

SOFT TISSUE ATTACHMENT TO TITANIUM IMPLANTS COATED WITH GROWTH FACTORS

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Chapter 2.

SOFT TISSUE ATTACHMENT TO TITANIUM IMPLANTS COATED WITH GROWTH FACTORS

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2.1 ABSTRACT

Background: Peri-implant tissues form a crucial but fragile seal between the oral environment, the bone and the implant surface. Enhancing the seal formed by the peri-implant soft tissues at the titanium/connective tissue interface may be an important factor in implant survival. Additionally, enhancing soft tissue adherence to the implant surface when implants are placed in dehiscence type defects may mean that simultaneous osseous grafting procedures will not always be required.

Objective: The aim of this study was to investigate the effect of implant surface modification with either platelet-derived growth factor (PDGF) or enamel matrix derivative (EMD) on the connective tissue attachment to moderately roughened titanium implants.

Material and Methods: 18 moderately roughened titanium implants were subcutaneously implanted into 14 rats. 6 implants each were coated with PDGF and EMD immediately prior to implantation and 6 implants were left uncoated. The implants were retrieved with a sample of surrounding tissue at 4 and 8 weeks. The specimens were resin-embedded and sections viewed under confocal microscopy for collagen autofluorescence and prepared for qualitative and histomorphometric analysis under light microscopy. ANOVA and t-tests were used to compare the thickness of fibroblast encapsulation on the implant surface and the depth of connective tissue penetration onto the implant grooves.

Results: Qualitative analysis under confocal and light microscopy showed encapsulation of all implants by fibroblasts and good soft tissue integration at the end of 4 and 8 weeks. Coating of the implants with growth factors did not alter the orientation of fibroblasts and collagen fibres. Histomorphometric analysis demonstrated that the depth of connective tissue penetration into the implant grooves was significantly greater for the implants coated with PDGF at 4 weeks (ANOVA, P value 0.0014). The thickness of the fibroblast encapsulation on the implant surface was significantly less for the implants coated with PDGF at 8 weeks (ANOVA, P value 0.0012).

Conclusion: Good soft tissue integration can be achieved on a moderately roughened titanium implant surface. Coating the implant surface with rhPDGF-BB could increase the speed of soft tissue healing around an implant surface but this increased rate of healing with rhPDGF-BB coating could also result in a less robust titanium/connective tissue interface.

2.2 INTRODUCTION

Osseointegrated dental implants are transmucosal “masticatory devices” that penetrate the oral mucosa, with the peri-implant tissues expected to exercise a protective function (Weber & Cochran 1998). Research and clinical focus in dental implantology in the past two decades has primarily concentrated on the bone-to-implant interface and the peri-implant mucosa, with the soft tissue seal around implants investigated to a much lesser degree. Both bone and soft tissue integration to dental implants are wound healing processes involving several stages of tissue formation and degradation (Berglundh *et al* 2003, Abrahamsson *et al* 2004, Berglundh *et al* 2007). Osseointegration is the result of the modelling and remodelling of bone tissue that occurs after implant placement, whilst the wound healing that occurs following the closure of mucoperiosteal flaps during implant surgery results in the establishment of a mucosal attachment (transmucosal attachment) to the implant. The establishment of the mucosal barrier around the implant is characterised by a gradual shift from a coagulum to granulation tissue followed by the formation of a barrier epithelium and the formation of connective tissue (Berglundh *et al* 2007).

Several studies using animal and human models have investigated the structure and function of the peri-implant mucosa (Berglundh *et al* 1991, 1992, 1994, 2003, 2007; Buser *et al* 1992, Ericsson *et al* 1996, 1997; Abrahamsson *et al* 1996, 1997, 1998, 1999, 2002, 2004; Berglundh & Lindhe 1996, Cochran *et al* 1997, Moon *et al* 1999, Glauser *et al* 2005, Schüpbach & Glauser 2007, Welander *et al* 2007, 2008, Allegrini Jr *et al* 2008, Nevins *et al* 2008). In an early study in dogs, Berglundh *et al* (1991) compared the gingiva around teeth and the mucosa around two-stage implants (Branemark System®, Nobel Biocare, Göteborg,

Sweden). It was found that the peri-implant mucosa consisted of a 2 mm long barrier epithelium and a zone 1-1.5 mm high where the connective tissue was in direct contact with the TiO₂ layer of the implant. This area was termed a zone of “connective tissue integration”.

Histologically, the peri-implant epithelium and the surrounding connective tissue of dental implants have similar characteristics to those structures surrounding teeth (Abrahamsson & Soldini 2006) but differ in terms of the orientation of collagen fibres (Buser *et al* 1992), the composition of the connective tissue (Moon *et al* 1999, Abrahamsson *et al* 2002), and the distribution of the vascular system of the peri-implant mucosa (Berglundh *et al* 1994). The connective tissue in the zone of integration has a low density of blood vessels but a large number of fibroblasts and collagen fibres appearing to originate from the periosteum of the bone crest and extending towards the margin of the soft tissue in a direction parallel to the long axis of the abutment. More detailed analyses of the soft tissue/implant interface using transmission electron microscopy found that the zone of connective tissue directly adjacent to the implant surface has a large number of round and flat-shaped fibroblasts with their long axes parallel with the implant surface but virtually no blood vessels. Further away from this zone the number of fibroblasts decreases but there are more collagen fibres and there is an increase in vascularity (Moon *et al* 1999, Abrahamsson *et al* 2002). Berglundh *et al* (1991) stated that the main difference between the mesenchymal tissues present at a tooth and at an implant site is the occurrence of cementum (acellular or cellular) on the root surface.

There is no doubt that the peri-implant soft-tissues form a crucial seal between the oral environment, the bone and the implant surface (Cochran *et al* 1994, 1997). The seal is fragile and due to the absence of periodontal ligament fibres when subjected to bacterial or mechanical challenge the destruction of peri-implant tissues can be a faster and more devastating process than in periodontal tissues (Salcetti *et al* 1997, Maksoud 2003). Thus, enhancing the seal formed by the peri-implant soft tissues, especially that of the titanium/connective tissue interface, may be an important factor in implant survival.

The titanium/connective tissue interface, certainly for smooth, machined surface dental implants, lacks a mechanical attachment of inserting collagen fibres, unlike that of periodontal tissues of teeth. Whether this lack of mechanical attachment differs for roughened surface implants has not been extensively investigated. A small number of recent *in vivo* studies have indicated that microtexturing of the implant can be used to control the soft tissue response (Glauser *et al* 2005, Schüpbach & Glauser 2007, Nevins *et al* 2008). Until recently most dental implants were designed such that the transmucosal portion of the implant was of a smooth or polished nature. Recently, these design concepts have changed, with several implant designs allowing crestal placement and incorporating roughened surfaces into the coronal portion of the body of the implant, up to the level of the implant-abutment platform (eg. Nobel Replace, Straumann Bone-level, Astra Osseospeed). Some clinicians have advocated that roughened surfaces may in fact be conducive to very good soft tissue adherence in dehiscence type defects and therefore placement of implants into these defects may not always require osseous grafting procedures to correct these defects (Dragoo, personal communication).

Surface modification of titanium implants may improve the ability of connective tissue components in the peri-implant mucosa to attach to the implants. Currently, most dental implant types incorporate a “roughened” surface as part of their macro-design. Many of these surfaces are able to absorb proteins and thus act as a reservoir or carrier for attachment proteins, growth factors and other biological agents which may be of assistance for soft or hard tissue integration. *In vitro* studies have shown that epithelial cell adhesion to titanium surfaces coated with biological agents such as fibronectin, laminin and collagen was enhanced in comparison with uncoated titanium (Dean *et al* 1995, Tamura *et al* 1998, Park *et al* 1998, Roessler *et al* 2001, Nagai *et al* 2002). However, in a recent study investigating soft tissue healing around implants in a canine model, it was found that the vertical dimensions of the epithelial and connective tissue components as well as the composition of the connective

tissue zone directly adjacent to the implant were similar for collagen-coated and non-coated implants at 4 and 8 weeks of healing (Welander *et al* 2007).

2.2.1 Platelet-Derived Growth Factor (PDGF)

PDGF is a natural protein sequestered by blood platelets and bone matrix secreted locally during clotting at the site of soft- or hard-tissue injury, stimulating a cascade of events that leads to the wound healing response. The primary effect of PDGF is that of a mitogen, initiating cell division. It has been shown, particularly with the PDGF-BB form, to be a potent stimulator of many types of connective tissue cells, including periodontal ligament fibroblasts, cementoblasts and osteoblasts (Lynch *et al* 1989, Piche & Graves 1989, Matsuda *et al* 1992, Dennison *et al* 1994, Boyan *et al* 1994). Improvement in periodontal wound healing leading to significant bone, cementum and periodontal ligament regeneration has been observed after applying PDGF-BB in combination with either insulin-like growth factor-1 (IGF-1), β -tricalcium phosphate (β -TCP) or a bovine derived xenograft (Lynch *et al* 1989, 1991a, 1991b, 2006; Rutherford *et al* 1992, 1993; Giannobile *et al* 1994, 1996; Howell *et al* 1997, Camelo *et al* 2003, Nevins *et al* 2003, 2005, 2007; McGuire *et al* 2006, Simion *et al* 2006). These results illustrate the beneficial effects of PDGF on both soft and hard tissue healing and regeneration. Recently, a growth-factor enhanced matrix (GEM) has become available for clinical use. This graft material consists of a concentrated solution of pure recombinant human platelet-derived growth factor (rhPDGF-BB), mixed with an osteoconductive matrix composed of β -TCP and is marketed as GEM 21S (Osteohealth, Shirley, New York, USA).

2.2.2 Enamel Matrix Protein Derivatives

The biologic concept behind enamel matrix-induced periodontal regeneration is based on the discovery that enamel matrix proteins, are not only involved in enamel formation but also play a key role in the formation of the root and attachment apparatus. Enamel matrix protein

derivatives (EMD), of which 90% are amelogenins, are secreted during tooth root development by Hertwig's epithelial root sheath and play a crucial role in the formation of acellular root cementum which is the most important tissue for the insertion of collagen fibres (Slavkin and Boyde 1975, Slavkin 1976, Slavkin *et al* 1988, Lindskog 1982a, 1982b; Lindskog & Hammarstrom 1982, Brookes *et al* 1995, Fong *et al* 1996). These proteins are thought to induce the formation of periodontal attachment during tooth formation and it is believed that EMD used in periodontal lesions mimic the development of the tooth supporting apparatus (Hammarström 1997). Emdogain® (Biora AB, Straumann, Malmö, Sweden), a porcine-derived material, is the only commercially available product using EMD.

