likely to have an increased recession depth (30). The etiology of gingival recession is multi-factorial. If the factors contributing to recession defects are not controlled, they will degrade over time.

Mucogingival Therapy

The term mucogingival surgery was proposed by Friedman in 1957 to describe any surgery "designed to preserve attached gingiva, to remove frena or muscle attachment, and to increase the depth of the vestibule" (31). The term periodontal plastic surgery, proposed by Miller in 1988, has also been adopted (32). In the 1996 consensus report, the American Academy of Periodontology expanded the definition of mucogingival therapy to include the "non-surgical and surgical correction of the defects in morphology, position and/or amount of soft tissue and underlying bone" (33). This definition is still used today (14).

The success of mucogingival therapy for the treatment of gingival recession defects depends on many factors, including the desired outcome, surgical technique, experience of the surgeon, and patient-related factors including systemic disease, smoking status, periodontal biotype and compliance to post-operative instructions. A crucial determinant for the success of therapy is the initial severity of the defect (32). As demonstrated in Table 1-1, several different classification systems for gingival recession exist, including those proposed by Sullivan and Atkins (34), Miller (35) and Cairo (36).

Table 1-1. Classification systems for gingival recession.

	Sullivan & Atkins (1968)	**Miller (1985)**	**Cairo (2011)**
Categories	*Deep-wide* • > 3mm in both dimensions (37)	*Class I* • Does not extend to MGJ • No interproximal AL	*Recession Type 1 (RT1)* • No interproximal AL
	Shallow-wide	*Class II* • Extends to MGJ • No interproximal AL	*Recession Type 2 (RT2)* • Interproximal AL ≤ facial AL
	Deep-narrow	*Class III* • Extends to MGJ • Interproximal AL	*Recession Type 3 (RT3)* • Interproximal AL > facial AL
	Shallow-narrow • < 3 mm in both dimensions (37)	*Class IV* • Extends to MGJ • Severe interproximal AL	
Therapeutic Root Coverage Anticipation	*Deep-wide*: Partial *Shallow-wide*: Partial *Deep-narrow*: Complete *Shallow-narrow*: Complete	*Class I*: Complete *Class II*: Complete *Class III*: Partial *Class IV*: No root coverage	*RT1*: Complete *RT2*: Possibility for complete *RT3*: No possibility for complete

Table 1-1. Continued.

	Sullivan & Atkins (1968)	**Miller (1985)**	**Cairo (2011)**
Limitations	• Subjective • No consideration for interproximal AL	• Not applicable to palatal recession defects • Can be difficult to distinguish Class I and II • No category for defects with interproximal AL that do not extend to MGJ	

MGJ: Mucogingival Junction
AL: Attachment Loss

The 1968 Sullivan and Atkins system classifies recession defects according to depth and width with 4 main categories: "deep-wide", "shallow-wide", "deep-narrow" and "shallow-narrow" (34). The "deep-wide" defect is considered the most difficult to treat due to the biologic limits of the blood supply required for healing (34). This classification system, as well as being subjective, does not include the presence or degree of interproximal attachment loss. The interproximal gingiva is of the utmost importance in predicting the outcome of therapy because it provides stability and is the coronal limit for blood supply to the graft (35), (36).

Miller's 1985 classification system also has 4 categories (35). As demonstrated in Figure 1-1, Miller Class I recession defects do not extend to the MGJ and present with no interproximal bone or soft tissue loss. Miller Class II recession defects extend to or beyond the MGJ, also with

no interproximal bone or soft tissue loss. Miller Class III recession defects extend to or beyond the MGJ with interproximal bone or soft tissue loss. Miller Class IV recession defects extend to or beyond the MGJ with severe interproximal bone or soft tissue loss (35). According to Miller, 100% root coverage is expected for Class I and II recession defects following mucogingival therapy (35). Partial root coverage up to the level of the interproximal tissue is anticipated when treating Miller Class III recession defects (35). No root coverage is anticipated for Miller Class IV recession defects (35). Surgical therapeutic success as defined by Miller is marginal tissue at the level of the CEJ, sulcus depth of 2 mm or less, and no bleeding upon periodontal probing (38).
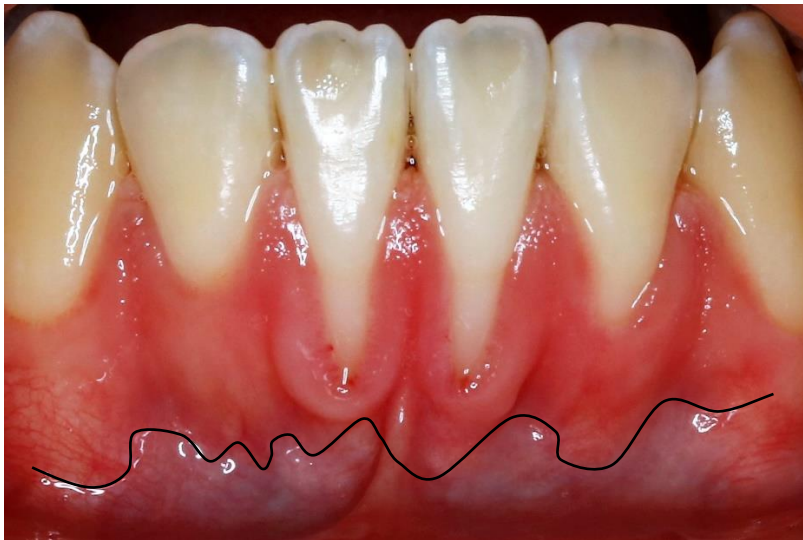
Figure 1-1. Miller Class 1 recession defects. Involved sites are the buccal surfaces of teeth #s 23, 24 and 25. Black line represents the mucogingival junction.

Miller's classification system, while useful, presents with several limitations (39). The first is that not all types of recession are included. For example, it does not include cases of recession with interproximal bone loss that do not extend to the MGJ, nor does it include palatal

recessions (39). Additionally, since the MGJ is sometimes difficult to identify, it can be difficult to distinguish between Class I and II recession defects (39). It is also important to note that Miller's prognostic guidelines for anticipated root coverage were based on the free gingival graft, a procedure (described below) which is not intended for root coverage. With advances in gingival grafting procedures, later studies show that complete root coverage can be achieved for Class III gingival recessions (40).

The more recent 2011 Cairo classification system overcomes many of these limitations (14). It categorizes recession defects into 3 main types according to the presence and severity of interproximal attachment loss (36). Recession Type 1 (RT1) defects have no loss of interproximal attachment. Recession Type 2 (RT2) defects have a loss of interproximal attachment that is less than or equal to the facial attachment loss. Recession Type 3 (RT3) defects have a loss of interproximal attachment that is greater than the facial attachment loss (36). For Cairo RT1, 100% root coverage is anticipated following mucogingival therapy (36). For Cairo RT2, 100% root coverage is possible, and for Cairo RT3, complete root coverage is not achievable (36). This classification system has been shown to be very reliable between examiners with a high predictive value for final root coverage outcomes (36).

Wound Healing

The clinical parameters used to evaluate therapeutic success, such as percentage of root coverage, width of keratinized tissue, and depth of the gingival sulcus, are indirect measures of the fundamental process required for success: wound healing. Wound healing is a complex and dynamic process that involves three main phases: (a) hemostasis and inflammation, (b) proliferation, and (c) maturation and remodelling (41), (42).

In the hemostasis and inflammation phase, the exposure of collagen to platelets results in platelet aggregation, degranulation, and activation of the coagulation cascade (41). The fibrin clot promotes hemostasis and acts as a scaffold for infiltrating inflammatory cells, including polymorphonuclear leukocytes (PMNs), macrophages and fibroblasts (41). The release of chemotactic substances by platelets, including platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), transforming growth factor-alpha (TGF-α) and transforming growth factor-beta (TGF-β), combined with an increase in vascular permeability, stimulate large quantities of these cells to migrate to the region (42). Other chemotactic factors, including complement factors, interleukin-1 (IL-1), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α), further promote PMN migration (41). PMN levels peak at 24 to 48 hours, followed by macrophages, peaking at 48 to 96 hours (41). PMNs primarily function to phagocytose bacteria and tissue debris to limit infection (41). Macrophages have a significant influence on cell proliferation, matrix synthesis and angiogenesis via the release of cytokines and growth factors (41). Epithelialization of the wound surface begins in the first 24 hours as epithelial cells respond to TGF-α to migrate and proliferate (41), (42). Epithelialization continues as the underlying matrix is established until a barrier forms and matures (41).

The second phase of wound healing is the proliferative phase, occurring approximately from day 4 to day 12 following injury (41). During this phase, fibroblasts replicate and synthesize collagen and endothelial cells participate in angiogenesis (41). Fibroblasts, derived from mesenchymal cells, are the most common cells in the connective tissue. These cells produce the extracellular matrix that includes collagen, elastin, glycosaminoglycans, proteoglycans and glycoproteins (43). Fibroblasts are "spindle-shaped" because their cytoplasm tapers off in both directions (43). Active fibroblasts can be distinguished from quiescent

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fibroblasts because they are larger and have a larger nucleus and prominent nucleolus, a branched cytoplasm, increased rough endoplasmic reticulum and a well-developed Golgi apparatus (43). Active fibroblasts accelerate wound healing because they appear quickly and proliferate rapidly (44). In addition to production of matrix components, fibroblasts also produce growth factors and cytokines, including fibroblast growth factor (FGF), TGF-β, vascular endothelial growth factor (VEGF) and keratinocyte growth factor (41), (44). Fibroblasts regulate extracellular matrix formation, angiogenesis and epithelialization. Furthermore, they also produce proteases for collagen degradation and turnover during maturation and remodeling phases of wound healing (1), (42).

Collagen turnover constantly occurs but the rate significantly increases during wound healing (41). In gingival and oral mucosal connective tissue the major collagen is type I with less of types III and IV (45). Type I collagen bundles are highly cross-linked, imparting insolubility and structural and mechanical stability (1), (45), (46). Type III collagen forms the reticular fibers which arrange into a network for cell support and are important in the wound repair process (41), (45). Type IV collagen is in the basal lamina, functioning to anchor epithelial cells to the underlying connective tissue or lamina propria (1), (45).

When stimulated by signaling factors, transcription for specific collagen mRNA synthesis begins in the fibroblast nucleus. The mRNA is translated into α-chain polypeptides called preprocollagen molecules in the rough endoplasmic reticulum (rER) (4). Several posttranslational modifications also occur in the rER. The N-terminus of the peptide is cleaved and vitamin C-dependent hydroxylation of proline and lysine residues occurs (4). There is also glycosylation of specific hydroxyl-lysine residues (4). Three α-helical chains combine to form a triple helix called procollagen, which is packaged into secretory vesicles in the Golgi apparatus

(4). Procollagen is exocytosed from the fibroblast and undergoes further processing

extracellularly (4). This includes cleavage, polymerization and cross-linking to form mature

collagen fibrils, which often consist of more than one type of collagen (4).

Along with collagen synthesis, significant angiogenesis occurs during the proliferative

phase of wound healing. Cytokines derived from platelets and macrophages, especially TNF-α,

TGF-β, PDGF and VEGF, stimulate the migration and proliferation of endothelial cells (41),

(42). VEGF is considered the most important mediator for wound angiogenesis since its

production significantly stimulates capillary growth (47). Cell disruption and hypoxia appear to

be the initial inducers of these angiogenesis factors (48). Using the fibrin and collagen scaffold

for support, new endothelial capillary tubes sprout from nearby venules to form mature vascular

basement membrane (42), (48). These new capillaries merge and anastomose to form a network

or microvascular bed, carrying oxygen, nutrients and mediators (cytokines) required for healing

(42), (48), (49). Once normal oxygen levels are achieved both the production of VEGF and new

capillaries decreases (47).

The final phase of wound healing is maturation and remodeling, which begins during the

second week following injury and can continue for several months (41). During this phase the

wound matrix reorganizes as new collagen is synthesized and existing collagen is broken down

by enzymes called matrix metalloproteinases (41). As with collagen synthesis, growth factors

and cytokines induce the activity of these collagenases (41). The early matrix consists mostly of

fibronectin, type III collagen, glycosaminoglycans and proteoglycans while the mature matrix

consists mostly of type I collagen (41). As collagen accumulates, the density of blood vessels

decreases (48). The tensile strength of the wound continues to increase even after the amount of

collagen being produced plateaus (41). Wound contraction occurs when myofibroblasts migrate,

pulling the collagen network together (41), (42). These specialized fibroblasts have intracellular α-smooth muscle actin, allowing the wound to close (41), (42).

Although these major processes occur during both cutaneous and oral wound healing, several significant differences exist. The major difference is that oral wound healing occurs more rapidly than cutaneous wound healing. This has recently been attributed to differences in gene regulation, affecting the inflammatory response by causing faster proliferation and differentiation of cells as well as resulting in more efficient epithelial remodeling and keratinization (50). Gingival myofibroblasts in particular have been reported to positively contribute to the speed of gingival wound healing (51). A less robust wound angiogenesis has also been observed in oral versus cutaneous wounds, suggesting that an optimal level of angiogenesis may exist for efficient wound healing (47). Additionally, saliva, which contains growth factors, increases the rate at which oral mucosal wounds heal (52). Another important difference between oral and cutaneous wound healing is that oral wounds generally heal with less scar formation. A potential mechanism for this is that oral mucosal fibroblasts have significantly increased ability to reorganize the extracellular matrix compared to dermal fibroblasts (53). Differences in apoptosis pathways have also been reported to contribute to the increased healing rate and reduced scar formation of mucosal wounds (54).

