The use of human placental connective tissue matrix for the enhancement of collagen synthesis and acceleration of wound healing in a diabetic rat model

Running title: Placental biomaterial in diabetic wound repair

Stelios Koutsoumbelis[†], MD, Zalak Parikh[*], MD, Ian R Sigal[*], BS, Daniel A Grande[†*], PhD

[†]Department of Orthopedic Surgery, North Shore LIJ Health System

[*]Orthopedic Research Lab, Feinstein Institute for Medical Research

Corresponding Author:

Daniel A Grande

Feinstein Institute for Medical Research

350 Community Drive

Manhasset, NY 11030

dgrande@nshs.edu

(516) 562-1138

(516) 562-1022

Key words: wound repair, tissue engineering, novel biomaterials, placental biomaterials

Abstract

Diabetic patients have impaired wound repair due to decreased fibroblast infiltration, growth factor production, and angiogenesis. Decellularized human placental biomaterials have high levels of extra-cellular matrix components, are rich in growth factors that promote angiogenesis, and have anti-inflammatory properties, so it was hypothesized that placental biomaterial could enhance healing in diabetic mice. Fibroblasts were cultured in a 10% placental biomaterial solution, and controls were unaugmented. Real-time PCR and thymidine and proline uptake were used to determine collagen type I synthesis and fibroblast proliferation respectively. Two dorsal lesions were subsequently induced in eighteen diabetic rats. One lesion was untreated; the other was covered with placental biomaterial or porcine small intestinal submucosa and secured with sutures. Rats were sacrificed 1, 2, or 3 weeks postoperatively and analyzed on macroscopic appearance, fibrosis, epithelialization, and inflammation. In vitro placental augmentation resulted a six-fold increase in collagen synthesis and a significant increase in fibroblast proliferation. In vivo placental biomaterial treatments significantly increased epithelialization without increasing inflammation. Placental biomaterial-treated wounds had re-epithelialized within two weeks, whereas submucosa treatments required three. These results indicate that placental biomaterial accelerates wound recovery due to enhanced fibroblast proliferation and collagen type I production. Diabetes is a challenging wound-repair model; as such placental biomaterial may have implications for treating injuries in both diabetic and healthy patients.

INTRODUCTION

Diabetes is a chronic disease characterized by high blood glucose level. Due to the rising obesity epidemic, diabetes has become a substantial problem in the United States, affecting 29.1 million people—9.3% of the population (1)—at a cost of $245 billion each year (2). Complications stemming from diabetes are now the seventh leading cause of death in the US, and as 86 million Americans are now at risk of developing the disease, these human and financial burdens will continue to increase (1,2).

Among the direst effects of diabetes is a substantial impairment in wound healing. Natural wound healing occurs in three phases. During the inflammatory phase, a clot forms at the injury site, and the blood vessels subsequently dilate to allow access to nutrients, white blood cells, and growth factors (3,4). This is followed by the proliferative phase, in which fibroblasts recruited to the injury site initiate angiogenesis and deposit collagen type III and other extracellular matrix components (5). Finally, during the maturation phase, collagen type III is broken down and replaced with collagen type I fibers which are arrayed along lines of tension to provide additional mechanical strength (3). These processes are substantially disrupted in diabetic patients, often leading to chronic, non-healing wounds. Vascular insufficiency diminishes the delivery of oxygen, nutrients, and white blood cells to the injury site (6). Decreased oxygen impairs the function of cells necessary from proper wound repair, and the lack of white blood cells may increase the risk of infection. Moreover, diabetes is associated with a substantial decrease in fibroblast infiltration, inhibiting the proliferative and maturation phases of healing (7). Finally, diabetes lowers the production of basic fibroblast growth factor and vascular endothelial growth factor (8), which are vital to the process of angiogenesis (9, 10, 11). For these reasons, treatment of diabetic wounds costs between nine and thirteen billion dollars

per year, and these injuries substantially lower the quality of life of those suffering from the disease (12, 13).

Placental biomaterial (PBM) is an attractive substrate for use as a scaffold for a number of reasons. It contains numerous natural growth factors, many of which have been shown to enhance tissue repair (10). It contains a large amount of extracellular matrix, which includes collagen, elastin, laminin, and proteoglycans. Furthermore, it also has natural antibacterial and anti-inflammatory properties and may prevent infections from occurring. For this reason, it was predicted that the administration of placental biomaterial on external wounds of diabetic rats would accelerate and improve the quality of wound repair when compared with small intestine submucosa (SIS)—a common wound treatment—and untreated controls. The material chosen for this study was Aedicell (Aedicell, Jersey City, NY), a decellularized, lyophilized placental biomaterial which may act as both a scaffold and metabolism enhancer for fibroblasts. The purpose of this experiment was to determine whether PBM could be a cost-effective wound dressing for use in diabetic patients, promoting rapid wound healing and decrease complications related to the injury.