Apart from its original use as an agent to enhance and promote regeneration, Emdogain® has also been reported to be effective in the management of recession defects by enhancing soft tissue adherence to root surfaces. Its use in these situations may promote collagen synthesis, the formation of cementum, periodontal ligament and bone and may therefore increase the width of keratinised tissue (Hägewald *et al* 2002, McGuire & Nunn 2003, McGuire & Cochran 2003, Nemcovsky *et al* 2004, Spahr *et al* 2005, Castellanos *et al* 2006, Moses *et al* 2006, Sato *et al* 2006, Shin *et al* 2007). This finding may be of particular relevance to the previously-stated opinion of some clinicians that moderately roughened surfaces may be conducive to good soft tissue adherence and that placement of implants into dehiscence-type defects may not always require osseous correction. Whether an agent such as EMD would enhance soft tissue adhesion to exposed implant surfaces in dehiscence type defects remains to be established.

A number of studies have examined the changes in soft tissue level after implant placement (Bengazi *et al* 1996, Grunder 2000, Ekfeldt *et al* 2003). Despite significant differences in experimental designs, the majority of studies conclude that gingival recession that varies between 0.6 mm to 1.5 mm is unavoidable. Whilst multiple factors can influence gingival recession around transmucosal dental implants, there is little doubt that the low level of connective tissue attachment to implant surfaces is important (Rompen *et al* 2006). Various

methods have been proposed to improve the quality of the soft tissue interface, including micro and macro design features of the transmucosal portion of the implant (Glauser *et al* 2005, Schüpbach & Glauser 2007, Nevins *et al* 2008).

To date there have been few studies investigating the effect of surface modification with EMD but none with PDGF on the connective tissue attachment to titanium implants. A number of studies have investigated the effects of PDGF and EMD on bone healing around dental implants. Whilst PDGF has been shown to be influential in improving the regeneration of peri-implant bone (Lynch *et al* 1991a, Becker *et al* 1992, Meraw *et al* 2000), the use of EMD has not been shown to contribute to the amount of bone-to-implant contact around titanium implants (Franke Stenport & Johansson 2003, Cangini & Cornellini 2005). Recently, a pilot study reporting on the effects of autogenous periodontal cell grafts, with and without the application of EMD, on the implant-connective tissue interface found that an implant-connective tissue interface morphologically consistent with a periodontal connective tissue attachment was not observed in sections from any of the implant or autogenous cell grafts (Craig *et al* 2006).

2.3 HYPOTHESIS AND AIM

The hypothesis for this study is that surface modification of roughened surface (TiUnite) titanium implants with PDGF or EMD results in improved bioactivity of the implant surface, thereby promoting cell attachment and CT formation, which is expected to result in an improved attachment.

The aim of this study is to investigate if surface modification of roughened surface (TiUnite) titanium implants with PDGF or EMD has the potential to enhance connective tissue attachment to titanium implants.

2.4 SIGNIFICANCE OF THE AIM

Enhancing the soft tissue seal formed by the peri-implant soft tissues will be of clinical significance, particularly with regards to preventing recession, enhancing aesthetic outcomes and even enhancing placement into sites with significant dehiscence defects.

2.5 MATERIAL AND METHODS

2.5.1 Animals

Fourteen female Dark Agouti (DA) rats, each about 6 to 8 weeks old were used. These were acquired through the Animal Services Division, Institute of Medical and Veterinary Science (IMVS), Adelaide. The research protocol related to the use of animals in this study was approved by the animal ethics committees of both the University of Adelaide and the IMVS.

2.5.2 Implants

Eighteen Branemark System® Mk III Groovy NP (3.3 mmØ x 10 mm) (Nobel Biocare AB, Göteborg, Sweden) implants were used. Six test implants were coated with enamel matrix protein derivative (Emdogain®, Biora AB, Straumann, Malmö, Sweden) and 6 test implants were coated with reconstituted recombinant human platelet-derived growth factor-BB (rhPDGF-BB, Pepro Tech, Rocky Hill, New Jersey, USA). The 6 control implants were uncoated. Two of these control implants were used for an initial pilot study to verify the feasibility of the experimental protocol.

2.5.3 Pilot Study

For the pilot study, two single uncoated implants were surgically placed subcutaneously into the backs of two rats. One animal was sacrificed at 4 weeks and the other at 8 weeks. The implants and surrounding tissue were retrieved at the time of sacrifice and then processed for future analysis. This experiment was conducted to check on the viability of the study, i.e. the

ability to get a meaningful sample by ensuring that the animals tolerated the implants and that there were no ill effects over the time course planned for this study.

2.5.4 Major Experimental Study

For the major experimental study, there were three groups:

Group 1 – Animals with uncoated implants

In this group, two rats each had two uncoated implants surgically implanted, making a total of four uncoated implants for the group.

Group 2 – Animals with enamel matrix protein derivative (Emdogain®) coated implants

Six implants coated with Emdogain® were placed into four animals. Two rats received two coated implants and the other two rats had only one coated implant placed into their backs.

Group 3 – Animals with platelet-derived growth factor (rhPDGF-BB) coated implants

This group also had four animals with two rats receiving two rhPDGF-BB coated implants while the other two had only one coated implant surgically implanted, making a total of six rhPDGF-BB coated implants.

Animals in each group were sacrificed at 4 weeks and 8 weeks. At the time of sacrifice, the implants and surrounding tissues were surgically retrieved, processed and analysed. Two implants were retrieved from Group 1 at each time point while 3 implants were retrieved from each of the other groups at each time point. A total of 16 implants were used for the major study.

2.5.5 Preparation of Growth Factors

2.5.5.1 Enamel Matrix Protein Derivative

A commercially available enamel matrix protein derivative (Emdogain® (Lot no. E1636A), Biora AB, Straumann, Malmö, Sweden) with a concentration of 30 mg/ml in a propylene glycol alginate carrier was opened at the time of implantation.

2.5.5.2 rhPDGF-BB

On the day of implantation, 500µg of recombinant human platelet derived growth factor-BB (rhPDGF-BB, Pepro Tech, Rocky Hill, New Jersey, USA) was reconstituted in 1.67 ml of sterile saline in accordance to the manufacturer's instruction to produce a rhPDGF-BB concentration of 0.3 mg/ml and stored at 4°C until ready for use. This concentration is the same as GEM 21S® (Osteohealth, Shirley, New York, USA), a commercially available rhPDGF-BB used in conjunction with β -TCP in periodontal regenerative therapy.

2.5.6 Surgical Procedures

All surgical procedures were performed using inhalation anaesthesia induced with 2% v/v isoflurane with O₂ flow rates set at 2L/min.

A modification of the implantation model used by Bartold *et al* (1989) was employed. Following the administration anaesthesia, a subcutaneous incision measuring approximately 20 mm was made along the ventral midline between the left and right shoulders (Figure 1).

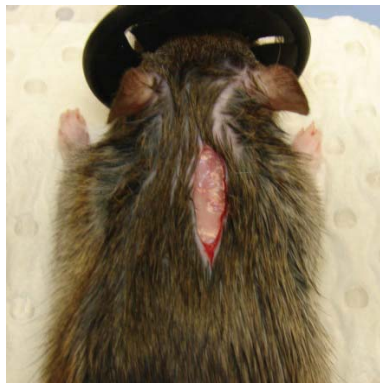


Figure 1. Initial subcutaneous incision in anaesthetised animal prior to implantation.



Figure 2. Implantation into subcutaneous pouch.

A subcutaneous pouch above either the right or left shoulder was created for the placement of the implant (Figure 2). If two implants were to be placed, then pouches were created below the left and right shoulder.

The control implants were placed uncoated and the test implants were either coated with Emdogain® or rhPDGF –BB by submerging them for 30 seconds in the freshly prepared

growth factor contained within an Ependorf tube (Figure 3) before immediate placement into the subcutaneous pouches.

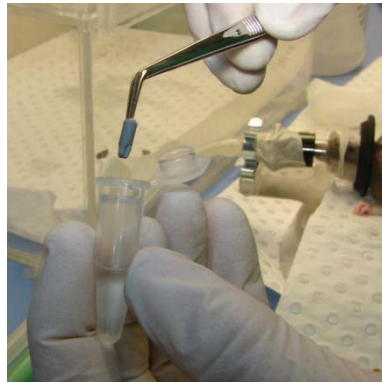


Figure 3. Coating of test implant with growth factor.

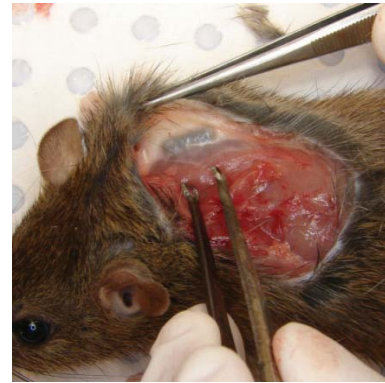


Figure 4. Implant in euthanized animal prior to retrieval.

After the implants were secured in their positions, the incision was closed using staples and swabbed with Betadine. Post-operatively the rats were administered 22.7 mg/ml enrofloxacin (Baytril®, Bayer AG, Leverkusen, Germany) orally for 1 week. The staples were removed 2 weeks after implant placement and the rats were monitored daily and weighed weekly during the healing period.

2.5.7 Implant Retrieval

The rats were euthanized by CO₂ asphyxiation and the implants were located through implantation records and palpation. For implant retrieval, a similar but larger ventral incision was made and the implant retrieved with a sample of surrounding tissue (Figure 4). The retrieved samples were placed in a fixative (10% PBS buffered formalin) for 48 hours prior to processing into resin blocks.

2.5.8 Resin Embedding

The retrieved implant/tissue biopsies were transferred from the fixative and dehydrated in serial steps of alcohol concentrations and subsequently embedded in a methyl-methacrylate resin (Merck Schuchardt OHG, Hohenbrunn, Germany) (Appendix 1 and 2). The resulting resin embedded implant/tissue blocks were cut using an Isomet slow-speed diamond saw

(Beuhler, Illinois, USA) along the long axis of the implant and maximising the volume of surrounding tissue to obtain two central sections. The distal portions of the implant/tissue blocks were cut along the same axis to create a resin block with parallel surfaces (Figure 5).