Surgical Techniques for Mucogingival Therapy

There are many different surgical techniques for mucogingival therapy, including the free gingival graft (34), the lateral pedicle flap (55), the coronally advanced flap (56), guided tissue regeneration (57) and the subepithelial connective tissue graft (58).

The Free Gingival Graft

The free gingival graft (FGG) was first described by Bjorn in 1963 and by Sullivan and Atkins in 1968 (34), (59). The primary indication for this technique is to increase the zone of keratinized tissue around single or multiple teeth or implants in areas with inadequate width. A secondary benefit is that a limited amount of root coverage may be achieved. The term "creeping attachment" describes the gradual coronal extension of the gingival margin in the months and years following surgery (60). Matter found that an average of 1.2 mm of creeping attachment occurs after 1 year (61).

The FGG technique involves first preparing the recipient bed to receive the graft. A split-thickness incision is made at the MGJ to create a flap that preserves the attachment of the periosteum to the alveolar bone (34). This ensures that the graft receives a blood supply from the adjacent gingiva as well as the underlying periosteum. A graft of desired thickness consisting of epithelium and underlying lamina propria is harvested from the palate and sutured to its recipient position (34). The donor site may be sutured with local hemostatic agents or covered with an acrylic stent for patient comfort.

The first step of healing of the FGG is the formation of the fibrin clot. The fibrin clot anchors the graft and serves as a matrix to allow for diffusion of metabolites and waste products (34). During the first two days of healing, the graft depends on diffusion of fluid from the vessels of the recipient bed, called plasmatic circulation (34). By day 2 or 3, recipient site capillaries start proliferating into the graft to anastomose with graft vessels and form a new capillary network (34). In most cases, the graft epithelium undergoes sloughing (1). By day 4 or 5, connective tissue starts developing (34). By day 10, a fibrous attachment and vascularization of the graft is complete (62). By 21 days, the connective tissue is well organized (62).

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The free gingival graft is a predictable option for soft tissue augmentation but presents with several drawbacks. The first is that tissue esthetics, color and texture, is poor because the graft is harvested from the palate. This technique is therefore not indicated in the maxillary esthetic zone and is used most frequently in the anterior mandible. The second is that the donor site heals by secondary intention which can lead to complications of pain and bleeding. Finally, there is a limitation to the number of teeth that can be treated due to non-availability of the donor site keratinized tissue.

The Lateral Pedicle Flap

The lateral pedicle or lateral sliding flap was first described by Grupe and Warren in 1956 (55). Unlike a graft which is detached from its original blood supply and transferred to a separate site, a flap remains attached to a native blood supply. The lateral pedicle flap technique is intended for a single tooth with an isolated recession defect and requires the adjacent donor site to have a broad and thick zone of attached gingiva. For this technique, a full-thickness flap is reflected at the adjacent tooth and rotated mesially or distally to cover the recession defect (55). Since this creates an area of exposed alveolar bone, the donor site is usually covered with a periodontal dressing for approximately two weeks (55).

The lateral pedicle flap, though not used as commonly today, can achieve successful root coverage results. Studies have shown it can achieve a mean root coverage in the area of 69% to 72% (63), (64). The lateral pedicle flap has the advantage of maintaining its own blood supply, tissue color matching and localizing the surgical site to one region. However, this comes at the expense of the donor site which must heal by secondary intention. The drawbacks of this technique are therefore similar to those of the free gingival graft as there can be pain and bleeding at the donor site. Additionally, due to tissue limitations the procedure is intended for

localized recession defects. At least 1 mm of recession is also expected to occur at the donor tooth (63).

The Coronally Advanced Flap

In 1975, Bernimoulin *et al* described a technique called the coronally positioned flap, also known as the coronally advanced flap (CAF) (56). In this study an FGG was performed at the treatment site two months in advance in order to increase the zone of keratinized tissue (56). For the CAF, a full-thickness flap is raised with vertical incisions at the teeth adjacent to the recession defect (56). Sub-marginal interproximal incisions are made to recreate the papillae (56). After flap reflection, the periosteum is released to allow the flap to be coronally positioned over the recession defects (56). The flap is sutured in place with or without a periodontal dressing (56), (65). This technique overcomes several of the drawbacks of the free gingival graft and the lateral pedicle flap. First, assuming adequate KT, there are no donor tissue quantity limitations. There is also only one surgical site, reducing post-operative morbidity. Additionally, multiple adjacent gingival recessions can be treated and the technique achieves good root coverage results. Allen and Miller demonstrated that for single tooth Miller Class I recession defects, the CAF achieved a mean root coverage gain of 97.8% at 6 months with complete root coverage achieved at 84% of sites (66). The root coverage achieved with the CAF has also been reported to be stable over time. For single tooth Miller Class I and II recession defects, Jepsen *et al* found a mean root coverage gain of 83.7% at 6 months and 82.8% at 3 years with complete root coverage achieved at 39% of sites for both time points (67).

Over the years the CAF has been modified in different ways. In 1986 Tarnow introduced the semilunar coronally positioned flap (68). For this technique, a semilunar incision is made at the free gingival margin of the tooth or teeth with recession defects (68). Through this incision, a

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split-thickness dissection is performed coronally and connected to an intrasulcular incision (68). The released keratinized tissue is then positioned coronally to cover the recession defect and held in place with pressure for several minutes (68). The donor site, which heals by secondary intention, can be covered with a periodontal dressing for patient comfort and the flap can be sutured or not sutured (68). An advantage of this technique is that unlike the original, the semilunar coronally positioned flap does not reduce the depth of the vestibule (68).

In 2000 Zucchelli and De Sanctis introduced a novel incision design using a split-full-split thickness envelope flap with oblique interproximal incisions (69). The oblique incisions allow varying degrees of adjacent recession to be treated simultaneously (69). Avoiding vertical incisions limits scarring, producing a more esthetic result. It also preserves more blood supply to the flap, as does the split-full-split thickness flap design (69). Zucchelli and de Sanctis found improved results compared to those reported for the original coronally positioned flap, with 88% of Miller Class I and II recession sites exhibiting complete root coverage at 1 year (69). When they modified their incision design to include a trapezoidal split-full-split thickness flap with vertical incisions, they found that 85% of Miller Class I and II recession sites exhibited complete root coverage at 3 years (70). They also noted an increase in KT width of 1.78 mm at 3 years (70).

Another modification to the CAF is combining it with a subepithelial connective tissue graft (CTG). In a 2008 systematic review, Cairo *et al* concluded that for Miller Class I and II recession defects, the use of a connective tissue graft with a CAF produces a greater percentage of mean root coverage compared to using a CAF alone (71). In their 5-year split-mouth study published in 2013, Kuis *et al* also found significantly better results when the CAF was combined with a CTG for Miller Class I and II defects for all parameters evaluated (72).

Guided Tissue Regeneration

Guided tissue regeneration (GTR) is a technique involving the use of resorbable or non-resorbable barrier membranes with the goal of regenerating the alveolar bone, periodontal ligament and cementum that comprise the periodontium. In contrast, most other types of root coverage procedures heal by formation of a long junctional epithelium with CT fibers that run parallel to the root surface (73). The barrier membrane is used to prevent rapidly dividing epithelial cells from forming an attachment to the root; instead allowing the formation of alveolar bone with new connective tissue and PDL fibers inserting into new cementum (73), (74).

Literature evaluating the outcomes of GTR indicate that it is generally a successful and stable procedure with results comparable to other types of mucogingival therapy (75), (76), (77). In a split-mouth study, Amarante *et al* performed a CAF with or without a resorbable barrier membrane and found no significant difference in root coverage, clinical attachment gain or probing depth at 6 months (75). In their 4-year study, Pini Prato *et al* showed that there was no significant difference in the mean root coverage achieved with a non-resorbable membrane versus a two-step surgery involving an FGG and CAF (76). GTR presents with an added material cost and increased risk for complications like membrane exposure or infection. Furthermore, if using a non-resorbable membrane, the second surgery required for membrane retrieval may traumatize the newly formed gingival tissue (77). Since the results obtained compare to traditional forms of therapy, at the present time there appears to be limited clinical advantage to using barrier membranes for mucogingival therapy (73), (75), (77).

Another technique with the potential to regenerate the periodontium and enhance root coverage involves a biologic material called enamel matrix derivative (EMD). EMD consists of enamel matrix proteins, mainly amelogenins, which are derived from the enamel layer of

developing porcine teeth (78). EMD has been demonstrated to regulate the expression of growth factors and cytokines involved in bone remodeling and to promote soft tissue regeneration and angiogenic activity (78). When comparing a CAF with or without EMD, studies have shown varying results. Del Pizzo *et al* found no significant difference in mean root coverage, complete root coverage or clinical attachment gain for Miller Class I and II recessions at 2 years (79). In a split-mouth randomized control trial, Spahr *et al* similarly found no significant differences in mean root coverage at 2 years for a CAF with or without EMD (80). However, they did note significantly better long-term results in the EMD group, with only 22% of sites deteriorating in the second year after therapy compared to 47% of control sites (80). In contrast, other studies have found significantly improved mean root coverage for Miller Class I and II recessions when EMD is combined with a CAF (81), (82). In 2015, the AAP Regeneration Workshop concluded that the combination does appear to be superior to the CAF alone (73).

The Subepithelial Connective Tissue Graft

The subepithelial connective tissue graft was described in 1985 by Langer and Langer (58). It is generally considered the gold standard for root coverage (83). This technique involves harvesting autogenous connective tissue from a donor site, usually the patient's hard palate or maxillary tuberosity, and suturing it to the prepared recipient site. Several techniques exist for both recipient bed preparation and graft harvest. Langer and Langer originally described a recipient site preparation technique wherein a partial thickness flap is reflected with horizontal incisions at the level of the CEJ and vertical releasing incisions (58). This split-thickness flap design provides a double blood supply to the graft with vascularity from both the underlying periosteum and the overlying flap (58). The Langers' donor site harvest consists of two parallel horizontal incisions on the palate separated by approximately 2 mm (58). The coronal incision is

scored to bone and the apical incision is released from the overlying epithelium to enable removal of a CT wedge (58). Variations to this harvest technique involve creating a "trap door" with the addition of one or two vertical incisions to facilitate connective tissue removal (58). The graft is sutured in place at the recipient bed and the flap is coronally positioned over it (58). The limitation of the Langer technique is that the vertical incisions at the recipient site can induce scar formation and reduce blood supply to the flap. Additionally, the double incision harvest removes approximately 2 mm of epithelium, resulting in healing by secondary intention since the wound edges are not re-approximated.

Several modifications to the Langer method have since been described. In 1985, Raetzke reported on a novel technique for single tooth recession defects in which a partial thickness sulcular incision is made at the recession site (84). An envelope is created without vertical incisions by undermining the tissue (84). The connective tissue is then tucked into the pouch and secured with a cyanoacrylate adhesive (84). In 1994, the Raetzke envelope was applied to multiple gingival recessions as the "supraperiosteal envelope" (85), later coined the "tunnel technique" (86). The Zucchelli envelope flap design for coronal advancement can also be used with a CTG very successfully (69). Variations to the harvest site preparation also exist, the most conservative of which is the single-incision harvest technique described by Hürzeler and Weng (87). This method avoids epithelium removal, allowing healing by primary intention and reducing patient morbidity.

Multiple benefits of the CTG for mucogingival therapy exist. An excellent tissue match can be achieved, multiple recession defects can be treated and there is less post-operative discomfort at the donor site compared to the free gingival graft (73). Root coverage is predictable, especially for Miller Class I and II recession defects (73). Mean root coverage

results as high as 89.9% have been reported for the CAF with CTG at 10-year follow-up evaluation for Miller Class I and II recession defects (88). A recent systematic review and meta-analysis evaluating tunnel technique outcomes reported an overall mean root coverage of 82% for single teeth and 87% for multiple teeth with Miller Class I or II gingival recessions (89). For Miller Class III defects, many studies demonstrate the most successful results when connective tissue is used (73). For example, Aroca *et al* reported a mean root coverage of 82% when a modified tunnel was combined with a CTG for Miller Class III recession defects, demonstrating the benefit of using autogenous CT for root coverage (40).

In 1975, before the CTG was described in humans, Karring *et al* performed a histologic study in the primate model in which sites previously grafted with connective tissue were de-epithelialized (90). Originally non-keratinized sites healed with keratinized epithelium, demonstrating that the CT is the genetic determinant for the phenotype of the overlying epithelium (90). Levine reported that gingivoplasty in sites previously grafted with CT resulted in an increase in the amount of keratinized tissue (91). However, no measurements were reported and limited data supporting this exists (91), (92). Maurer *et al* reported no difference in the width of KT in gingivoplasty versus non-gingivoplasty groups (92). They did, however, achieve a mean increase in KT width of 1.2 mm following the CTG, which is commonly reported in the literature (92), (73).