MATERIAL AND METHODS

In vitro

In vitro studies were conducted prior to animal testing to determine the metabolic effects of the PBM material on fibroblasts.

Quantitative RT-PCR: In this study, rat fibroblasts were cultured in DMEM + 10% FBS supplemented with antibiotics and antimycotics. In the experimental group, this was further supplemented with an intact PBM patch such that the patch made up 10% of the volume of the

resulting solution. The control group was not supplemented with the patch. Fibroblasts were harvested after 24, 48, 72, and 144 hours and extracted for RNA using the High Pure RNA Isolation Kit (Roche Applied Science, Nutley, NJ).

All samples were analyzed in triplicate using quantitative real-time, reverse transcriptase polymerase chain reaction (qRTPCR) using Eurogentec chemistry and the Universal Probe Library (Roche Applied Science). Collagen type I expression was measured using the primer pair listed below to assess fibroblast gene expression. All qRTPCR runs were normalized with GAPDH, HPRT1, and RPL13A reference genes, designed through Roche RealTime Ready Designs for *rattus norvegicus*. Calculations were performed using the $2^{-\Delta\Delta CT}$ method, as described by Arocho A, *et al* (14).

Collagen type I primers: 5'-GCTGAATCCTTCCGTGTT-3'

5'-AGGGAGGGGACTTATCTG-3'

Metabolic Studies: 160,000 fibroblasts were seeded into each well of six-well culture plates and suspended in DMEM + 10% FBS supplemented with antibiotic and antimycotic. Cells were allowed 24 hours to attach to the plate after which PBM was added to three of six wells in each culture plate such that the biomaterial comprised 10% of the volume of each well. On days 1,2,3 and 7, cell proliferation and collagen synthesis were determined via uptake of tritiated thymidine (H3-Thy) and tritiated proline (H3-Pro), respectively. To measure cell proliferation, a pulse media composed of 50µl H3-Thy/100 ml media was added to the culture well 24 hours prior to the experimental time point. Cells were washed with phosphate buffered saline to remove unincorporated isotope. These cells were subsequently lysed, and scintillation counting was used

to measure incorporated isotope. Collagen synthesis measurement by H3-Pro uptake was performed using pulse media of 250µl H3-Pro/ 10ml media added to the culture well 4 hours prior to the experimental time point. All scintillation counts were normalized by DNA content using Hoechst 33258. Data is expressed as cpm/ug DNA. Further preparation and scintillation counting was similar to H3-Thy.

Statistical analysis: Data was analyzed utilizing the unpaired Student's t-test. Statistical significance was defined as $p<0.05$.

In vivo

Animals and experimental design: For this experiment wound repair was examined in Zucker Diabetic Fatty rats, a type II diabetes animal model (Taconic Biosciences, Cranbury, NJ). All rats were 8-10 week-old males between 300 and 400 grams in mass. General anesthesia was induced via inhalation of 3% isoflourane with 1L O_2 for induction and was maintained using 1-2% isoflourane with 1L O_2**.** Each rat received 1cm full-thickness paramedial dorsal defects induced with a 1.5cm biopsy punch. Either a PBM or Oasis (Cook Biotech, West Lafayette, IN)—porcine-derived SIS matrix—patch was inserted into one defect, and the site was secured using 5-0 Vicryl sutures. The contralateral defect served as the control, and consequently was left untreated. Both wound sites were dressed with a transparent adhesive film protect the defect**.** The 18 rats were divided into three groups of six; these groups were sacrificed after 1, 2, and 3 weeks. All methods were approved by IACUC at the Feinstein Institute.

Macroscopic examination: Photographs of the defects were taken at 1, 2, and 3 weeks. The relative size, depth, and inflammation of the defects at each time point were used to compare the

healing rates of the control, PBM-treated, and Oasis-treated defects. The incidence of exudates, infections, or other complications were taken into consideration as well.

Histology: The animals were euthanized 1, 2, or 3 weeks postoperatively via CO_2 asphyxiation, and the defects were surgically excised. These samples and were fixed in formalin, dehydrated, embedded in paraffin, sectioned at 5 microns, and mounted onto slides, which were subsequently stained with hematoxylin and eosin. A pathologist blinded to the study graded the specimens on three criteria: inflammation, epithelialisation, and collagen formation using the Abramov 4-point scale: 0=none, 1=scant, 2=moderate, and 3=abundant.