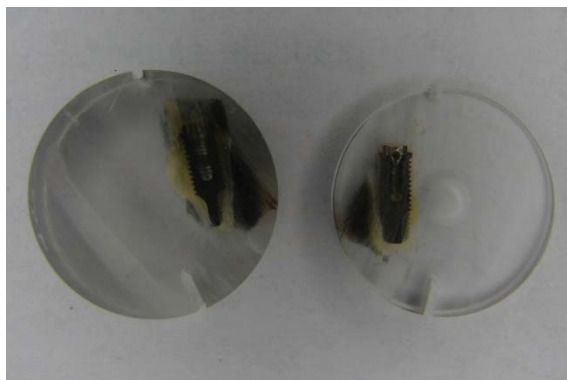


Figure 5. Sectioned implant/tissue resin embedded blocks.

2.5.9 Confocal Laser Scanning Microscopy Analysis

Confocal laser scanning microscopy of the sectioned implant/tissue resin embedded blocks was carried out using a Leica TCS SP5 Confocal Microscope System (Leica Microsystems, Heidelberg, Germany) (Clarke 2007). The implant/tissue block sections were viewed using a 20x IMM objective lens (magnification of x200) on the Leica DMI6000B inverted microscope (Leica Microsystems, Heidelberg, Germany), using the argon-neon laser set at a power setting of 40% and emitting at a wavelength of 458 nm, allowing confocal laser scanning microscopic analyses of collagen autofluorescence. The filter cube on the microscope was set for blue fluorescent light for excitation of the green fluorophores. The images were captured using the LAS AF software (Leica Microsystems, Heidelberg, Germany). Scanning speed was set at 400 Hz with a pixel format of 1024 x 1024. The default pinhole size of 1 Airy and line average of 4x were used.

2.5.10 Thin Sections

Six implant/tissue resin embedded block sections representative of each coated and control group were selected for thin sectioning. These were further sectioned using an Isomet slow-speed diamond saw (Beuhler, Illinois, USA) to 100µm sections and mounted with an adhesive

on a clear perspex slide. The sections were polished with progressively finer silicon carbide abrasive discs mounted on a Abramin micro-grinding system (Struers, Denmark) with the final polish using diamond paste to achieve a specimen thickness of 14-18 μ m measured by micrometer (Moore & Wright, Sheffield, England). The sections were re-imaged on confocal microscopy and then stained with haematoxylin and eosin (H&E).

2.5.11 Histomorphometric Measurements

The thin sections were used for measurement of the thickness of fibrous encapsulation taken at the apex of the implant threads (Figure 6) and the depth of connective tissue penetration into the implant grooves (Figure 7). The depth of penetration was measured perpendicularly from an imaginary line connecting the apices of two adjacent threads to the maximum depth of tissue penetration into the implant groove.



Figure 6. Measurement of thickness of fibrous encapsulation.

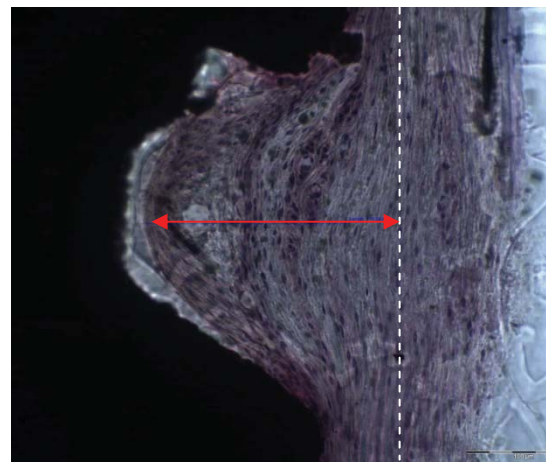


Figure 7. Measurement of depth of connective tissue penetration.

The measurements were carried out at a magnification of x200 in an Olympus BH-2 brightfield microscope (Olympus Optical Co. Ltd., Tokyo, Japan) equipped with an image system Altra 20 colour camera (Olympus Soft Imaging Solutions, Munster, Germany) and ANALYSIS imaging software package.

2.5.12 Statistical Analysis

Mean values for each variable were calculated for each implant unit. The differences within the 4 week and 8 week groups of control and coated implants were analysed using a one-way ANOVA and Bonferroni's Multiple Comparison Test was used as a post test. The null hypothesis was rejected at $P < 0.05$. The differences between the control implants at 4 weeks and 8 weeks and the coated implants at 4 weeks and 8 weeks were analysed using Student's *t*-tests. Once again the null hypothesis was rejected at $P < 0.05$. Statistical analysis was carried out using a Graph Pad Prism 5 for windows statistical software package (Graph Pad Software Inc, La Jolla, California, USA).

2.6 RESULTS

Healing following implant placement was uneventful for all animals involved in the study. The incision wounds appeared to have healed by 4 weeks. Although 18 implants were placed in the rats (including the pilot study), 16 were retrieved. Two implants were not recovered from two animals at the 8 week time point. One of these lost implants had been coated with Emdogain® and the other with rhPDGF-BB. This reduced the number for analysis to 5 for each coated groups at the 8 week time point.

2.6.1 Histological Assessment – Qualitative Analysis at Four weeks

Fibrous encapsulation of the control and growth factors coated implants was evident after 4 weeks. The fibroblast layer adherent to the implants and the surrounding connective tissues appeared well-organised with little indication of any residual inflammation. The images seen under confocal microscopy were well correlated with the images seen for the H&E stained thin sections viewed under light microscopy (Figures 8 - 17).

For the control uncoated implant viewed under confocal microscopy (Figure 8a), collagen autofluorescence indicated that the collagen fibres were aligned parallel with the long axis of the implant, with a high concentration of collagen. The same thin sections, when stained with

H&E, showed a dense distinct layer of fibroblasts over the implant threads and suspended over the implant grooves, surrounded by less dense connective tissue (Figure 8b). The fibroblasts also appeared to be aligned parallel to the long axis of the implant. A thin (1-3 cells thick) but distinct cellular layer, in intimate contact with the surface of the implant grooves, that autofluoresced for collagen was evident (Figure 8a).

The collagen fibre orientation and fibroblast alignment observed in the coated implants at 4 weeks was no different to that reported for the uncoated implants when viewed under confocal and light microscopy. However, from this qualitative histological analysis, there appeared to be a greater depth of connective tissue penetration into the implant grooves with the Emdogain® and PDGF coated implants and a thicker dense cellular/fibroblast layer with the Emdogain® coated implants (Figures 9a, b & c, Figures 10a, b & c).

The presence of an adipose-like cell layer almost devoid of other cell types surrounding the dense fibroblast layer was a distinctive feature of the rh-PDGF-BB coated implant at 4 weeks (Figures 10a, b & c).

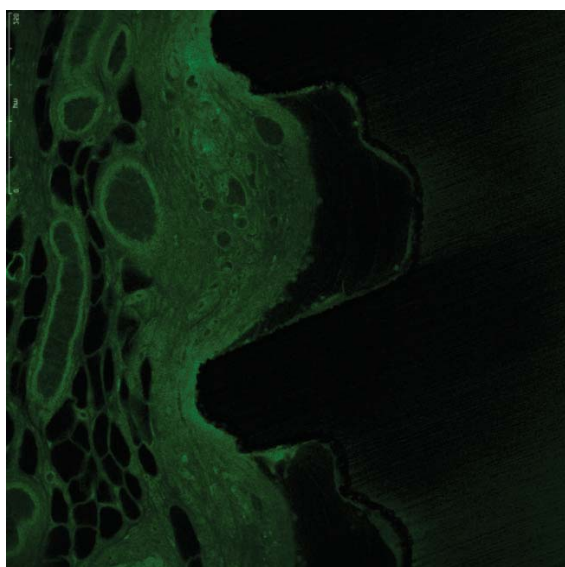


Figure 8a. Control (uncoated) implant at 4 weeks of healing. Thin section (16µm/unstained), confocal microscopy, thread 4 (LHS), original magnification x200.



Figure 8b. Control (uncoated) implant at 4 weeks of healing. Thin sections (16µm/H&E stained), light microscopy, threads 3-5 (LHS), original magnification x 200 (composite image).

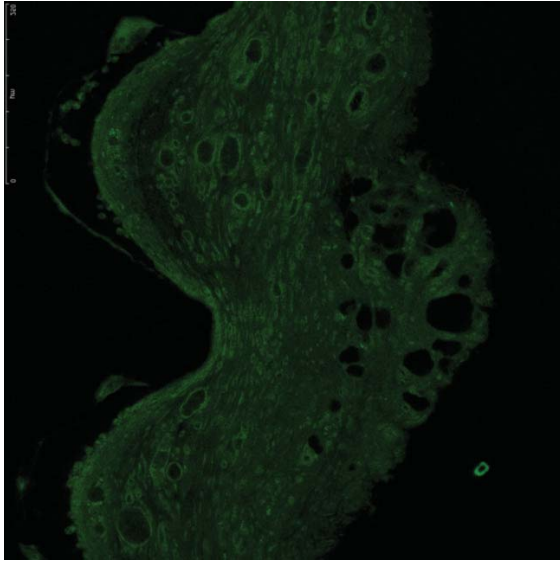


Figure 9a. Emdogain® coated implant at 4 weeks of healing. Thin section (16µm/unstained), confocal microscopy, thread 5 (LHS), original magnification x200.

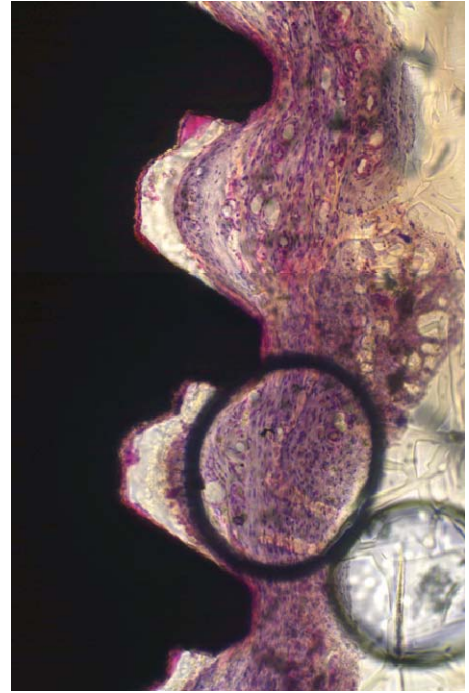


Figure 9b. Emdogain® coated implant at 4 weeks of healing. Thin sections (16µm/H&E stained), light microscopy, threads 4-6 (LHS), original magnification x 200 (composite image).