Perotto *et al* reported on the early healing of subepithelial connective tissue grafts in humans (93). Compared to baseline, at two months there was an increase in the amount of collagen matrix and a reduction in the number of inflammatory cells associated with the flap (93). Revascularization was evident, but the connective tissue appeared poorly organized and a boundary between the deep and superficial layers indicated that graft integration was not

completed (93). Longer-term histologic studies in humans report complete maturation between 6

and 12 months (94), (95), (96). In a human case report, Harris showed no evidence of

regeneration of the alveolar bone, periodontal ligament or cementum 6 months following a CTG

(96). Instead, repair occurred with formation of a long junctional epithelium or connective tissue

attachment (96).

Surgical complications unique to the connective tissue graft primarily involve the harvest

site. Palatal anatomy differs among individuals resulting in variable thickness and quality of

connective tissue which can create limitations for the grafted site. There is also varying

proximity to vital structures depending on the height of the palatal vault (97). Trauma to the

greater palatine artery and nerve can cause hemorrhage and paraesthesia, respectively, and

excessive removal of connective tissue can lead to necrosis of the palatal flap. A second surgical

site also inevitably results in increased post-operative pain and discomfort. Patients generally

prefer shorter, less traumatic procedures with one surgical site (83), (71), (98). To overcome the

limitations of the connective tissue graft, non-autogenous materials have been developed for

gingival augmentation procedures.

Acellular Dermal Matrix

Acellular dermal matrix is donated human cadaveric dermal tissue. The epidermal and

dermal cells have been removed in order to avoid tissue rejection. It has a basal lamina on one

side with an underlying dermal matrix that serves as a scaffold for the ingrowth of native

fibroblasts and endothelial cells to produce a *de novo* connective tissue matrix (99), (100). It

consists of types I, II, III and IV collagen, laminin, elastin, glycosaminoglycans and vascular

channels (99), (101). Unlike the gingiva, acellular dermal matrix contains an abundance of

elastin fibers, enabling clear visualization of the material histologically (99). The major

advantage of using an allograft over an autograft is eliminating the need for a second surgical

site. Additionally, an unlimited amount of donor tissue is available with uniform thickness that

can be made to have ideal dimensions. Several different commercial acellular dermal matrices

have been designed that are processed in different proprietary ways.

AlloDerm^{TM} Regenerative Tissue Matrix

AlloDerm^{TM} (ADM) Regenerative Tissue Matrix (RTM) (BioHorizons, Birmingham AL)

is the longest used and most extensively researched acellular dermal matrix. AlloDerm^{TM} RTM

was developed in 1994 by LifeCell^{TM} Corporation. It has been used in the medical field for a

variety of applications, including burn treatment, hernia repair and breast reconstruction surgery

(102). It was introduced into the dental field in 1997 for gingival augmentation procedures (102).

Donor tissue is recovered from independent Food and Drug Administration (FDA)-registered

tissue banks (102). Medical and social history screening must meet FDA and American

Association of Tissue Banks (AATB) criteria (102). Donor blood is screened for infectious

diseases including HIV, Hepatitis B and Hepatitis C, and tissue samples are screened for

microbial pathogens (102). The tissue is decellularized and incubated in a cryopreservative

solution (101). The LifeCell^{TM} patented freeze-drying process creates an amorphous ice that

maintains the structural integrity of the dermal matrix (101), (102). LifeCell^{TM} has reported that

their method is superior to conventional freeze-drying that creates ice crystals which disrupt the

matrix architecture leading to increased susceptibility to inflammation and rejection (101), (102),

(103). Antibiotics used during tissue processing include the penicillin group, streptomycin,

gentamicin, kanamycin, neomycin, bacitracin, and vancomycin (101). The tissue is stored at

room temperature and packaged in an inner Tyvek pouch within an outer foil bag (101), (104).

When opened for use, AlloDerm^{TM} RTM must be rehydrated twice in sterile saline or lactated

Ringer's, separating the backing from the tissue after the first rehydration step (104). It usually takes between 10 and 40 minutes for complete rehydration depending on tissue thickness, which ranges from 0.9 to 1.6 mm (104), (105). The tissue has an outer basement membrane side and a lower dermal side that is placed against the vascular wound bed (104).

Numerous clinical and histologic studies have been performed comparing AlloDerm[TM] to traditional CT grafting and most have demonstrated comparable results (98), (99), (106), (107), (108), (109). In a randomized controlled trial, Hirsch *et al* reported a mean root coverage of 98.8% for AlloDerm[TM] and 99.1% for CTG for Miller Class I and II recession defects with results that were stable over the 2-year follow-up period (106). A significantly greater KT gain was achieved for the CTG group, however, with a gain of 2.98 mm versus 2.21 mm for the AlloDerm[TM] group (106). A similar trend was reported by Paolantonio *et al* at 1-year follow-up, and by Novaes *et al* at 3 months (107), (108). However, for this study, by 6 months, there were no statistically significant differences between groups for KT gain (108). A meta-analysis performed by Gapski *et al* found no statistically significant differences between the use of AlloDerm[TM], CTG, CAF or FGG for any outcome measured, which included recession coverage, KT formation, probing depths and clinical attachment levels (98). In terms of comparative esthetic outcomes, superior esthetic results have been reported by clinicians and patients for AlloDerm[TM] versus CTG even when complete root coverage is not achieved, potentially due to the uniformity of allograft thickness (109).

When the CAF with AlloDerm[TM] is compared to the CAF alone, significantly better root coverage results are obtained with the allograft (73), (110), (111). There is also less recurrence of recession over time (111). This is in contrast with grafting with CT, which has been reported to undergo less relapse compared to AlloDerm[TM] (112), (113). Harris reported a mean root

coverage of 93.4% for AlloDerm™ at 12 weeks which decreased to 65.8% at 48 months, compared to the CTG which had a mean root coverage of 96.6% at 12 weeks that changed to 97.0% at 48 months (112). However, it was noted that 32% of the AlloDerm™ cases improved or remained stable over time, and, interestingly, treatment of multiple-teeth recession defects with AlloDerm™ performed significantly better than single-tooth recession defects over time (112). This was a retrospective, non-randomized private practice study which potentially introduced bias (112).

The number of human histologic studies using AlloDerm™ is limited due to ethical considerations. Cummings *et al* performed either a CT or AlloDerm™ graft at 12 teeth planned for extraction in 4 patients (99). Block biopsy sections obtained after 6 months of healing revealed that both grafts were well incorporated with no gross inflammatory reaction (99). New fibroblasts, vascular elements and collagen were present throughout the AlloDerm™ with retention of transplanted elastin fibers (99). Another study demonstrated signs of revascularization, epithelial cell colonization and new collagen synthesis at 2 weeks with complete graft substitution and re-epithelialization at 10 weeks (114). In this study, the existing collagen fibers of the AlloDerm™ were difficult to visualize as early as 6 weeks (114). A recent histologic study in 22 patients who underwent breast reconstruction surgery with ADM reported colonization of the matrix with fibroblasts, myofibroblasts, lymphocytes, macrophages, multinucleated giant cells and mast cells with a relatively rapid ingrowth of blood vessels at a mean of 6 months (115). They noted that the revascularization process of the ADM was more rapid than lymphangiogenesis; early signs of which were only evident in one patient at 9 months (115).

PerioDerm^{TM}

PerioDerm^{TM} (PEM) (Dentsply Implants, Watham MA) was developed by Dentsply Implants in 2010. Donor tissue is recovered and processed by the Musculoskeletal Transplant Foundation (MTF) which screens donors for hepatitis B, hepatitis C, HIV and syphilis (116), (117). The donor tissue is subjected to a three-phase process for decellularization and disinfection that does not compromise the integrity of the matrix (116). In the first phase, the tissue is soaked in a sodium chloride solution that removes the epidermis and disrupts the cells (116). In the second phase, the tissue is washed in a mild detergent called Triton that removes cellular debris, and in the third phase the tissue is disinfected to render PerioDerm^{TM} sterile (116). No antibiotics are used in tissue processing, reducing the risk for allergic reaction (117). The tissue is freeze-dried and stored at room temperature in an inner foil pouch within an outer Tyvek package (117). It must be rehydrated in sterile saline or lactated Ringer's until soft and pliable (117). Once hydrated the thickness ranges from 0.4 to 1.7 mm (116). Like AlloDerm^{TM}, PerioDerm^{TM} has a specific orientation with an outer basement membrane side and a lower dermal side that is positioned towards the tissue bed (117).

No peer-reviewed literature is currently available evaluating the clinical or histological results obtained with PerioDerm^{TM}. A case report sponsored by Dentsply demonstrates the successful use of PerioDerm^{TM} for soft tissue augmentation around an anterior implant provisional crown (118). Based on his clinical experience, Melker reported that the benefits of PerioDerm^{TM} over other ADMs include a short hydration period, good handling properties and good compatibility with existing tissue (119). In his Master's thesis, Richert demonstrated no significant differences in rat gingival fibroblast viability or distribution when seeded on either

AlloDerm™, PerioDerm™ or Puros Dermis™ ADMs at 24 hours, 1 week and 2 weeks (120).

More research is needed to evaluate the clinical and histologic performance of PerioDerm™.

Puros Dermis™

Another ADM product used for soft tissue augmentation is Puros Dermis™ (PDM)

(Zimmer Dental, Carlsbad, CA). The dermal collagen matrix is preserved through the proprietary

Tutoplast technique which has been used for over 40 years (121). It involves a multi-step

sterilization process that utilizes alkaline, osmotic and oxidative treatments to remove cells, kill

bacteria and reduce viral load (121). Unlike AlloDerm™ and PerioDerm™ which are preserved

through freeze-drying, the Tutoplast method involves solvent dehydration and low-dose gamma

irradiation (122). Allografts processed by solvent dehydration are reported to have greater

mechanical properties compared to those that are freeze-dried (123). Puros Dermis™ is stored at

room temperature, rehydrates quickly and does not contain residual antibiotics (121), (122). The

manufacturer advises trimming prior to hydration (122). It is available in thicknesses ranging

from 0.3 to 1.8 mm (121).

Barker *et al* performed a split-mouth study with 52 contralateral Miller Class I, II, or III

sites using either AlloDerm™ or Puros Dermis™ with Zucchelli incisions and a coronally

advanced flap (124). At 6 months both groups had a significant improvement in the percentage

of root coverage, with 81.4% for Puros Dermis™ and 83.4% for AlloDerm™ (124). There were

also no significant differences in probing depth, KT, or amount of postoperative pain (124).

Wang *et al* published two papers evaluating the 12-month results obtained for Miller Class I and

II recession defects using a CAF with either AlloDerm™ or Puros Dermis™ (125), (126). The

first, a multicenter study with 80 patients, reported no significant differences between groups for

any parameter measured except better ease of handling with Puros Dermis™ (125). The second

included an analysis of the data from one specific center (126). Again, both groups demonstrated significant improvements with 80.66% mean root coverage for AlloDerm[TM] and 80.97% for Puros Dermis[TM] (126). Comparable clinical results can therefore be obtained for these two acellular dermal matrices.

OrACELL[TM]

OrACELL[TM] (LifeNet Health, Virginia Beach VA) is an acellular dermal matrix product from LifeNet Health that is intended for use in guided tissue and bone regeneration as well as soft tissue augmentation (127). The tissue is decellularized with patented technology called Matracell[TM] which removes more than 97% of the DNA, reducing potential for immunogenicity (127). In addition to maintaining its collagen and elastin, OrACELL[TM] also retains its native growth factors (127). This increases the potential for faster healing and regeneration (127). The tissue is freeze-dried, stored at room temperature and unlike other commercially available ADMs does not require hydration, resulting in minimal preparatory time (127). It ranges in thickness from 0.76 to 1.75 mm (127).

As with PerioDerm[TM], limited histologic and clinical studies are currently available assessing OrACELL[TM]. Wallace performed a non-controlled case study in which 20 sites with Miller Class I and II recession defects received a CAF with EMD and OrACELL[TM] (128). At 3 months, 91.2% mean root coverage was achieved with gains in clinical attachment level (3.2 mm) and width of KT (0.9 mm) (128). However, this study had several limitations, including a short follow-up period, only 5 subjects, no control group, and concomitant use of enamel matrix derivative serving as a confounding variable. Vreeberg *et al* performed a randomized controlled trial in which 24 patients with single or multiple Miller Class I, II, or III recession defects received a CAF using Zucchelli incisions with either OrACELL[TM] or CT (129). At 6 months, the

mean vertical recession decreased significantly with no significant differences between groups

(129). 91.25% and 95.66% mean root coverage was obtained with the OrACELL^{TM} and CTG

groups, respectively (129).

CHAPTER II

STUDY DESIGN

Background to Issue

Several different acellular dermal matrices are commercially available for use in root coverage and soft tissue augmentation procedures as an alternative to connective tissue grafting. Although many peer-reviewed clinical studies have compared AlloDerm[TM] to connective tissue grafting, only three have compared AlloDerm[TM] to Puros Dermis[TM] (124), (125), (126), only one has compared OrACELL[TM] to CTG (129), and none have evaluated PerioDerm[TM]. Given that these ADMs have been used in clinical practice for years, there is a paucity of both clinical and histologic evidence comparing their characteristics and effectiveness.

As the key cells responsible for matrix remodeling and *de novo* collagen production, *in vitro* studies center on evaluating the distribution of gingival fibroblasts associated with these matrices. Rodrigues *et al* assessed human gingival fibroblast activity following seeding onto AlloDerm[TM] (44). At 7 days, a single-cell layer of fibroblasts was adherent to the surface but was unevenly distributed (44). At 14 days, the layer was distributed more uniformly but exhibited limited migration into the matrix even at 21 days (44). Maia *et al* noted a similar pattern of fibroblast distribution at 14 days (130). In contrast, Richert confirmed rat gingival fibroblast migration throughout the full thickness of the AlloDerm[TM] matrix as early as 24 hours (120). As the first study comparing three different types of ADMs, he also reported no differences in fibroblast viability or distribution when grown on AlloDerm[TM], Puros Dermis[TM] or PerioDerm[TM] (120).

We expanded Richert's research to determine if his findings translated to an *in vivo* model, the Sprague Dawley rat. We compared rat gingival fibroblast growth and distribution, *de novo* collagen production, and matrix degradation at two time points using AlloDerm™ and OrACELL™. A comparative analysis of matrix revascularization permitted a more in depth understanding of wound healing physiology. Histologic and molecular analyses was performed to assess for specific wound healing markers. Gingival fibroblast density and function was evaluated (1) histologically and (2) Col1a1 and Fn-1 mRNA expression. ADM degradation was assessed histologically, and angiogenesis was measured by (1) Vegf-A mRNA expression.

Due to the absence of cells and blood vessels and the high elastin content present in acellular dermal matrices, visualization of *de novo* connective tissue formation was possible over time. Although both ADM products were freeze-dried, they were processed in unique proprietary ways, leading to different requirements for preparation and handling. By comparing AlloDerm™, the most widely tested and utilized ADM, to an alternative product, we assessed whether differences in tissue processing produced differences in wound healing. The purposes of this study were to compare, *in vivo*, the relative amounts of gingival fibroblast density, collagen production, angiogenesis and commercial matrix degradation associated with AlloDerm™ and OrACELL™ at two time points.

Materials and Methods

This study was approved by the Texas A&M University College of Dentistry Institutional Animal Care and Use Committee (IACUC, Animal Use Protocol #2019-0061-CD) and was undertaken as a split-mouth prospective clinical trial.

A statistical description and power analysis (SAS System 9.4) were performed before initiating the study. The number of required animals was determined to be 4 for RT-PCR and 5

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for histology for each of two time points. The minimum number of animals required to determine a statistically significant difference between groups was therefore determined to be 18 ($\alpha = 0.05$, $1 - \beta = 0.8$).

Study Population and Outcomes

Twelve male and twelve female Sprague Dawley rats were selected for the study, weighing between 300 to 700 g. The primary study outcomes included gingival fibroblast distribution, collagen formation, ADM degradation and angiogenesis, and soft tissue thickness at 7 and 21 days. The secondary outcome was to determine if wound healing was gender dependent. The study subject distribution is outlined in Table 2-1.

Table 2-1. Study subject distribution and investigation sequence.

	7 days	**21 days**
Histology	3 male 3 female	3 male 3 female
RT-PCR	3 male 3 female	3 male 3 female

Surgical Procedure

A pilot surgery was performed on a deceased Sprague Dawley rat to verify the details of the surgical procedure. Live animals were weighed and anesthetized with 3% isoflurane inhalation for 3 to 5 minutes followed by an intraperitoneal injection of a combination of 40-80

mg/kg Ketamine and 5-10 mg/kg Xylazine. A 3 mm x 3 mm pouch was created at the buccal

gingival margin adjacent to the first molars in each maxillary quadrant using a spoon excavator

[Thompson Dental, 1.15 mm]. No vertical incisions were made. ADMs were prepared according

to manufacturer's instructions. AlloDerm^{TM} was rehydrated in sterile saline in two separate baths

for a total of 25-30 minutes until soft and pliable throughout. OrACELL^{TM} was hydrated in

sterile saline in a single step for 10-15 minutes. A 2 mm biopsy punch [Premier^{TM} Uni-Punch®]

was taken of each prepared ADM. Each surgical pouch received one of the following materials

as shown in Figure 2-1, with the exception of the control group, which served as an untreated

control. Any remaining ADM was discarded. The pouches were sealed with cyanoacrylate

adhesive [PeriAcryl®, GluStitch Inc.] and hemostasis was achieved. No sutures were used.

Quadrant 1 (Upper Right): AlloDerm^{TM} RTM	Quadrant 2 (Upper Left): OrACELL^{TM}
Quadrant 4 (Lower Right): Untreated control	

Figure 2-1. Implant design by quadrant.
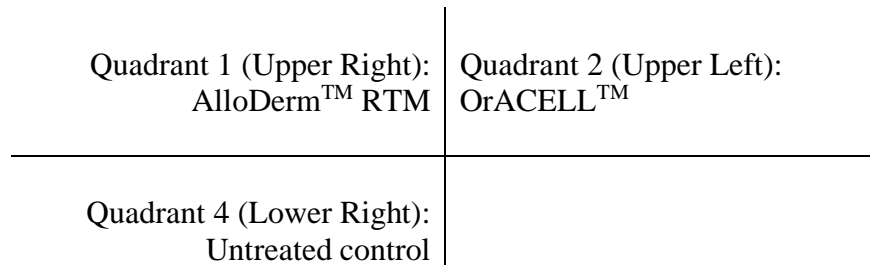
Post-Surgical Care

Animals were numbered and placed under a heating lamp for post-surgical monitoring. A

subcutaneous injection of 2-5 mg/kg Nalbuphine was administered immediately post-operatively.

Animals were observed daily for signs of distress indicating a need for humane intervention.

Criteria for early sacrifice followed IACUC recommendations, including but not limited to

bleeding that could not readily be stopped, an inability to rise or move about the cage, lethargy and labored breathing. No animals were sacrificed early. Animals were placed on a soft food diet [DietGel®, ClearH$_2$O] for the entire post-operative period, either 7 or 21 days.

Sample Collection

Animals were weighed prior to sacrifice. Intraoral photographs of each surgical site were taken immediately following sacrifice [Canon EOS Rebel T6, 100 mm macro lens]. Biopsy specimens were harvested using a 3 mm biopsy punch [Premier^{TM} Uni-Punch®] at the buccal gingiva adjacent to the first molars in the test and control quadrants. A microsurgical blade [Salvin® Dental Specialties, Mini Blade #69] was used to separate the specimen from the alveolar bone. Samples were placed in the appropriate medium depending on analysis method. All sample containers were immediately labelled according to animal number and material (AlloDerm^{TM}, OrACELL^{TM}, Control).

Histology

Biopsy specimens for histologic analysis were fixed in 10% formalin, processed and embedded in paraffin, and serially sectioned at 7 μm. Six sections were stained with hematoxylin and eosin (H&E), three sections were stained with Picro-sirius red, and three sections were stained with Verhoeff's solution. Picro-sirius red stain is specific for most types of collagen while Verhoeff's solution identifies the presence or absence of elastin. See Figures 2-2, 2-3 and 2-4 for representative slides.
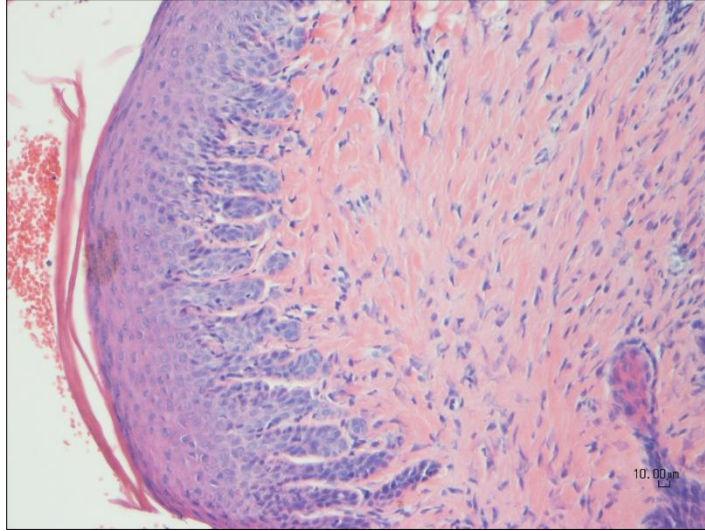
Figure 2-2. AlloDerm^{TM}, 21-day male. [20x magnification, H&E stain]

Figure 2-3. OrACELL^{TM}, 21-day female. [4x magnification, Picro-sirius red stain]
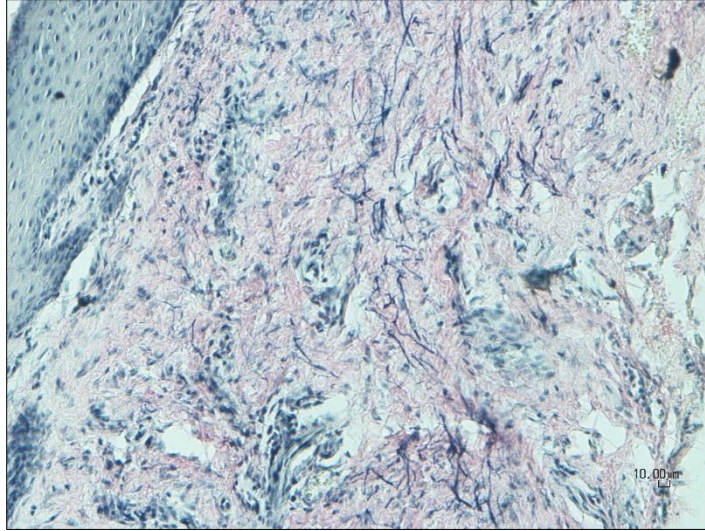
Figure 2-4. OrACELL[TM], 7-day male. [20x magnification, Verhoeff's solution]

Fibroblast Quantification

H&E stained slides were photographed using a light microscope with a 10x objective. Scientific imaging analysis software (ImageJ, NIH) was used to overlay a grid on each image measuring 100 µm^2. See Figure 2-5. Two adjoining boxes containing connective tissue only were then selected from the center of each sample. These two boxes were isolated from the remainder of the image and divided into four squares each to facilitate counting (ImageJ, NIH). The number of fibroblasts within each box were counted by two calibrated examiners who were blinded to the treatment group (IC, KX) and one calibrated examiner not blinded to the treatment group (SC).

A.

B.
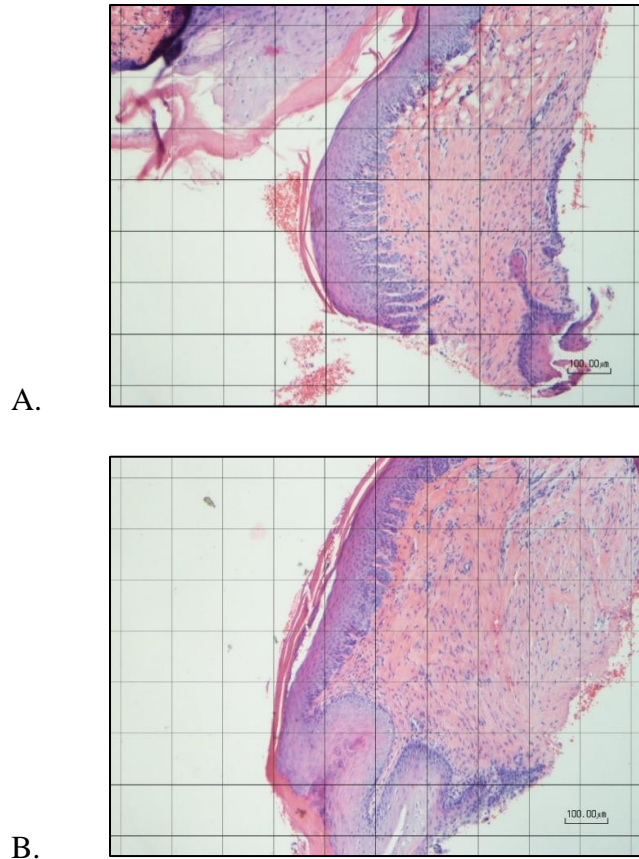
Figure 2-5. Analysis method for fibroblast quantification.
A. AlloDerm™, 21-day male group [10x magnification, H&E stain].
B. OrACELL™, 21-day male group [10x magnification, H&E stain].

Collagen Quantification

Picro-sirius red stained slides were photographed using a polarizing light microscope with a 4x objective. Scientific imaging analysis software (ImageJ, NIH) was used to create binary images to quantify the total number of black pixels representing the total tissue area. A second image was then produced by separating the original image into its red, blue and green channels, and converting the red channel to binary for quantification of the Picro-sirius red-stained collagen. This produced a ratio of birefringent collagen to the total sample, enabling calculation of the % of collagen present in each sample. See Figure 2-6.