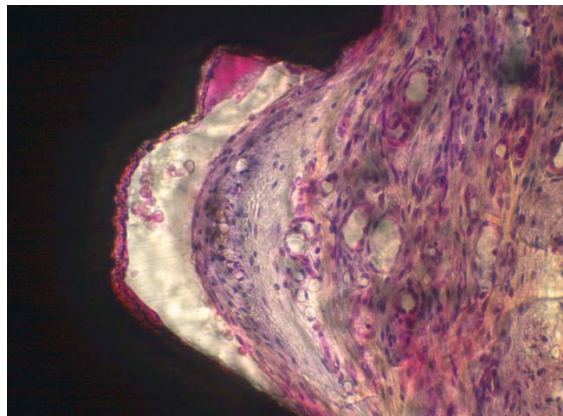


Figure 9c. Emdogain® coated implant at 4 weeks of healing. Thin section (16µm/H&E stained), light microscopy, groove 4 (LHS), original magnification x 200 (Close-up image).

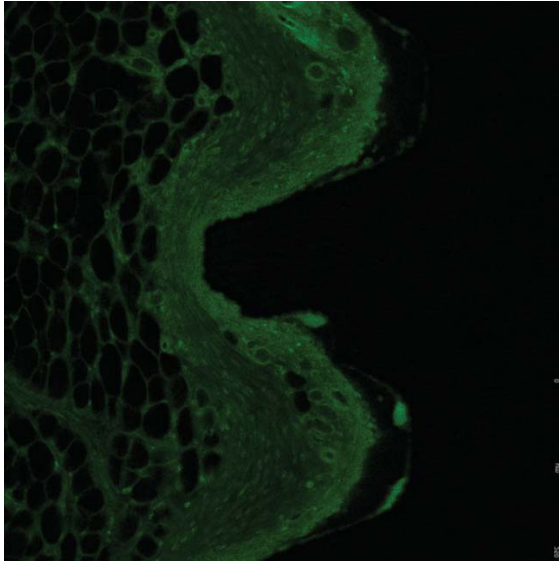


Figure 10a. rhPDGF-BB coated implant at 4 weeks of healing. Thin section (18 μ m/unstained), confocal microscopy, thread 7 (LHS), original magnification x200.

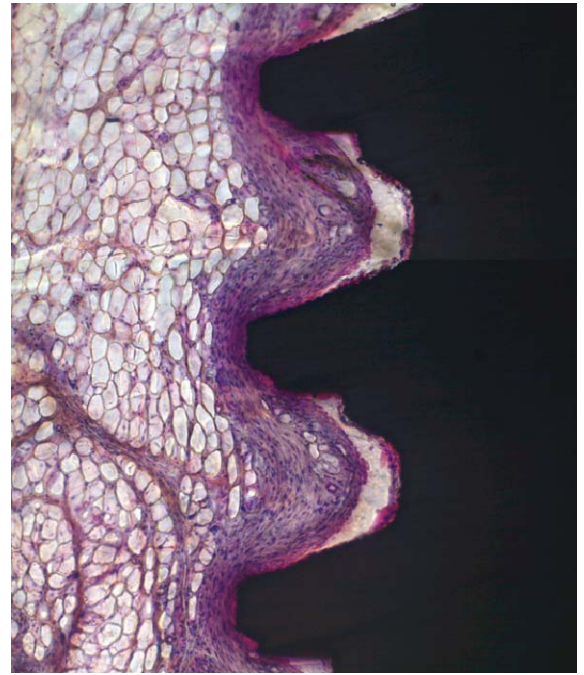


Figure 10b. rhPDGF-BB coated implant at 4 weeks of healing. Thin sections (18 μ m/H&E stained), light microscopy, threads 6-8 (LHS), original magnification x 200 (composite image).

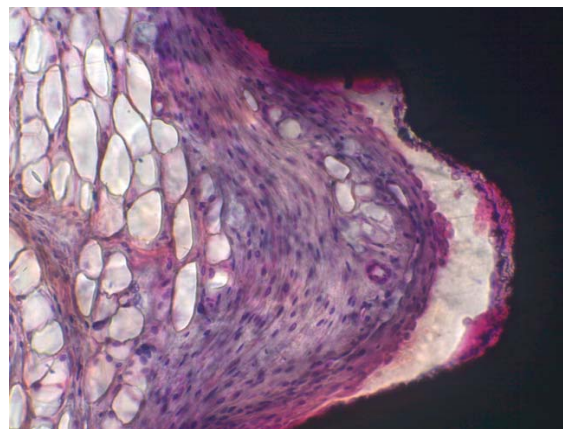


Figure 10c. rhPDGF-BB coated implant at 4 weeks of healing. Thin section (18 μ m/H&E stained), light microscopy, groove 7 (LHS), original magnification x 200 (Close-up image).

2.6.2 Histological Assessment – Qualitative Analysis at Eight weeks

When viewed at 8 weeks, neither the controls nor the growth factors coated implants showed any distinct change in collagen fibre orientation or fibroblast alignment when viewed under confocal and light microscopy. The collagen fibres and fibroblasts were still aligned parallel to the long axis of the implants (Figures 11 - 17).

For the control implant at 8 weeks, there appeared to be a greater depth of connective tissue penetration into the implant grooves than at 4 weeks but the thickness of the fibroblast

layer was inconsistent around the implant, with some areas having a thick dense fibroblast layer (Figures 11a & b) but in other areas, the dense fibroblast layer remained thin (Figures 12a, b & c).

The Emdogain® coated implant at 8 weeks also showed good depth of connective tissue penetration into the implant grooves, with a good, consistent thickness of the dense fibroblast layer over the threads and in the grooves of the implant (Figures 13 - 15). This fibroblast layer appeared more organised and closely-packed together than the Emdogain® coated implant at 4 weeks (Compare Figures 9 to 13 - 15).

The rhPDGF-BB coated implant at 8 weeks also showed good depth of connective tissue penetration into the implant grooves. However, with the rhPDGF-BB coated implant, the dense fibroblast layer was consistently thin all around the implant, especially at the implant threads, surrounded by a pronounced adipose-like tissue response (Figures 16 & 17). The thickness of the dense fibroblast layer in the rhPDGF-BB coated implants appeared to have decreased from 4 to 8 weeks (Compare Figures 10 to 16 & 17).

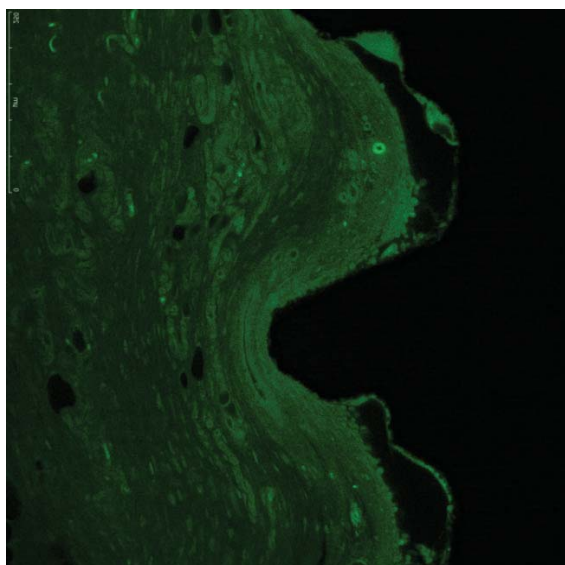


Figure 11a. Control (uncoated) implant at 8 weeks of healing. Thin section (14µm/unstained), confocal microscopy, thread 8 (LHS), original magnification x200.

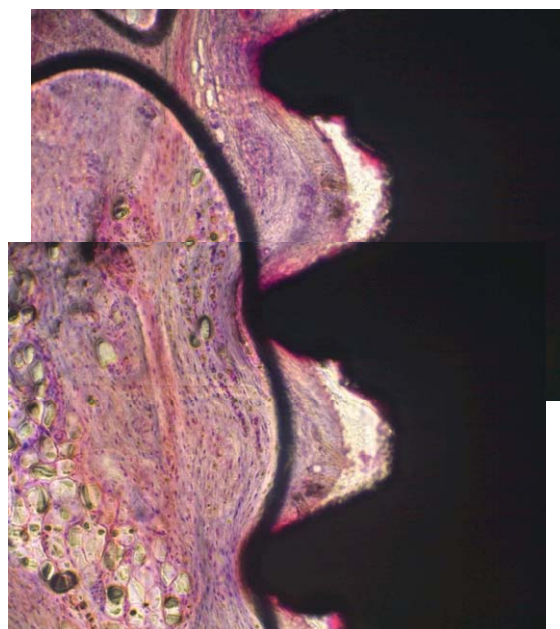


Figure 11b. Control (uncoated) implant at 8 weeks of healing. Thin sections (14µm/H&E stained), light microscopy, threads 7-9 (LHS), original magnification x 200 (composite image).

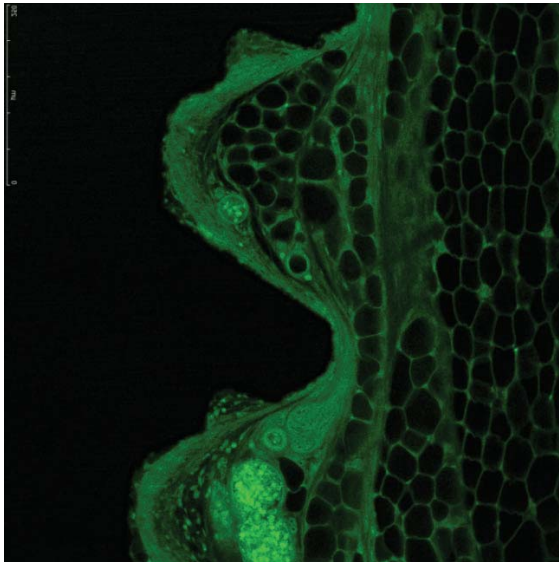


Figure 12a. Control (uncoated) implant at 8 weeks of healing. Thin section (14 μ m/unstained), confocal microscopy, thread 4 (RHS), original magnification x200.

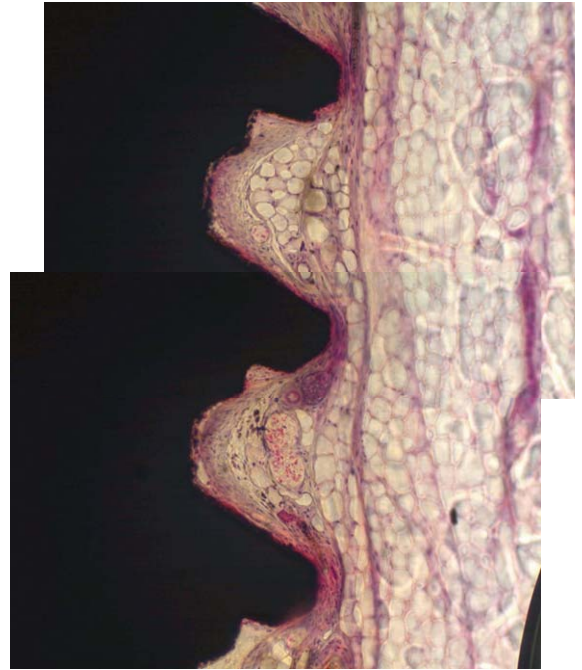


Figure 12b. Control (uncoated) implant at 8 weeks of healing. Thin sections (14 μ m/H&E stained), light microscopy, threads 3-5 (RHS), original magnification x 200 (composite image).