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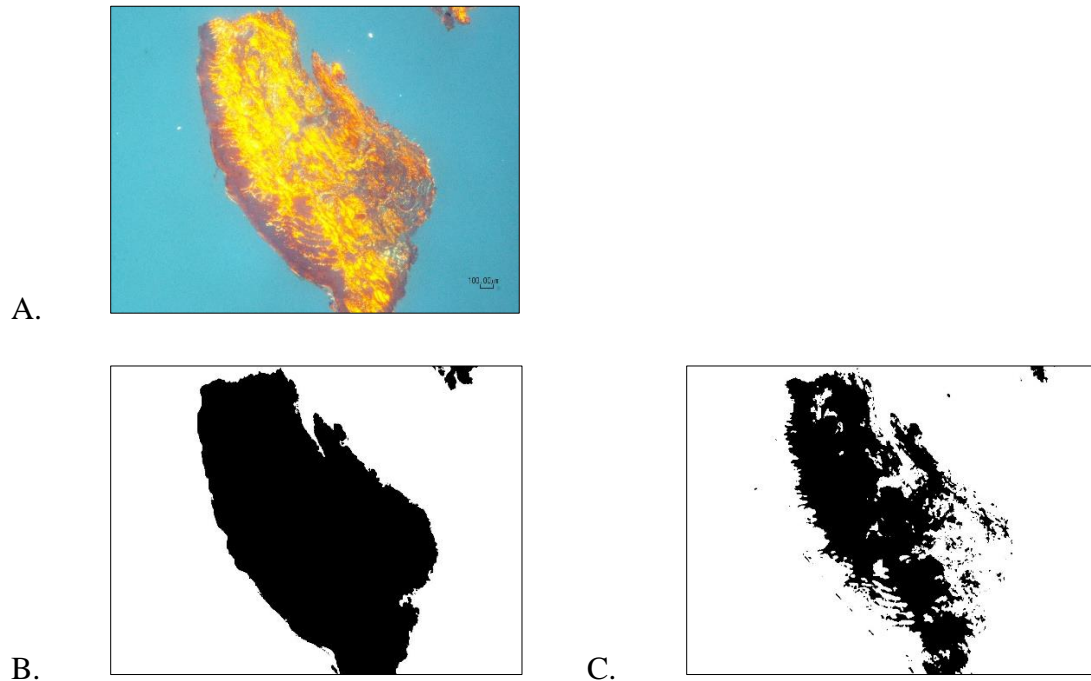
Figure 2-6. Analysis method for collagen quantification.
A. AlloDerm^TM, 7-day female group [4x magnification, Picro-sirius red stain].
B. Binary image created to quantify the total number of black pixels (i.e. 430,003).
C. Image separated into red channel and made binary to quantify the number of black pixels (i.e. 260,236). This enabled calculation of the % of birefringent collagen in the sample (i.e. 60.52%).

Elastin Degradation

Verhoeff's stained slides were photographed using a light microscope with a 10x objective. Scientific imaging analysis software (ImageJ, NIH) was used to create binary images to quantify the total number of black pixels representing the total area of connective tissue. A second image was then produced by separating the original image into its red, blue and green channels, and converting the blue channel to binary for quantification of the Verhoeff-stained elastin. This produced a ratio of elastin and cell nucleus elements to the total connective tissue, enabling calculation of the % of elastin present in each sample. See Figure 2-7.
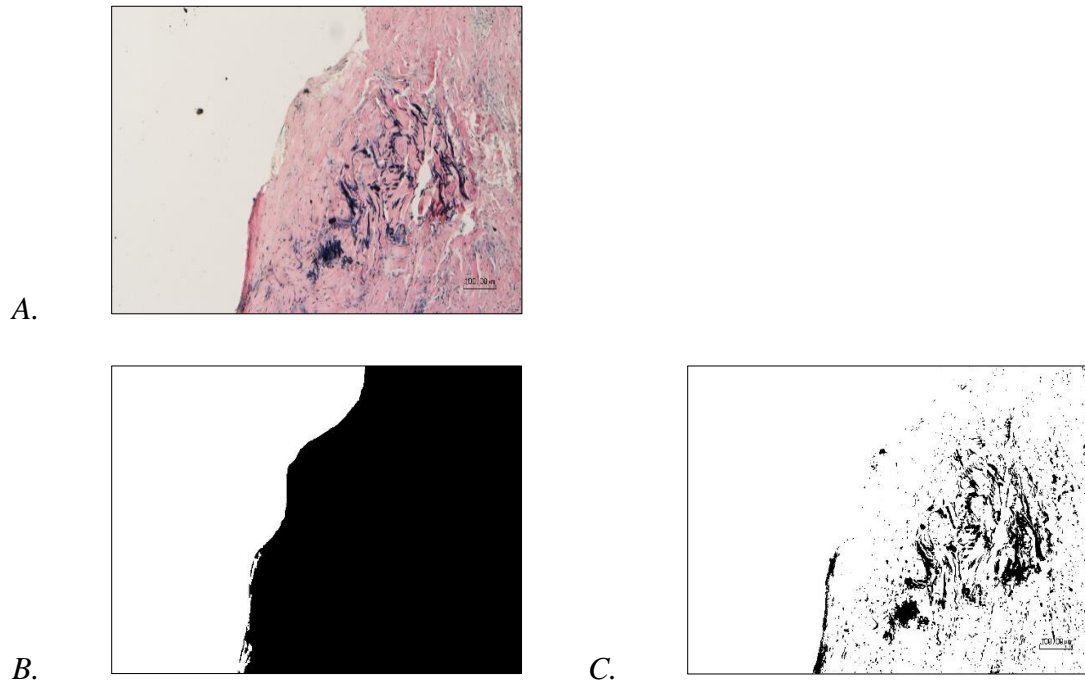
Figure 2-7. Analysis method for elastin degradation

A. OrACELL™, 21-day male group [10x magnification, Verhoeff's stain].

B. Binary image created to quantify the total number of black pixels (i.e. 705,066).

C. Image separated into blue channel and made binary to quantify the number of black pixels (i.e. 87,189). This enabled calculation of the % of elastin in the sample (i.e. 12.37%).

RT-PCR

The relative expression of genes for alpha-1 type 1 collagen (Col1a1), vascular endothelial growth factor (Vegf-A), and fibronectin (Fn-1) was assessed by RT-PCR. Biopsy specimens, collected using a 3 mm biopsy punch including both epithelium and connective tissue, were stored at -80 degrees Celsius. Total RNAs were isolated from mouse periodontal tissues and human PDL cells using the RNeasy® Plus Mini Kit according to the manufacturer's instructions [Qiagen®]. Two micrograms of total extracted RNA were applied toward cDNA generation with RNA to cDNA EcoDry Premix [Takara Bio Inc.]. Real-time PCR was performed

using sequence specific primers (Rat Col1a1 Forward 5' aatggtgctcctggtattgc 3', Reverse 5'

ggttcaccactgttgccttt 3'; Fn Forward 5' catgaaggggggtcagtccta 3', Reverse 5' gtccattccccttttccatt 3';

Vegf-A Forward 5' cgaacagagagagggacagg 3', Reverse 5' cgactggtccgatgaaagat 3'; β-actin

Forward 5' agccatgtacgtagccatcc 3', Reverse 5' accctcatagatgggcacag 3'; GAPDH Forward 5'

aagggctcatgaccacagtc 3', Reverse 5' ggatgcagggatgatgttct 3') using the SYBR green Master Mix

and the ABI Prism 7000 Sequence detection system [Applied Biosystems, Thermo Fisher

Scientific]. Reaction conditions were as follows: 2 min at 50°C (1 cycle), 10 min at 95°C (1

cycle), 15s at 95°C, and 1 min at 60°C (40 cycles). Samples were normalized to levels of

GAPDH or β-Actin. To quantify relative differences in mRNA expression, the comparative CT

method (ΔΔCT) was used to determine relative quantity. Values were graphed as the mean

expression level ± standard deviation (SD).

Clinical Photograph Survey

Intraoral photographs of each test site were taken immediately following sacrifice [Canon

EOS Rebel T6]. See Figure 2-9 for sample photographs. Seven blinded examiners consisting of

1st, 2nd, and 3rd year Graduate Periodontics residents evaluated each photograph in two separate

sessions; the first session for male specimens and the second for female specimens. Examiners

completed a survey (Figure 2-8) assessing soft tissue thickness using a scoring system ranging

from 0 to 3 and the totals were calculated for each specimen.
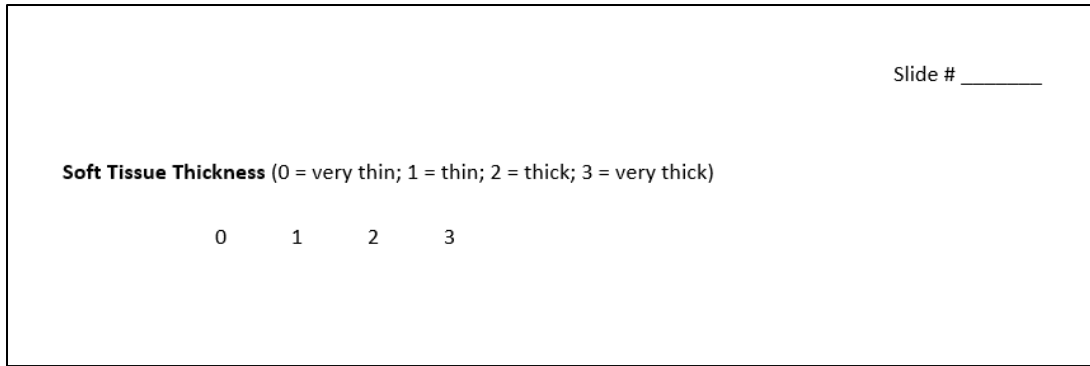
Figure 2-8. Soft tissue thickness survey.

A.   B.
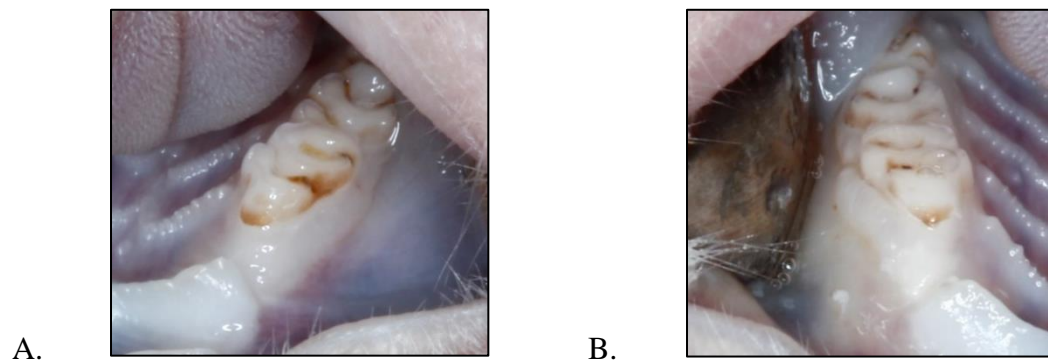
Figure 2-9. Sample clinical photographs.
A. AlloDerm™, 7-day female
B. OrACELL™, 7-day female
C. AlloDerm™, 7-day male
D. OrACELL™, 7-day male
E. AlloDerm™, 21-day female
F. OrACELL™, 21-day female
G. AlloDerm™, 21-day male
H. OrACELL™, 21-day male

C.

D.

E.

F.

G.

H.
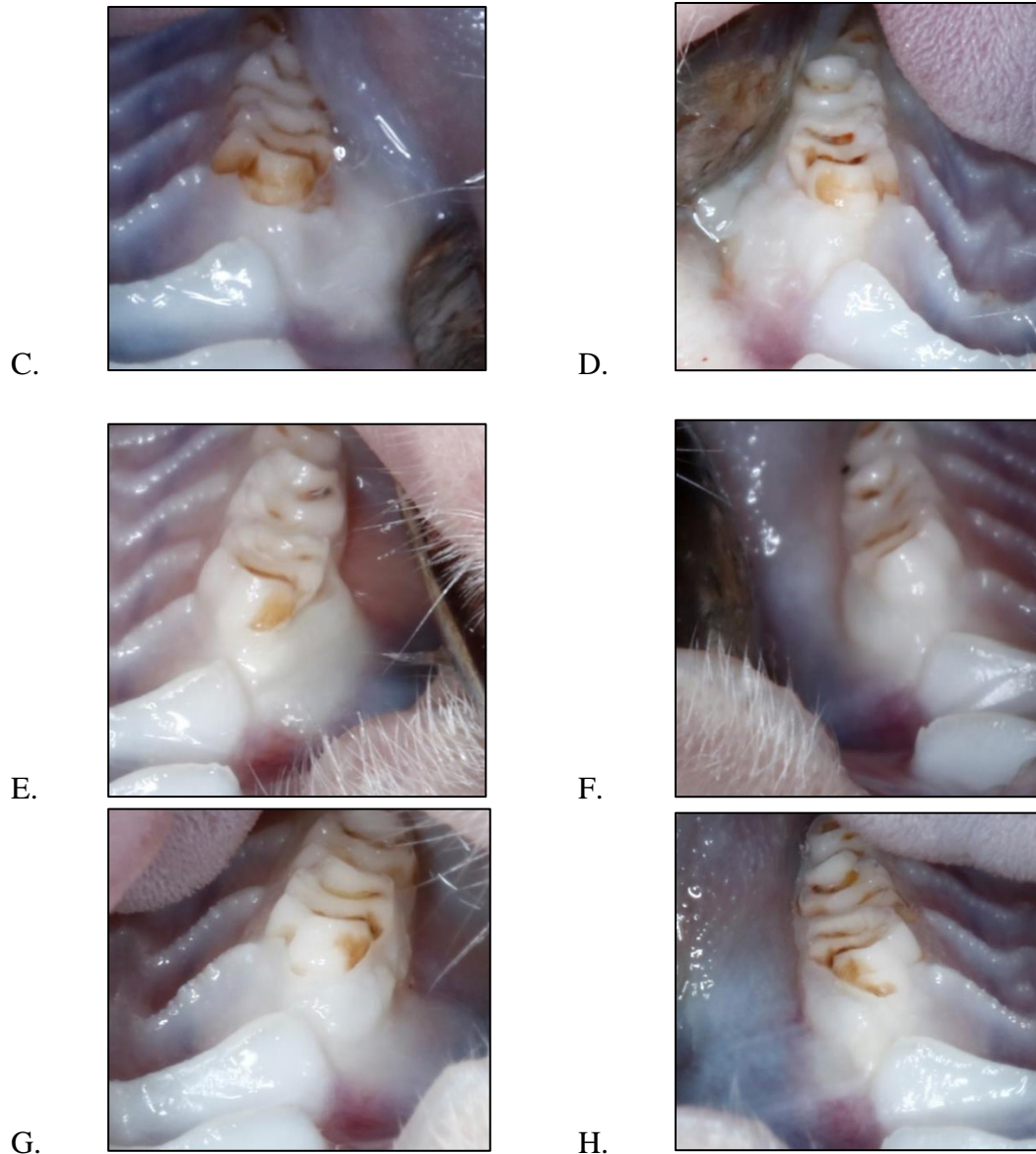
Figure 2-9. Continued.

Statistical Analysis

We used linear mixed effects models to study differences in average fibroblast cell count/μm^2, % birefringent collagen, % elastin, Col1a1 mRNA, Vegf-A mRNA, Fn-1 mRNA, and perceived soft tissue thickness between three treatment groups: AlloDerm™, OrACELL™,