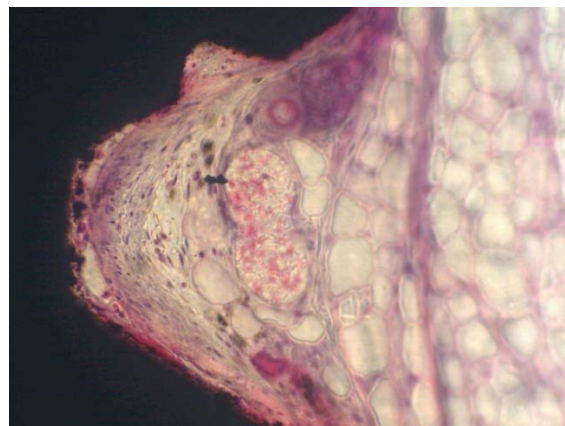


Figure 12c. Control (uncoated) implant at 8 weeks of healing. Thin section (14 μ m/H&E stained), light microscopy, groove 4 (RHS), original magnification x 200 (close-up image)

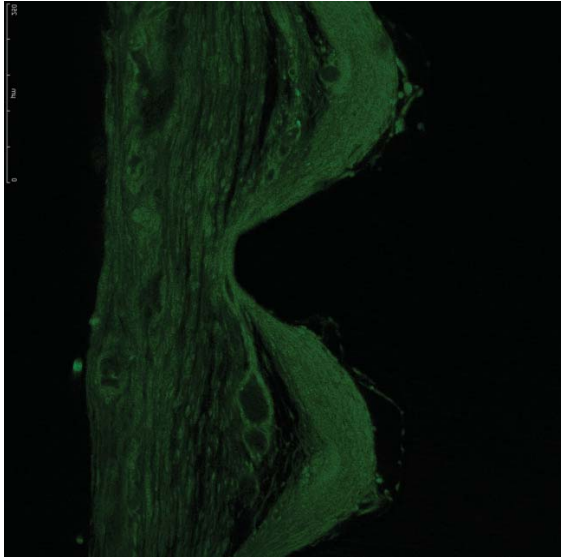


Figure 13a. Emdogain® coated implant at 8 weeks of healing. Thin section (14 μ m/unstained), confocal microscopy, thread 11 (LHS), original magnification x200.

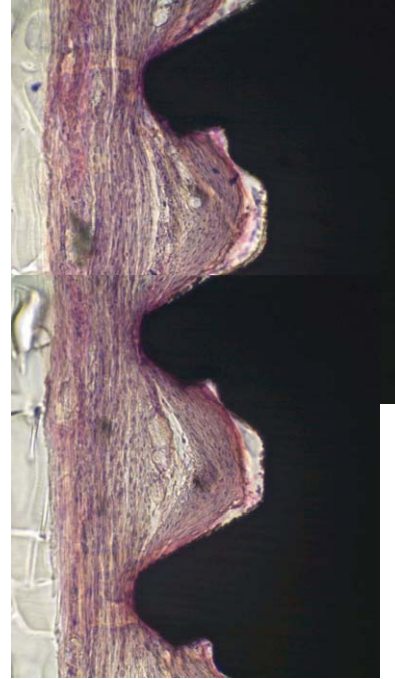


Figure 13b. Emdogain® coated implant at 8 weeks of healing. Thin sections (14 μ m/H&E stained), light microscopy, threads 10-12 (LHS), original magnification x 200 (composite image).

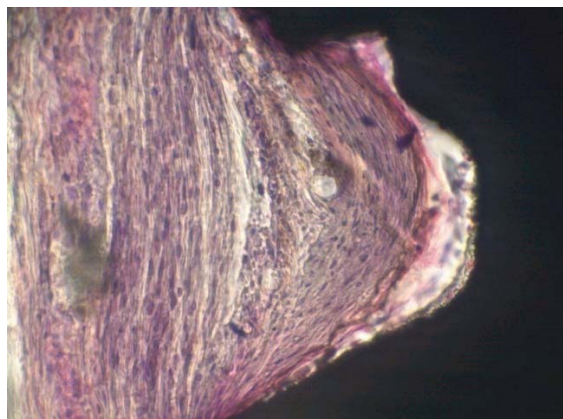


Figure 13c. Emdogain® coated implant at 8 weeks of healing. Thin sections (14 μ m/H&E stained), light microscopy, groove 10 (LHS), original magnification x 200 (close-up image).

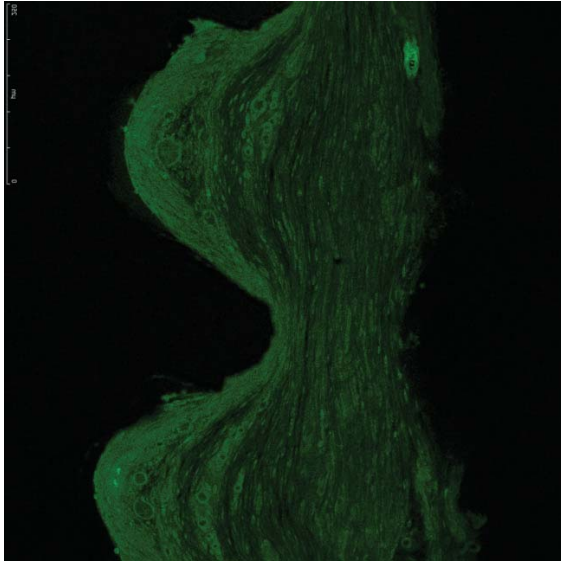


Figure 14a. Emdogain® coated implant at 8 weeks of healing. Thin section (14µm/unstained), confocal microscopy, thread 7 (RHS), original magnification x200.

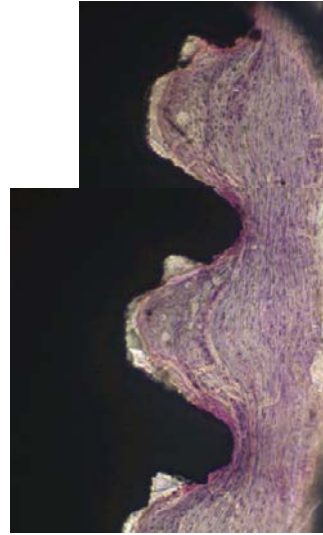


Figure 14b. Emdogain® coated implant at 8 weeks of healing. Thin sections (14µm/H&E stained), light microscopy, threads 6-8 (RHS), original magnification x 200 (composite image).

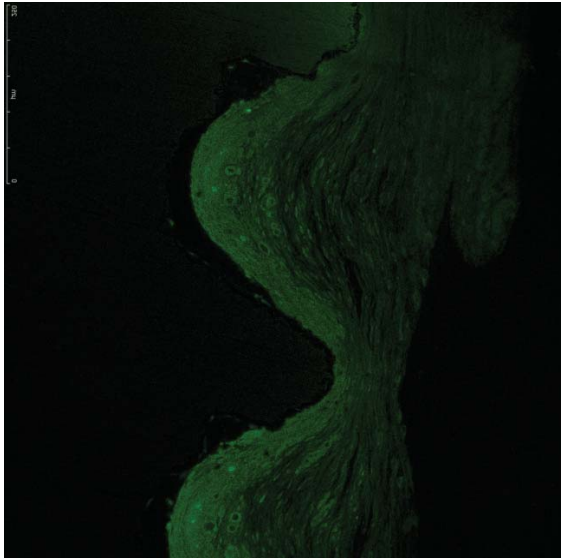


Figure 15a. Emdogain® coated implant at 8 weeks of healing. Thin section (14 μ m/unstained), confocal microscopy, thread 9 (RHS), original magnification x200.

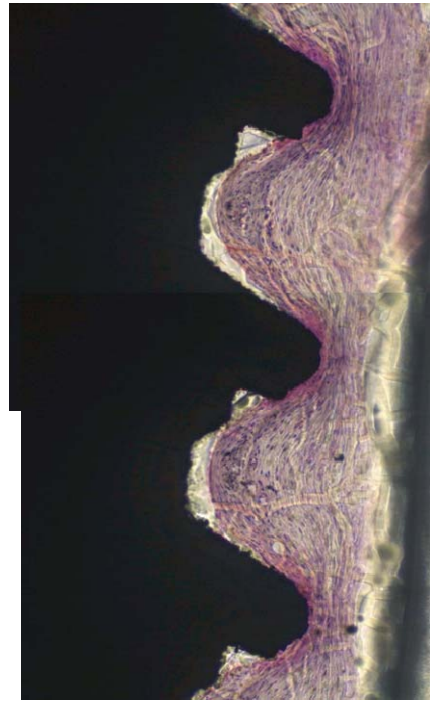


Figure 15b. Emdogain® coated implant at 8 weeks of healing. Thin sections (14 μ m/H&E stained), light microscopy, threads 8-10 (RHS), original magnification x 200 (composite image).

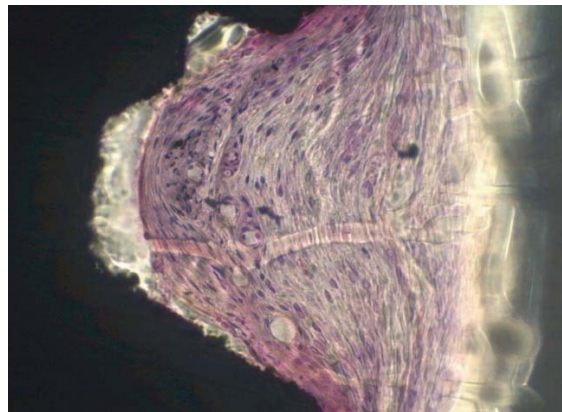


Figure 15c. Emdogain® coated implant at 8 weeks of healing. Thin section (14 μ m/H&E stained), light microscopy, groove 9 (RHS), original magnification x 200 (close-up image).