and control at two timepoints (7 days versus 21 days post-treatment). Significance was achieved at p-value < 0.05. For linear mixed effects models that met significance, post-hoc Tukey's HSD tests were performed to determine which group(s) had differences; significance was achieved at p-value < 0.0167 (0.05 ÷ 3) to correct for multiple comparisons (Bonferroni). Further, we used linear mixed effects models to consider differences in average fibroblast count / μm^2, % birefringent collagen, % elastin, Col1a1 mRNA, Vegf-A mRNA, Fn-1 mRNA, and soft tissue thickness between genders (male and female) at two time points (7 days versus 21 days) for each group. Significance was achieved at p-value < 0.05. Significant models were followed-up with post-hoc Welch's two sample t-tests to determine average differences between genders for each group; significance was achieved at p-value < 0.05.

CHAPTER III

RESULTS

12 animals were sacrificed at 7 days and 12 animals were sacrificed at 21 days.
The final breakdown of the number of histology slides and PCR samples available for analysis is
shown in Table 3-1.

Table 3-1. Number of slides (histology) and samples (PCR) analyzed per group.

Method	Stain/Gene	Group	7 days	21 days
Histology	H&E	ADM	2 M 3 F	3 M 2 F
		ORA	2 M 2 F	3 M 3 F
		CON	2 M 3 F	3 M 2 F
	Picro-sirius	ADM	2 M 3 F	3 M 3 F
		ORA	2 M 3 F	3 M 3 F
		CON	2 M 3 F	3 M 3 F
	Verhoeff's	ADM	1 M 3 F	3 M 1 F
		ORA	2 M 2 F	3 M 2 F
		CON	2 M 3 F	3 M 2 F

Table 3-1. Continued.

Method	Stain/Gene	Group	7 days	21 days
RT-PCR	Col1a1	ADM	3 M 3 F	3 M 3 F
		ORA	3 M 3 F	3 M 3 F
		CON	3 M 3 F	3 M 3 F
	Vegf-A	ADM	3 M 3 F	3 M 3 F
		ORA	3 M 3 F	3 M 3 F
		CON	3 M 3 F	3 M 3 F
	Fn-1	ADM	3 M 3 F	3 M 3 F
		ORA	3 M 3 F	3 M 3 F
		CON	3 M 3 F	3 M 3 F

ADM: AlloDerm™
ORA: OrACELL™
CON: Control

Histology

Fibroblast Quantification

Table 3-2. Number of fibroblasts per timepoint (average per box).

	7-day subjects	**21-day subjects**
AlloDerm™	34.03 ± 11.49	38.28 ± 8.87
OrACELL™	45.83 ± 19.56 *	44.50 ± 7.06 *
Control	34.90 ± 9.96	36.07 ± 4.84

* Indicates a trend towards a difference between groups

Table 3-3. Number of fibroblasts per timepoint and gender (average per box)

	7-day males	7-day females	21-day males	21-day females
AlloDerm^{TM}	34 ± 12.07	34.05 ± 13.59	33.06 ± 9.18	43.5 ± 5.53
OrACELL^{TM}	57 ± 24.07 **	34.67 ± 1.81	43.72 ± 9.12	45.28 ± 6.30
Control	37.58 ± 14.94	32.22 ± 1.78	39.56 ± 3.56	32.58 ± 3.06

** Indicates a significant difference between genders ($p < 0.05$)
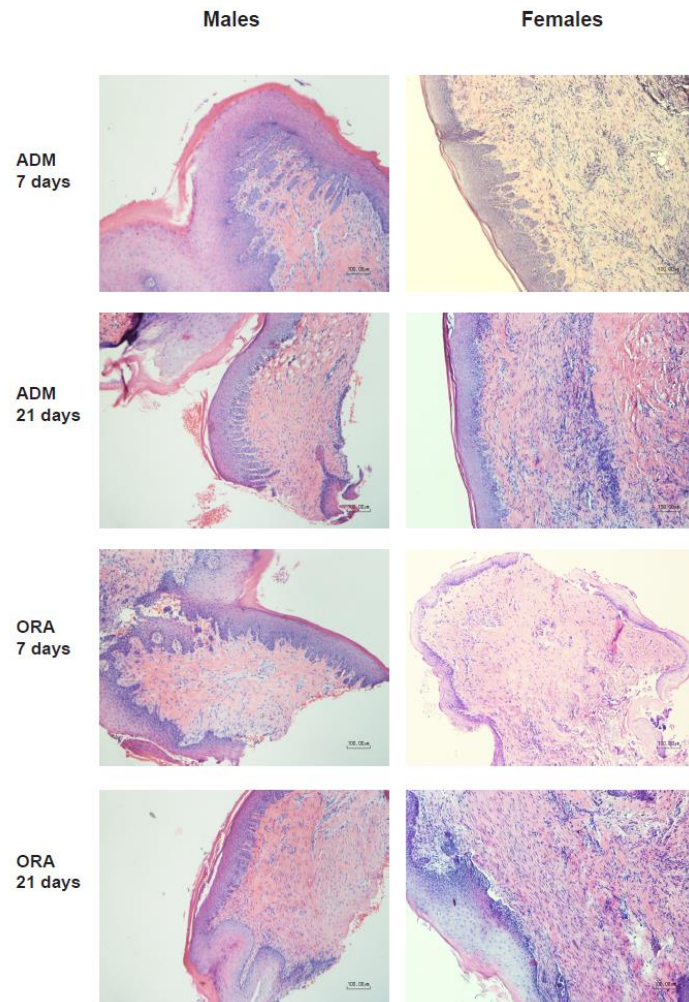
Figure 3-1. Sample slides for fibroblast quantification. [10x magnification, H&E stain]

There was a significant difference in the number of fibroblasts present between the AlloDerm™, OrACELL™, and control groups at 7 days (p = 0.0495) and at 21 days (p = 0.0494). Following correction for multiple comparisons, there were no significant differences between specific groups at either time point (p > 0.0167) but there was a trend towards a greater number of fibroblasts for OrACELL™ compared to AlloDerm™ at both 7 days (p = 0.072) and 21 days (p = 0.058).

There was a strong trend for difference in fibroblast number between the 7-day male and female subjects (p = 0.052). Post-hoc analysis revealed a significantly greater number of fibroblasts present for males in the OrACELL™ group compared to females (p = 0.041). There were no significant differences in the number of fibroblasts present between the 21-day male and female subjects in any group (p = 0.569).

Collagen Quantification

Table 3-4. Percentage of birefringent collagen (% BRC) per timepoint.

	7-day subjects	**21-day subjects**
AlloDerm™	44.55 ± 27.93%	55.71 ± 19.75%
OrACELL™	53.96 ± 23.37%	52.05 ± 21.94%
Control	41.42 ± 14.34%	29.53 ± 22.50%

Table 3-5. Percentage of birefringent collagen (% BRC) per timepoint and gender.

	7-day males	7-day females	21-day males	21-day females
AlloDerm[TM]	$20.92 \pm 19.83\%$	$60.31 \pm 20.79\%$	$49.25 \pm 17.42\%$	$62.16 \pm 23.38\%$
OrACELL[TM]	$41.09 \pm 1.63\%$	$62.54 \pm 22.25\%$	$55.16 \pm 20.01\%$	$48.93 \pm 27.82\%$
Control	$45.12 \pm 4.67\%$	$38.96 \pm 19.43\%$	$29.23 \pm 14.12\%$	$29.82 \pm 32.66\%$
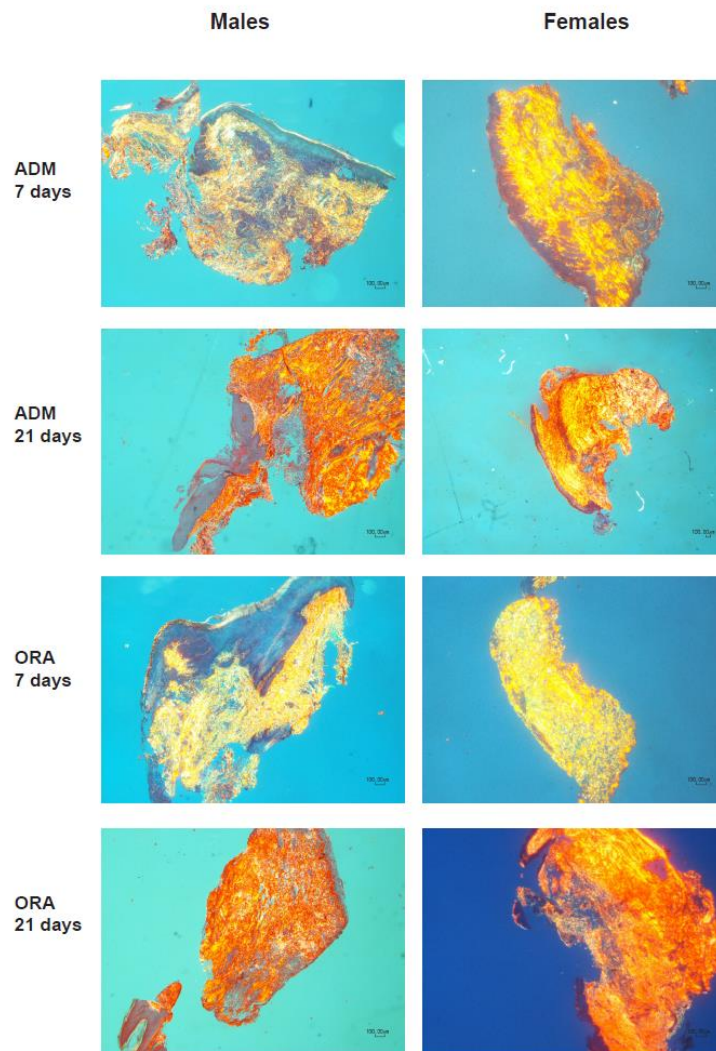
Figure 3-2. Sample slides for % BRC analysis. [4x magnification, Picro-sirius red stain]

There were no differences in % BRC between the AlloDerm™, OrACELL™, and control groups at 7 days (p = 0.634) or between the three groups at 21 days (p = 0.121).

There were no differences in % BRC between the 7-day male and female subjects in any group (p = 0.131) or between the 21-day male and female subjects in any group (p = 0.820).

Elastin Degradation

Table 3-6. Percentage of elastin per timepoint.

	7-day subjects	**21-day subjects**
AlloDerm™	16.85% ± 6.77% **	7.70% ± 2.63%
OrACELL™	11.12% ± 3.41%	8.09% ± 3.38%
Control	6.71% ± 2.68%	6.99% ± 2.21%

** Indicates a significant difference between groups (p < 0.0167)

Table 3-7. Percentage of elastin per timepoint and gender.