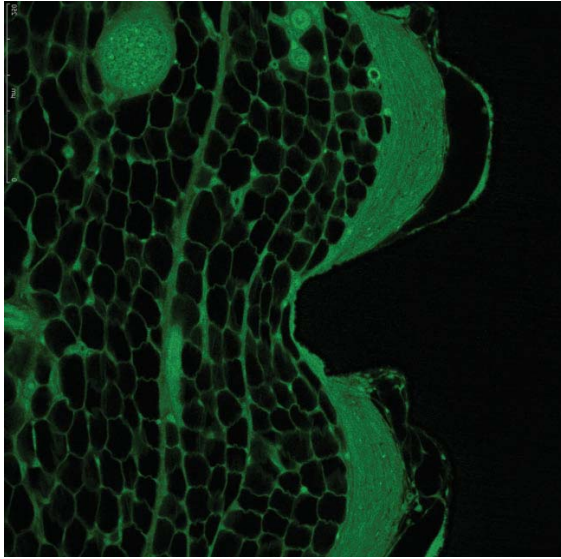


Figure 16a. rhPDGF-BB coated implant at 8 weeks of healing. Thin section (14 μ m/unstained), confocal microscopy, thread 4 (LHS), original magnification x200.



Figure 16b. rhPDGF-BB coated implant at 8 weeks of healing. Thin sections (14 μ m/H&E stained), light microscopy, threads 3-5 (LHS), original magnification x 200 (composite image).

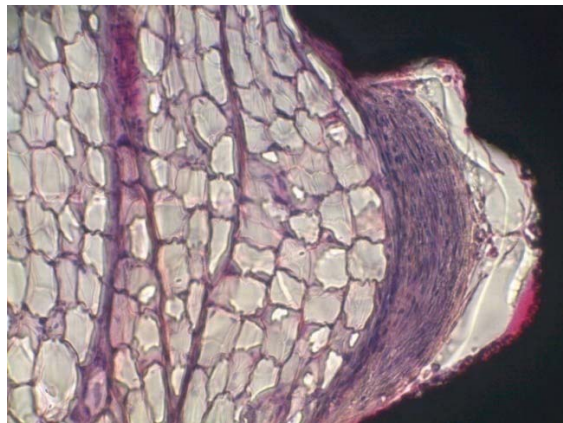


Figure 16c. rhPDGF-BB coated implant at 8 weeks of healing. Thin section (14 μ m/H&E stained), light microscopy, groove 4 (LHS), original magnification x 200 (close-up image).

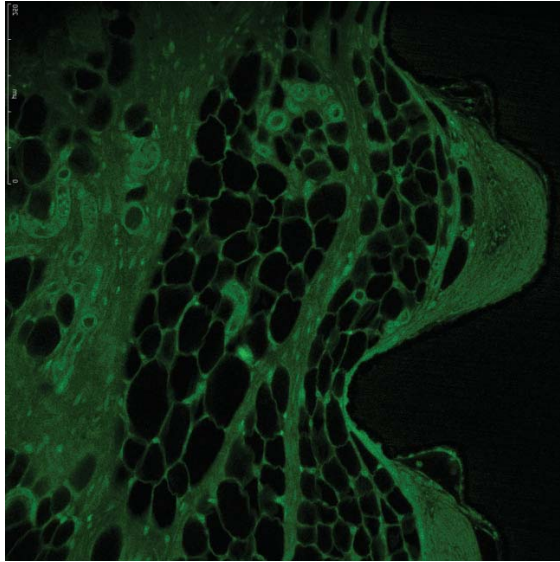


Figure 17a. rhPDGF-BB coated implant at 8 weeks of healing. Thin section (14 μ m/unstained), confocal microscopy, thread 2 (LHS), original magnification x200.

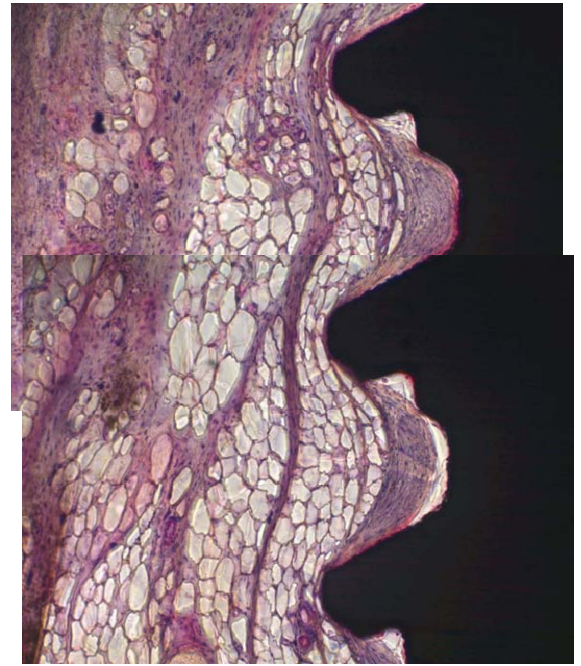


Figure 17b. rhPDGF-BB coated implant at 8 weeks of healing. Thin sections (14 μ m/H&E stained), light microscopy, threads 1-3 (LHS), original magnification x 200 (composite image).

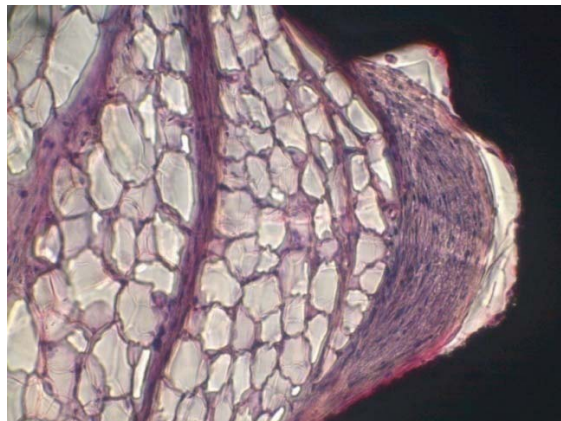


Figure 17c. rhPDGF-BB coated implant at 8 weeks of healing. Thin section (14 μ m/H&E stained), light microscopy, groove 2 (LHS), original magnification x 200 (close-up image).

2.6.3 Histomorphometric Measurements

The aims of the histomorphometric measurements were to:

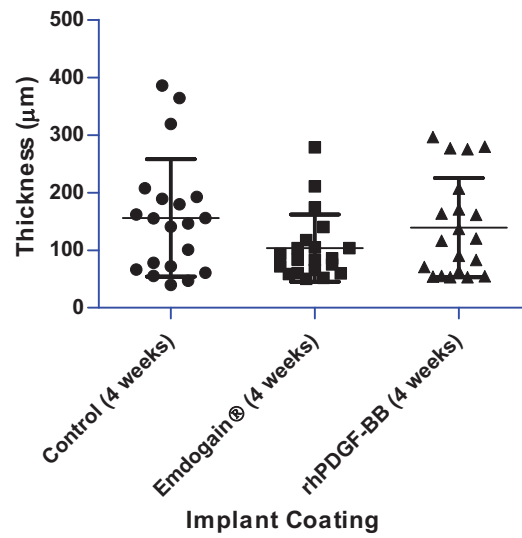
- Determine whether there were any significant differences between the uncoated, Emdogain® and rhPDGF-BB coated implants in terms of the thickness of the dense fibroblast layer at the two time points;

- Determine the depth of connective tissue penetration into the implant grooves and whether there were any significant differences between the uncoated, Emdogain® and rhPDGF-BB coated implants at the two time points and;
- Determine if the changes in thickness of the dense fibroblast layer and the depth of connective tissue penetration for each implant type as time was significant as time progressed.

The measurement of the thickness of the fibroblast layer was taken from the apex of the implant threads (Figure 6) of the first 10 threads from the implant collar on both the left and right hand sides. The measurement of the depth of connective tissue penetration into the implant grooves (Figure 7) were taken from the first 10 grooves from the implant collar on both the left and right hand sides.

The results of these measurements are presented graphically in Figures 18a, b and Figures 19a, b (raw measurements are presented in Appendices 2.3-2.6), with the mean thicknesses and depths of connective tissue penetration for the uncoated and coated implants at 4 and 8-weeks presented in Tables 1 & 2.

Thickness of fibroblast layer - 4wk measurements



Thickness of fibroblast layer - 8wk measurements

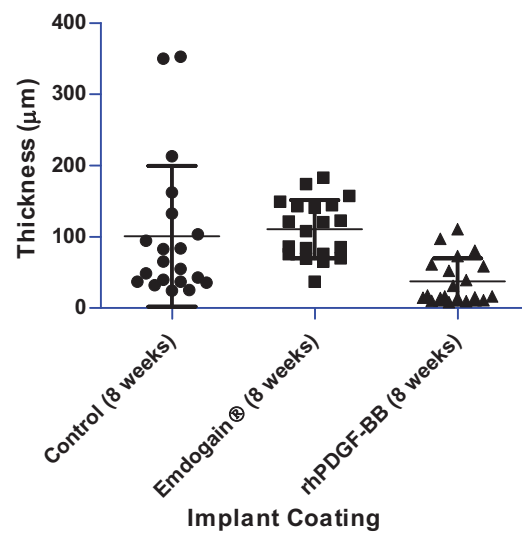
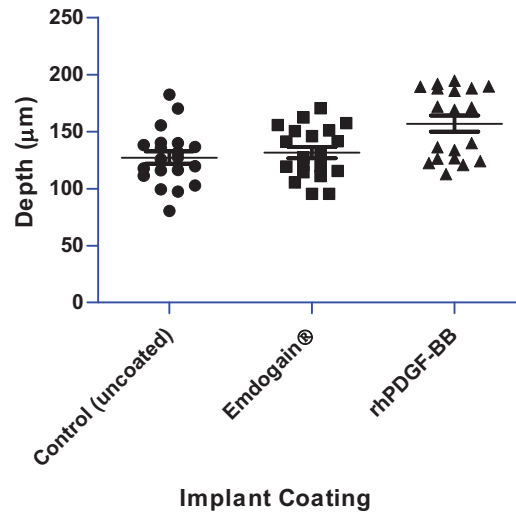


Figure 18a & b. Vertical scatter plots illustrating the thickness of the fibroblast layer at implant threads 1 -10 (LHS and RHS) of uncoated and coated implants at (a) 4 weeks and (b) 8 weeks.