	7-day males	**7-day females**	**21-day males**	**21-day females**
AlloDerm™	7.04% ± 0.00%	20.12% ± 2.15% **	7.75% ± 3.21%	7.55% ± 0.00%
OrACELL™	8.72% ± 1.40%	13.52% ± 3.14% **	8.76% ± 3.29%	7.08% ± 4.54%
Control	5.03% ± 0.64%	7.83% ± 3.08%	7.50% ± 1.92%	6.23% ± 3.20%

** Indicates a significant difference between genders (p < 0.05)
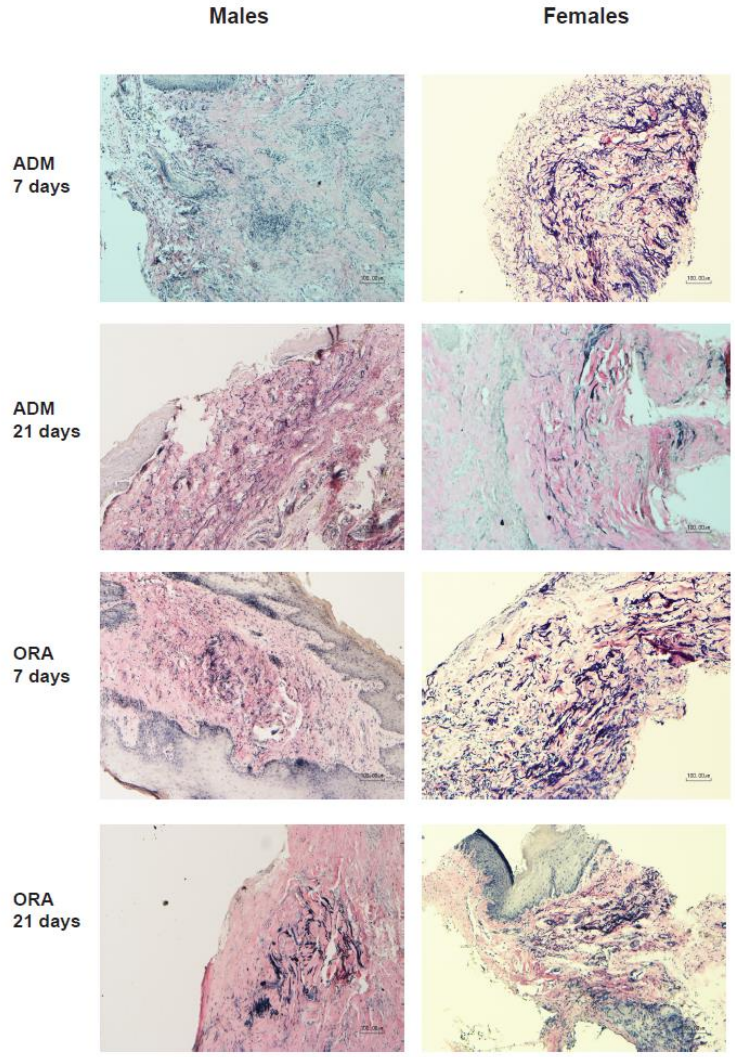
Figure 3-3. Sample slides, % elastin. [10x magnification, Verhoeff's solution]

There was a significant difference in the % of elastin between the AlloDerm[TM], OrACELL[TM], and control groups at 7 days (p = 0.0036). Post-hoc analysis revealed that the % of elastin present was significantly greater for the AlloDerm[TM] group compared to the control group (p = 0.003). There was also a trend for greater elastin present for the AlloDerm[TM] group compared to the OrACELL[TM] group (p = 0.074). There was no difference in the % of elastin between groups at 21 days (p = 0.831).

There was a significant difference for the % of elastin between the 7-day male and female subjects (p = 0.010). Following post-hoc analysis, this gender difference was only significant for the test groups combined (AlloDerm™ + OrACELL™); i.e. there was a significantly greater % of elastin present for the test groups of the 7-day females compared to those of the 7-day males (p = 0.006). There was no difference in the % of elastin between the 21-day male and female subjects in any group (p = 0.484).

RT-PCR

Col1a1
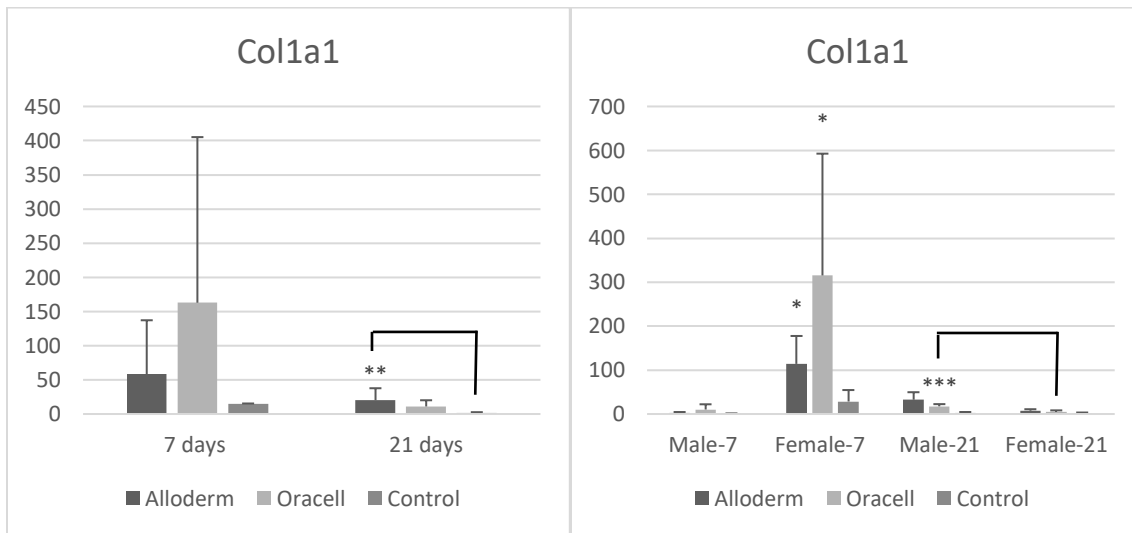
A.   B.

* Indicates a trend towards a difference between groups
** Indicates a significant difference between groups (p < 0.0167)
*** Indicates a significant difference between genders (p < 0.05)

Figure 3-4. Relative Col1a1 expression.
A. Relative Col1a1expression per timepoint
B. Relative Col1a1expression per timepoint and gender

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There were no differences in the expression of Col1a1 between the AlloDerm™, OrACELL™, and control groups at 7 days (p = 0.176), but there was a significant difference between groups at 21 days (p = 0.017). Post-hoc analysis revealed increased Col1a1 expression in the AlloDerm™ group compared to the control group at 21 days (p = 0.013).

There was a significant difference in the expression of Col1a1 between the 7-day male and female subjects (p = 0.028). When post-hoc analysis was performed, this difference was not statistically significant though a trend was noted when comparing the two test groups combined (AlloDerm™ + OrACELL™); there was greater expression of Col1a1 for the test groups in the 7-day female subjects compared to the test groups in the 7-day males (p = 0.060). There was a significant difference in the expression of Col1a1 between the 21-day male and female subjects (p = 0.013). Following post-hoc testing, this difference was seen for the OrACELL™ group, with greater expression of Col1a1 in 21-day males compared to 21-day females (p = 0.027).

Vegf-A

A.   B.

* Indicates a trend towards a difference between genders

Figure 3-5. Relative Vegf-A expression.
A. Relative Vegf-A expression per timepoint
B. Relative Vegf-A expression per timepoint and gender

There were no differences in the expression of Vegf-A between the AlloDerm[TM],

OrACELL[TM], and control groups at 7 days (p = 0.3496) or between the three groups at 21 days

(p = 0.415).

There was a trend towards a significant difference in the expression of Vegf-A between the

7-day male and female subjects (p = 0.0529). Following post-hoc testing, there was a trend when

comparing the two test groups combined (AlloDerm[TM] + OrACELL[TM]); there was greater

expression of Vegf-A for the test groups in the 7-day male subjects compared to the test groups

in the 7-day females (p = 0.078). There were no differences in the expression of Vegf-A between

the 21-day male and female subjects in any group (p = 0.620).

Fn-1

A.   B.
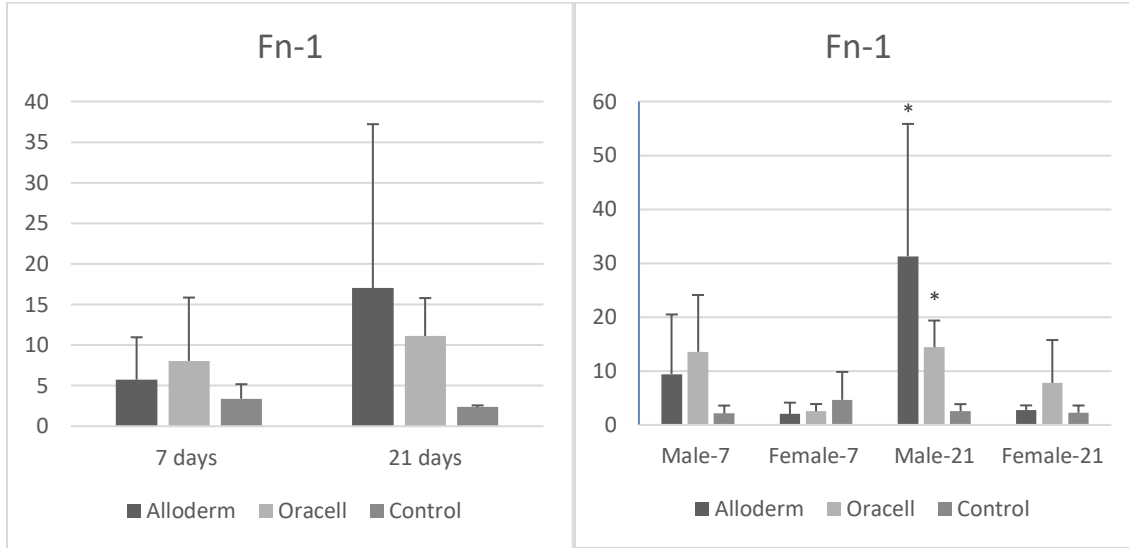
* Indicates a trend towards a difference between genders
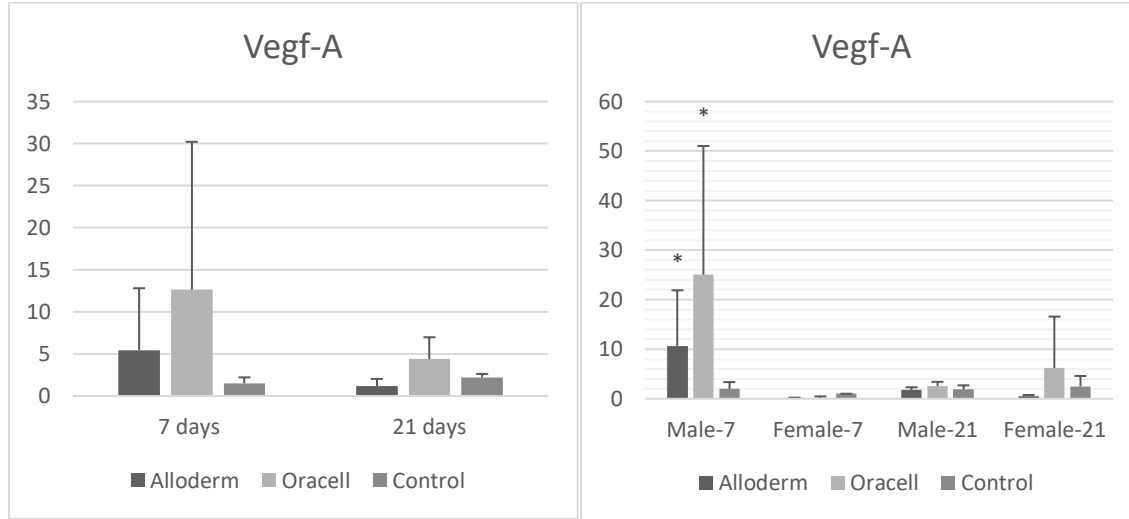
Figure 3-6. Relative Fn-1 expression.
A. Relative Fn-1 expression per timepoint
B. Relative Fn-1 expression per timepoint and gender

There were no differences in the expression of Fn-1 between the AlloDerm™, OrACELL™, and control groups at 7 days (p = 0.529) or between the three groups at 21 days (p = 0.1452).

There was no difference in the expression of Fn-1 between the 7-day male and female subjects (p = 0.110) or in the expression of Fn-1 between the 21-day male and female subjects, though a trend toward significance was noted (p = 0.058). Following post-hoc analysis, a trend was noted when comparing the test groups combined (AlloDerm™ + OrACELL™), with greater expression of Fn-1 for the test groups in the 21-day males compared to the 21-day females (p = 0.072).

Clinical Photograph Survey

Soft Tissue Thickness
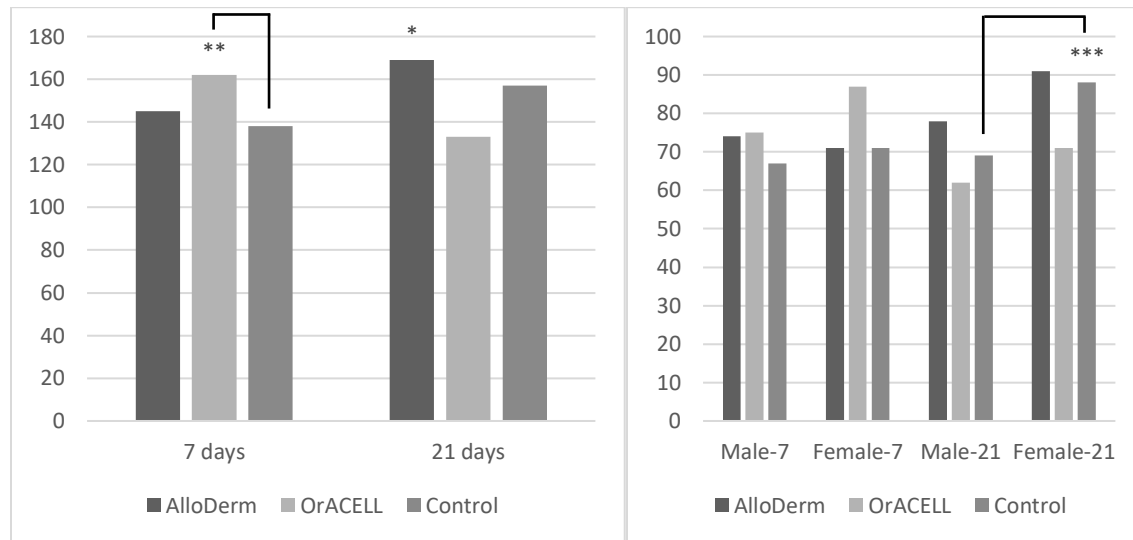
A.   B.