Depth of connective tissue penetration - 4 week measurements



Depth of connective tissue penetration - 8 week measurements

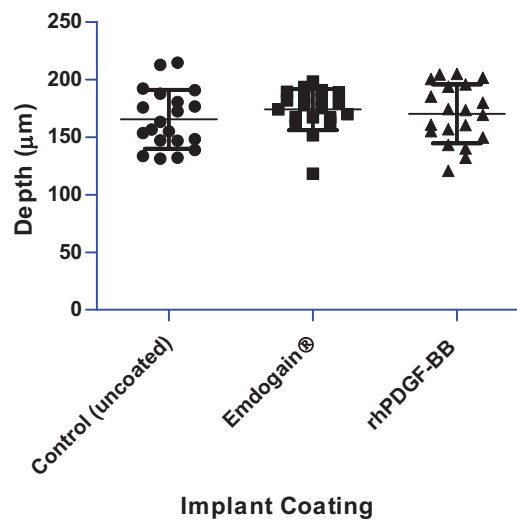


Figure 19a & b. Vertical scatter plots illustrating the depth of connective tissue penetration at implant grooves 1-10 (LHS and RHS) of the coated and uncoated implants at (a) 4 weeks and (b) 8 weeks.

	<u>Control</u> (uncoated)	<u>Emdogain®</u>	<u>rhPDGF-BB</u>
Number of values	20	20	20
<u>4 Weeks</u>			
Minimum	39.86	50.17	52.82
Maximum	386.0	278.4	296.3
Mean	155.9	103.7	139.2
Std. Deviation	102.2	58.21	86.33
One-way ANOVA <i>P</i> value 0.1431 (ns)			
Number of values	20	20	20
<u>8 Weeks</u>			
Minimum	24.25	36.88	8.310
Maximum	352.5	183.1	110.6
Mean	100.9	110.6	37.54
Std. Deviation	98.72	40.74	32.24
One-way ANOVA <i>P</i> value 0.0012 (s)			
<i>t</i> -test (4 wks. Versus 8 wks.) <i>P</i> value	0.0912 (ns)	0.06638 (ns)	<0.0001 (s)

Table 1. Thickness of fibroblast layer at the implant thread – 4 and 8 week measurements (µm).

	<u>Control</u> (uncoated)	<u>Emdogain®</u>	<u>rhPDGF-BB</u>
Number of values	20	20	19
<u>4 Weeks</u>			
Minimum	80.40	95.35	112.6
Maximum	182.4	170.4	194.7
Mean	127.2	131.5	156.9
Std. Deviation	24.52	22.46	30.53
One-way ANOVA <i>P</i> value 0.0014 (s)			
Number of values	20	20	20
<u>8 Weeks</u>			
Minimum	131.2	118.3	120.6
Maximum	214.6	198.3	205.0
Mean	165.4	174.0	170.1
Std. Deviation	25.44	17.89	25.51
One-way ANOVA <i>P</i> value 0.5092 (ns)			
<i>t</i> -test (4 wks. versus 8 wks.) <i>P</i> value	<0.0001 (s)	<0.0001 (s)	0.1515 (ns)

Table 2. Depth of connective tissue penetration into the implant grooves – 4 and 8 week measurements (µm).

The mean thicknesses of the fibroblast layer at implant threads 1 – 10 (LHS & RHS) for the uncoated, Emdogain® and rhPDGF-BB coated implants were $155.9 \pm 102.0\mu\text{m}$, $103.7 \pm 58.2\mu\text{m}$ and $139.2 \pm 86.3\mu\text{m}$ respectively at 4 weeks and $100.9 \pm 98.7\mu\text{m}$, $110.6 \pm 40.7\mu\text{m}$ and $37.5 \pm 32.2\mu\text{m}$ at 8-weeks.

One-way ANOVA found that there were no significant differences between the thicknesses of the fibroblast layer at the implant threads between the uncoated and coated implants at 4-weeks ($P = 0.1431$) (Table 1). However at 8 weeks, a significant difference in the thicknesses of the fibroblast layer ($P = 0.0012$) (Table 1) was detected. Using Bonferroni's Multiple Comparison Test as a post-test, the rhPDGF-BB coated implant was found to have a significantly thinner fibroblast layer at the implant threads than the uncoated ($t = 3.112$) and Emdogain® coated ($t = 3.589$) implants (Appendix 2.12). There was no significant difference in the thickness of the fibroblast layer between the Emdogain® coated and uncoated implants at 8 weeks, confirmed by the post-test ($t = 0.4763$) (Appendix 2.12).

Student's *t*-tests indicate that there was no significant change in the thicknesses of the fibroblast layer for the uncoated and Emdogain® coated implants between 4 and 8 weeks (Table 1). This was not the case for the rhPDGF-BB coated implants where the thickness of the fibroblast layer significantly decreased between 4 and 8 weeks ($P < 0.0001$) (Table 1).

The mean depth of connective tissue penetration into implant grooves 1 – 10 (LHS & RHS) for the uncoated, Emdogain® and rhPDGF-BB coated implants were $127.2 \pm 24.5\mu\text{m}$, $131.5 \pm 22.4\mu\text{m}$ and $156.9 \pm 30.5\mu\text{m}$ respectively at 4-weeks and $165.4 \pm 25.4\mu\text{m}$, $174.0 \pm 17.9\mu\text{m}$ and $170.1 \pm 25.51\mu\text{m}$ at 8-weeks.

One-way ANOVA detected a significant difference depth of connective tissue penetration into the implant grooves at 4 weeks ($P = 0.0014$) (Table 2). Once again, using Bonferroni's Multiple Comparison Test as a post-test, the depth of connective tissue penetration into the implant grooves was found to be significantly greater for the implant coated with rhPDGF-BB than for the uncoated ($t = 3.573$) and Emdogain® coated ($t = 3.048$) implants at 4 weeks (Appendix 2.13). However, at 8 weeks this difference was no longer significant ($P = 0.5092$)

(Table 2). Coating the implant with Emdogain® was not found to significantly alter the depth of connective tissue penetration into the implant grooves over an uncoated implant, confirmed by the post-test ($t = 0.5319$) (Appendix 2.14).

Student's t -tests indicate that the depth of connective tissue penetration into the implant grooves increased for the uncoated ($P < 0.0001$) and Emdogain® coated ($P < 0.001$) implants between 4- and 8-weeks but remained the same for the rhPDGF-BB coated implants (Table 2).

2.7 DISCUSSION

The adherence of the peri-implant tissues to the implant/abutment surface is crucial to its function as a barrier between the oral environment and the bone and implant surfaces. Enhancing this adherence by surface modification with biological agents can serve to improve implant survival, as well as potentially contribute to an improvement in implant success rates by preventing recession and improving aesthetic outcomes.

The aim of this study was to investigate the connective tissue attachment to the roughened surface of (TiUnite) titanium implants and roughened surfaces modified with rhPDGF-BB or EMD. Although there are distinct differences between gingival and subcutaneous connective tissues in terms of remodelling, turnover rates and architecture, both of these connective tissue types contain principally type I and III collagen as the most abundant biochemical component. As this study investigates connective tissue attachment by examining the collagen fibre orientation to the implant surface, the use of a subcutaneous murine model, whilst not mimicking the conditions in the oral cavity as accurately as would a buccal dehiscence model in a larger animal, does provide an appropriate cost-effective means to test the hypothesis of this study.

To our knowledge, this is the first study to utilise confocal laser scanning microscopy (CLSM) and collagen autofluorescence to image connective tissue attachment to titanium implants. The basic rationale behind CLSM is that illumination of tissues with a short

wavelength light from a monochromatic punctiform laser source leads to excitation of endogenous substances, resulting in the emission of fluorescence light of longer wavelengths. The resulting emission energy is detected by a spatially filtered optical system, the pinhole, which filters out light signals from out-of focus planes (Lucchese *et al* 2008). Amongst the molecules, called fluorophores, responsible for this tissue autofluorescence include collagen (DaCosta *et al* 2002, 2003). Recent studies investigating the collagen fibre orientation in the peri-implant mucosa have used a variety of methods including: decalcified ground sections stained with toluidine blue (Abrahamsson & Cardaropoli 2007, Berglundh *et al* 2007, Welander *et al* 2007, 2008; Nevins *et al* 2008), decalcified ground sections stained with methylene blue/Azure II (Schüpbach *et al* 2007), “fracture technique” sections stained in PAS and toluidine blue (Welander *et al* 2007), scanning electron microscopy (Schüpbach *et al* 2007, Welander *et al* 2007, Nevins *et al* 2008, Tete *et al* 2009), transmission electron microscopy (Schüpbach *et al* 2007) and circular polarised light microscopy (Allegrini Jr *et al* 2008, Tete *et al* 2009). Collagen fibre orientation in bone around osseointegrated implants have also been investigated using circular polarised light microscopy with tissue incorporated fluorescent dyes in human peri-implant bone (Traini *et al* 2005) and peri-implant bone from minipigs (Neugebauer *et al* 2006). These methods, whilst effective and allowing for high quality imaging of collagen, require complex and time-consuming sample preparation techniques.

Recently, Lucchese *et al* (2008) analysed collagen fibre distribution in human crown dentine using CLSM and found an intense autofluorescence that was ascribed to collagen fibres in all their samples. In our study, we were able to correlate the collagen autofluorescence seen in the CLSM images to the fibroblasts observed in the same thin sections when stained with H&E and viewed under light microscopy. The use of CLSM thus appears to provide a less time-consuming method of preparing tissue samples and therefore is a useful auxiliary tool for investigating the presence, distribution and collagen fibre orientation in the peri-implant soft tissues.

In this study, two of the coated implants, one coated with Emdogain® and the other coated with rhPDGF-BB, were not recovered from two animals at the 8 week time point. The reasons for this exfoliation are unknown. We suspect that this exfoliation would have occurred early on in the experiment as healing following implant placement was uneventful for all animals involved in this study. A distinct encapsulation by a layer of fibroblasts occurred around all the retrieved implants, regardless of whether the implants were coated or uncoated. This is similar to what occurs with an osseointegrated implant intraorally, whereby the connective tissue forms a non-vascularised, circular, scar-like structure around the transmucosal portion of the implant. The qualitative analysis and histomorphometric measurements of the uncoated implants indicate that resolution of inflammation and connective tissue formation appeared to be completed by 4 weeks. However the healing process, which included tissue maturation and organisation continued between the 4 and 8 week period, as evidenced by the significant change in depth of connective tissue penetration into the implant grooves. Our observations in a murine model appear to be consistent with the conclusions made recently by Berglundh *et al* (2007). In their investigation of the morphogenesis of the peri-implant mucosa in a canine model, they concluded that the peri-implant mucosa exhibited minor signs of inflammation during the first 2 weeks of healing but from 4 weeks, the mucosa was stable and well attached to the bone. Berglundh *et al* (2007) further concluded that the soft tissue barrier adjacent to titanium implants placed in a non-submerged protocol takes about 6 to 8 weeks to establish a soft tissue barrier with proper dimensions and tissue organisation.