* Indicates a trend towards a difference between groups
** Indicates a significant difference between groups (p < 0.0167)
*** Indicates a significant difference between genders (p < 0.05)

Figure 3-7. Soft tissue thickness
A. Soft tissue thickness per timepoint
B. Soft tissue thickness per timepoint and gender

There was a significant difference in the observed soft tissue thickness between the

AlloDerm[TM], OrACELL[TM], and control groups at 7 days (p = 0.0066) and at 21 days (p =

0.0367). When post-hoc analyses were performed, there was a significantly greater observed soft

tissue thickness for the OrACELL[TM] group compared to the control group (p = 0.007) and a

trend towards greater thickness for the OrACELL[TM] group compared to the AlloDerm[TM] group

(p = 0.029) at 7 days. At 21 days, there was a trend for greater observed soft tissue thickness for

the AlloDerm[TM] group compared to the OrACELL[TM] group (p = 0.032).

There was no difference in the observed soft tissue thickness between the 7-day male and female subjects (p = 0.892). There was a significant difference between the 21-day male and female subjects (p = 0.019). Following post-hoc analysis, this difference was found to be significant for the control group only, with female subjects having a greater thickness than male subjects (p = 0.047).

Table 3-8. Summary of statistically significant findings

	Group		Gender	
7 days	**Fibroblasts**	---	**Fibroblasts**	ORA: M > F
	% Elastin	ADM > CON	**% Elastin**	ADM + ORA: F > M
	Soft tissue thickness	ORA > CON	**Soft tissue thickness**	---
21 days	**Col1a1**	ADM > CON	**Col1a1**	ORA: M > F
	Soft tissue thickness	---	**Soft tissue thickness**	CON: F > M

ADM: AlloDerm™
ORA: OrACELL™
CON: Control

CHAPTER IV

DISCUSSION

Rats are a well-established animal model with anatomy suitable for experimental investigation. The molar region was selected instead of the more accessible incisor region for several reasons. As noted during the pilot surgery, the gingival tissue adjacent to the incisors was challenging to elevate. Rat incisors continually erupt which may impact wound stability (131). There is also a tendency for fur and bedding to become impacted into the sulcus of these prominent anterior teeth. In contrast, the buccal gingiva of the first molar region was straightforward to elevate and protected from the external environment and tongue. To minimize trauma to surgical sites, all rats were placed on a soft food diet for the post-surgical duration of the study.

Both male and female rats were included to evaluate gender differences in wound healing. In rats as well as humans there were gender distinctions in important components of wound healing (132). For example, gender differences existed in relation to the coagulation system, specifically prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT) and fibrin values (132). Adult female rats were also significantly smaller than males, resulting in fewer cellular elements available for matrix colonization and remodeling (131).

The timepoints of 7 and 21 days were chosen because existing *in vitro* studies examining acellular dermal matrices observed gingival fibroblast migration at these times (44), (120), (130). For example, Rodrigues *et al* seeded AlloDerm™ with human gingival fibroblasts and evaluated cell distribution at 7, 14 and 21 days (44). Although there was limited migration into the matrix, cell adhesion and spreading were evident as early as 7 days (44). Existing *in vivo* histologic

studies on AlloDerm[TM] had greater variation in study duration, examining surgical sites as early as 3 days and as late as 9 months (99), (115).

It is important to note that wound healing in rats was more rapid than humans (132). The clotting time in rats was three times faster than humans, leading to faster wound stability (132). Rats, unlike humans, can synthesize their own Vitamin C, a necessary component for collagen synthesis (132). These properties made the relative differences between groups (AlloDerm[TM], OrACELL[TM], control) more meaningful for translational research.

A comprehensive literature search revealed no existing studies comparing these two products. In this unique study, three histologic stains were used to evaluate and quantify the relative amounts of fibroblasts, collagen, and elastin between ADM groups. Furthermore, relative gene expression of Col1a1, Fn-1 and Vegf-A was assessed. RT-PCR is a rapid technique with high sensitivity that has numerous applications in biomedical science (133). It allowed for an objective depiction of the quantities of type 1 collagen, fibronectin, and VEGF-A, key components of wound healing.

12 animals were sacrificed at 7 days and 12 animals were sacrificed at 21 days. One male subject from the 7-day histology group was excluded from all methods of histologic analysis due to evidence of infection (pus) in the AlloDerm[TM] quadrant at the time of biopsy. The final sample size was 23 subjects (11 male, 12 female).

One H&E slide was excluded from the 7-day female OrACELL[TM] group and one H&E slide each was excluded from the 21-day female AlloDerm[TM] and 21-day female control groups due to poor quality of biopsy specimens.

One Verhoeff's slide was excluded each from the 7-day male AlloDerm group, 7-day female OrACELL[TM] group, 21-day female OrACELL[TM] group, and 21-day female control group

due to poor quality of either the stain or the biopsy specimen. Two Verhoeff's slides were excluded from the 21-day female AlloDerm^{TM} group due to poor quality.

At 7 and 21 days, there was a trend towards a greater density of fibroblasts for the OrACELL^{TM} group compared to the AlloDerm^{TM} group. Since both materials were acellular, all cells present histologically were derived from native tissue. Higher numbers of fibroblasts, the primary cells responsible for extracellular matrix production, signified rapid migration into the wound area and an active proliferative phase for faster wound healing. LifeNet Health, the manufacturer of OrACELL^{TM}, advertised that the product retains its native growth factors (127). Though they do not specify which growth factors were present, multiple growth factors attract fibroblasts, including fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and transforming growth factor β (TGF-β) (134). Greater collagen production by these cells provide evidence to support the observation that the soft tissue was thicker in the OrACELL^{TM} group at 7 days. Interestingly though, there were no differences between test groups in the percentage of birefringent collagen or in the expression of Col1a1 at either timepoint. Including the epithelial layer in the specimens used for RT-PCR may have diluted the gene expression. Therefore, tissue processing or more time would be needed to distinguish significant differences between groups in collagen production.

In addition to the potential growth factors associated with OrACELL^{TM}, inherent structural differences of the two products may have contributed to these results. For example, the OrACELL^{TM} may have been thicker than the AlloDerm^{TM}. A range of thickness exists for both matrices, with AlloDerm^{TM} ranging from 0.9 to 1.6 mm and OrACELL^{TM} ranging from 0.76 to 1.75 mm thick (105), (127). However, greater tissue thickness means a greater distance for native cells to migrate. The connective tissue scaffold of OrACELL^{TM} may be denser, with an enhanced

framework for cell migration; or more rigid, with better space maintenance than AlloDerm[TM].

An ultrastructural or biomechanical study of both products would aid in elucidating these

features.

At 7 days there was trend towards greater density of elastin present for the AlloDerm[TM]

group compared to the OrACELL[TM] group. Unlike dermal tissue, gingival tissue does not

contain elastin (99). The presence of elastin in our slides meant that (1) the acellular dermal

matrices had been successfully implanted and remained in place until the time of sacrifice, and

(2) we could assess matrix remodeling over time. The non-keratinized oral mucosa does contain

elastin, however, explaining why it was present in the control group (135).

It is possible that we observed more elastin for the AlloDerm[TM] group because the

original product may have contained more. Alternatively, OrACELL[TM] may have faster early

matrix remodeling, also supported by the greater number of fibroblasts. By 21 days, there were

no significant differences between groups, indicating that both products reach the same stage of

remodeling in three weeks. In their human study, Cummings *et al* noted retained elastin fibers

after 6 months (99). It was unclear if elastin from implanted acellular dermal matrices becomes

completely remodeled (99).

An additional objective of this study was to assess differences between groups in

angiogenesis and revascularization. Therefore, we measured the gene expression of vascular

endothelial growth factor A (VEGF-A). VEGF is a cytokine produced by platelets, endothelial

cells, neutrophils and macrophages that stimulates the migration and proliferation of endothelial

cells for angiogenesis (41), (134). Since OrACELL[TM] was prepared to retain native growth

factors, we expected a higher expression of Vegf-A in the OrACELL[TM] group. At both

timepoints there was slightly greater Vegf-A expression for OrACELL™ compared to AlloDerm™ but the levels were not significantly different.

Fibronectin, an extracellular matrix protein produced by fibroblasts important in all phases of wound healing, was not significantly different between groups (134). It stabilized the initial clot, guided cell migration to the site of injury, and was a large component of the early extracellular provisional wound matrix (41), (134). Fibronectin gene expression was expected to increase through the maturation phase. In this study both AlloDerm™ and OrACELL™ displayed elevated Fn-1 expression relative to the control, with greater expression at 21 days compared to 7 days.

A secondary objective of this study was to determine if wound healing was gender dependent. However, there were no significant gender differences for either ADM product in the percentages of birefringent collagen or elastin, soft tissue thickness, or expression of Vegf-A and Fn-1. In the OrACELL™ group, there was a significantly greater number of fibroblasts for males at 7 days, and a significantly greater expression of Col1a1 for males at 21 days. The average weight of the male rats was 615 g compared to the average weight of female rats at 333 g. Although a standardized diameter tissue punch was used for all biopsies, the specimen thicknesses were not uniform. Larger rats would be expected to have thicker tissue with more numerous cellular components, producing greater expression of Col1a1. Since no differences were observed for elastin quantities, we can conclude that the rate of matrix remodeling was similar in males and females.

This study had several limitations. The first was that the sample size was small. The second was that the control group was untreated. A treated control was attempted during the pilot surgery by taking a punch biopsy of palatal connective tissue but there was insufficient tissue for

use. A suitable alternative for future studies would be to create a surgical pouch without implanted tissue, producing tissue injury to more accurately assess wound healing differences. A third limitation was that test and control quadrants were not randomized; test quadrants were always located in the maxilla and control quadrants in the mandible. Because the buccal gingiva of the mandible was thinner and more difficult to access, test groups were standardized to the maxilla.

No attempt was made for acellular dermal matrix orientation, despite both companies recommending a specific orientation of the dermis. For example, the manufacturer's instructions for OrACELL[TM] state that the reticular side should be placed against the surgical wound or most vascularized tissue with the papillary side facing up (127). Though only tested using AlloDerm[TM], it has been demonstrated in human clinical studies that the orientation of the dermis has no bearing on the amount of root coverage achieved (136). Whether this produces histologic differences remains unknown.

Future studies to build on this research should address the above limitations. They should also include an ultrastructural analysis of both products in order to assess whether structural or biological differences could explain these results. It would be interesting, for example, to test for the presence of growth factors in OrACELL[TM]. Excluding the epithelium in the samples for RT-PCR would aid in a more accurate analysis of the expression of these connective tissue-specific genes. Furthermore, a clinical study comparing these two products would certainly help determine if these differences have clinical implications in humans.

The inspiration for this research project was the *in vitro* study by Richert *et al* which found no significant differences in gingival fibroblast growth and distribution between AlloDerm[TM], Puros Dermis[TM], and PerioDerm[TM] (120). This *in vivo* study found several

differences between AlloDerm™ and OrACELL™, two commercially available acellular dermal matrices used for periodontal soft tissue grafting procedures. OrACELL™ had higher numbers of fibroblasts at 7 and 21 days, and greater soft tissue thickness and less elastin at 7 days. OrACELL™ appears to have faster early wound healing characteristics than AlloDerm™.

CHAPTER V

CONCLUSION

This split-mouth histologic and molecular study found differences in the wound healing

of two acellular dermal matrices available for use in periodontal surgery; AlloDerm^{TM}, which has

been widely researched and used in clinical practice for decades, and OrACELL^{TM}, a relatively

newer product with limited available peer-reviewed literature. This was the first study to date

comparing these two materials. Both products were freeze-dried but were processed by distinct

proprietary technologies leading to different preparation steps. It appears that this difference in

processing methodology may have effects at the cellular and molecular levels, with OrACELL^{TM}

displaying faster early wound healing than AlloDerm^{TM} in the rodent model. This product

exhibited more rapid migration of fibroblasts to the site of injury as well as faster matrix

remodeling. A potential explanation for this may be the presence of retained growth factors

associated with OrACELL^{TM}.

This study also found a gender difference in the wound healing of these implanted

materials. Males displayed more rapid matrix remodeling and wound stabilization, and greater

angiogenesis than females. This reinforces current understanding of the gender distinctions that

exist in wound healing.

Whether these results have positive or negative clinical implications for soft tissue

grafting procedures in humans remains to be determined. AlloDerm^{TM} was a time-tested product

supported by a large body of evidence. Future studies should focus on comparing OrACELL^{TM}

to its predecessor to determine if it can produce equivalent or superior long-term clinical results.

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