A number of previous *in vitro* studies have investigated the effect of surface modification by the coating of titanium with biological agents on epithelial and connective tissue attachment to titanium surfaces. Dean *et al* (1995) observed that coating machined, plasma-sprayed and hydroxyapatite titanium surfaces *in vitro* with fibronectin and laminin-1, a component of epithelial cell membranes, enhanced gingival fibroblast and epithelial cell attachment respectively by about threefold. Tamura *et al* (1997) observed, also *in vitro*, that coating titanium alloy with laminin-5 enhanced gingival epithelial cell attachment and

hemidesmosome assembly. Park *et al* (1998) observed that type IV collagen provided an excellent substrate for epithelial cell attachment to titanium surfaces and later *in vitro* studies have shown that cell adhesion to titanium discs coated with collagen was enhanced compared with uncoated titanium (Roessler *et al* 2001, Nagai *et al* 2002). However, in a recent study investigating soft tissue healing around implants in a canine model, Welander *et al* (2007) found that the vertical dimensions of epithelial and connective tissue components, as well as the composition of the connective tissue zone directly adjacent to the implant were similar at collagen-coated and non-coated implants after 4 and 8 weeks of healing.

This study is the first study to investigate the effect of surface modification with the growth factor PDGF on connective tissue attachment to titanium implants. Recently, a pilot study conducted on a minipig reported on the effects of autogenous periodontal cell grafts (periodontal ligament and gingival connective tissue cultures), with and without the application of EMD, on the implant-connective tissue interface (Craig *et al* 2006). This pilot study was based on the hypothesis that a periodontal connective tissue attachment could be formed on dental implants provided a source of periodontal regeneration competent cells was present in the wound healing environment and that the application of EMD might aid in the formation of this attachment. However, in this pilot study, with and without the application of EMD, an implant-connective tissue interface morphologically consistent with a periodontal connective tissue attachment was not observed in sections from any of the implant or autogenous cell grafts (Craig *et al* 2006).

In this study, surface modification of the TiUnite surface of titanium implants with Emdogain® or rhPDGF coating was not found to change the orientation of the fibroblasts or collagen fibres in the encapsulating fibroblast layer. The orientation of the fibroblasts and collagen fibres when viewed under light microscopy and confocal laser scanning microscopy respectively appeared parallel to the long axis of the implant. Although reorientation of the fibroblasts and collagen fibres did not occur, there was good adaptation of the fibroblast layer onto the TiUnite surface and implant grooves for both the uncoated and growth factor coated

implants at the end of the study period. This could indicate a degree of soft tissue integration onto the TiUnite surface that is more adherent than previously thought.

The mechanical attachment at the titanium/connective tissue interface for roughened surface implants has not been extensively investigated. Abrahamsson *et al* (2002) compared the composition of soft tissue barriers onto implant abutments with a machined surface with abutments with a dual, thermal acid-etched surface using a canine model over a 6-month period. It was found that the roughness of the titanium surface did not influence the soft-tissue attachment that formed on commercially pure titanium in terms of the dimensions of the epithelial-connective tissue barrier and the composition of the connective tissue attachment. However, two recent *in vivo* studies provide evidence that microtexturing of the implant surface can influence the soft tissue response (Glauser *et al* 2005, Schüpbach & Glauser 2007). The influence of surface modifications on interactions between the implant surface on both the junctional epithelium and connective tissue was evaluated in a human study using one-piece experimental mini-implants (Nobel Biocare AB, Gothenburg, Sweden) with either a machined surface, acid-etched surface, or a surface with an oxidised and microporous TiO₂ layer, essentially a TiUnite surface. A shorter epithelial attachment and a longer connective tissue seal was observed with the acid-etched and oxidised implants compared to the machined surface implants (Glauser *et al* 2005). Furthermore, it was found that with the machined and acid-etched mini-implants, the adherence of the junctional epithelium to the implant surface was characterised by a basal lamina and numerous hemidesmosomes but the interface between the connective tissue and the implant surface was smooth, with collagen fibres running a course more or less parallel to the implant surface, indicating poor mechanical resistance. However, with the microtopographically complex oxidised TiUnite implant surface, the junctional epithelium exhibited attachment by hemidesmosomes together with mechanical interdigitation of the innermost cell layer with the open pores of the implant surface, with the connective tissue showing functionally oriented collagen fibrils towards the implant surface under polarised light microscopy, indicating a less vulnerable seal

(Schüpbach & Glauser 2007). Nevins *et al* (2008), employing a single-stage protocol using implants with 'Laser-Lok' microchannels at the collar (Biohorizons Implant Systems, Birmingham AL, USA), observed under light microscopy that the junctional epithelial cells were in close contact with the implant surface and that the microgrooved area of the implants were covered with connective tissue. Polarized light and scanning electron microscopy of the microgrooved area showed functionally oriented collagen fibres running toward and attaching to the grooves of the implant surface (Nevins *et al* 2008).

In our study, close adaptation of the fibroblast layer onto the TiUnite implant surface was seen at the implant threads histologically, and a good depth of connective tissue penetration into the implant grooves was observed for both uncoated and growth factor coated implants at the end of the study period. Coating the implant with rhPDGF-BB significantly increased the depth of connective tissue penetration into the implant grooves at 4 weeks over that in the Emdogain® coated and uncoated implants. Whilst the depth of connective tissue penetration for the rhPDGF-BB coated implants did not change after 4 weeks, it increased significantly for the Emdogain® coated and uncoated implants from 4 to 8 weeks. Thus, at 8 weeks all the uncoated and growth factor implants exhibited similar depths of connective tissue penetrance. Therefore, coating the TiUnite implant surface with rhPDGF-BB seems to increase the speed of soft tissue healing, but ultimately the same degree of soft tissue integration occurs around the TiUnite implant surface regardless of whether it has been coated or not.

Nevins *et al* (2005) in a large multi-centre, randomized blinded human clinical trial of 180 participants investigated the effectiveness of PDGF-BB with a porous β -tricalcium phosphate (TCP) matrix. The subjects had at least one interproximal periodontal defect ≥ 4 mm after debridement and were divided into three treatment groups: Group 1 – β -TCP plus 0.3 mg/ml rhPDGF-BB (GEM 21S); Group 2 – β -TCP plus 0.1 mg/ml rhPDGF-BB; and Group 3 – β -TCP and buffer alone. At 3 months post surgically, GEM 21S showed a significantly greater CAL gain than the β -TCP alone but at 6 months although the CAL gain for GEM 21S continued to be greater than the β -TCP alone, this was found not to be statistically significant.

One of the conclusions that made by Nevins *et al* (2005) was that growth factors such as rhPDGF-BB have short half-lives and so after a sharp initial increase in clinical attachment gain, no significant gains are observed in the long-term. The results from our study seem to support this statement.

On closer inspection, there appears to be a degree of tissue separation between the fibroblast layer and a thin cellular layer on the implant surface for a large number of the implant grooves. This thin cellular layer when present appears to be continuous with areas where the fibroblast layer is in close contact with the implant surface. Although tissues processed for embedding in acrylic or epoxy resins are considered less prone to artefacts (Bosshardt *et al* 2005), due to the concave morphology of the implant grooves, tissue shrinkage has probably occurred in a number of the implant grooves in our samples and that the degree of connective tissue penetration into the implant grooves for the coated and uncoated implant in this study is probably greater than that observed histologically.

Coating of the implants with rhPDGF-BB did not result in any significant difference in fibroblast thickness at the implant threads when compared to the Emdogain® coated and uncoated implants at 4 weeks, but the more intense appearance of the collagen autofluorescence under confocal microscopy could have indicated a higher concentration of collagen in the dense fibroblast layer adjacent to the implant surface with the rhPDGF-BB coated implants at this time point (Figure 10a). However, a significant decrease in the thickness of the fibroblast layer at the implant threads with the rhPDGF-BB coated implant was evident at the end of the 8 week study period. A noticeable amount of adipose-like tissue deposited around the rhPDGF-BB coated implants was also observed, both at the 4 week and 8 week implants. Preadipocytes convert to adipocytes through the process of adipogenesis, a process that is catalysed biochemically by glycerol-3-phosphate dehydrogenase (GPDH) activity. GPDH is a specific marker for adipogenesis and catalyses the formation of glycerol-3 phosphate, an important step in the synthesis of triacylglycerines (Wise & Green 1979). PDGF, a mitogen, is known to stimulate the proliferation of human preadipocytes (Haurer *et*

al 1995) but inhibits their differentiation to adipocytes by inhibiting the activity of GDPH (Hauner *et al* 1995, Koellensperger *et al* 2006). Thus, we propose two possible explanations to explain this phenomenon observed in our study. The first plausible explanation is that the increased amount of adipose tissue observed is a coincidental artefact from the implants being surgically implanted in a rat, or part of a rat that simply had more adipose tissue deposits. The second explanation is that the rhPDGF-BB initially stimulated the proliferation of preadipocytes in the immediate vicinity of the implant, but because rhPDGF-BB is a short-lived growth factor (Nevins *et al* 2005), its inhibitory effects towards preadipocyte differentiation were also short-lived and over the experimental period these increased numbers of preadipocytes differentiated to adipocytes.

The thickness of the fibroblast layer on the implant threads of the Emdogain® coated implants were not significantly different from the uncoated implants throughout the whole study period. Qualitative analysis of the confocal and light microscopy images however, indicate a more consistent thickness and density of the fibroblast layer with the Emdogain® coated implants compared to the uncoated implants. Although there is no statistical significance between the two, the much smaller standard deviation (s.d.) values at 4 and 8 weeks seen with the Emdogain® coated implants as compared to the uncoated implants for fibroblast layer thickness help reinforce the observations of the qualitative analysis. One of the characteristic difference between EMD and rhPDGF-BB is that whilst the half-life of rhPDGF-BB is short, EMD acts over a long-period of time and further clinical gains have been observed in the short (3 years) and longer term (5 years) post- operatively (Heijl *et al* 1997, Heden & Wennstromm 2006). The short duration of this study may have therefore precluded any significant differences being observed between the Emdogain® coated and uncoated implants.

In conclusion, this study shows that good soft tissue integration can be achieved on a moderately roughened TiUnite surface. Surface modification of the TiUnite surface by coating with rhPDGF-BB could increase the speed of soft tissue healing around the implant

surface. However, the increased speed of healing with rhPDGF-BB coating could result in a less robust titanium/connective tissue interface. The positive influence of implant surface modification with Emdogain® on soft tissue attachment and maturation around the implant surface should not be discounted and more research into this area is warranted.

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