Statistical analysis: Changes in histologic scores over time were analyzed using an ANOVA two-way test. Comparisons between treatment groups were conducted using the unpaired Student's t-test. Statistical significance was defined as $p<0.05$.

RESULTS

In vitro

qRTPCR: Tenocytes cultured with placental material demonstrated a progressive, significant increase in collagen type I gene expression at 1, 3, and 6 days. The increase in gene expression was 6 fold above the control for collagen type I after day 6.

Metabolic studies: Cell proliferation as measured by incorporation of tritiated proline ($p=0.016$) and thymidine ($p=0.016$) had increased significantly compared to the control by one week in culture (Figures 1 & 2). Collagen synthesis was also significantly enhanced by co-culture with placental biomaterial.

In vivo

Macroscopic: Gross macroscopic observation showed that PBM- and SIS-treated defects were similar in size at week one and were substantially smaller than the controls. Both PBM and Oasis reduced inflammation and exudates relative to the control, although both parameters were further reduced in the PBM treatment group. Granulation tissue was also evident in the wound base of PBM-treated lesions (Figure 3).

By week two, the PBM-treated wounds had been almost completely covered with epithelium. Oasis-treated lesions were substantially smaller than the untreated controls, but were still exposed (Figure 4).

By week three, the PBM-treated defects had been completely covered in epithelium and appeared to have healed. Oasis lesions had developed an epithelial layer as well (Figure 5).

Histology: PBM-treated wounds had more extensive epithelialization at all time points than the Oasis and control treatments. These differences were significant at week two ($p=0.017$) and week three ($p=0.025$) between the PBM and control wounds (Figure 6). Epithelialization increased significantly throughout the trial ($p<0.001$). PBM treatments accelerated cartilage synthesis as well; at weeks one and two, PBM-treated wounds demonstrated significantly more fibrosis than controls ($p<0.02$), although there was no significant difference in fibrosis at week 3 (Figure 7). Moreover, PBM did not increase inflammation; there were no significant differences in inflammation between PBM and controls or between PBM and SIS treatments at any timepoint (Figure 8).

DISCUSSION

The purpose of this experiment was to determine if PBM could accelerate wound healing when used as a wound covering. Placental amnion/chorion tissue contains abundant ECM

proteins, such as fibronectin and laminin, and high concentrations of growth factors, including PDGF-AA, PDGF-BB, TGFa, TGF-B1, bFGF, and EGF, which may augment wound healing (11, 15-16). It has been demonstrated to improve migration and proliferation of fibroblasts in vitro (14) and to improve angiogenesis in vivo (11). For this reason, it was hypothesized that PBM would accelerate wound healing in a diabetes rat model.

This treatment may substantially improve the prognosis of diabetic patients suffering from foot ulcers. Even using costly treatments, such as autografts and xenografts, these wounds heal slowly, causing prolonged pain, discomfort, and increasing the risk of complications. In a clinical trial involving 120 patients, only 55% percent of ulcers treated with acellular porcine intestinal submucosa had healed within twelve weeks (17). In similar studies involving fibroblast- and keratinocyte- embedded bovine collagen, 56-59% of ulcers had healed within twelve weeks (17). Autograft materials were relatively ineffective as well; only 42% of diabetic lesions treated with autologous keratinocytes embedded on a silicon membrane had healed after 18 weeks. Using an analogous treatment, only 78% of lesions had healed after nine months (17). In contrast, 85% of diabetic patients treated with EpiFix, an amnion/chorion allograft, had recovered after only four weeks, and 95% had recovered after six weeks (18). These results indicate that PBM can substantially improve outcomes for diabetic patients.

The results of our in vitro studies demonstrated that PBM supports the proliferation and collagen type I production of fibroblasts. Collagen is a vital component of scar tissue; as such, a large increase in collagen expression may induce rapid wound healing. This was confirmed by in vivo testing. Histological examination revealed that PBM accelerated the synthesis of collagen type I in the injury site. Moreover, the material substantially increased the rate of epithelialization; PBM-treated injuries were re-epithelialized within two weeks, whereas SIS and

control treatments took three weeks to heal. This may reduce complications associated with these injuries; open wounds are more susceptible to infiltration by pathogens, so the rapid wound covering promoted by PBM may prevent infections from occurring. This is especially significant for diabetics, as diabetic neuropathy reduces sensation in the extremities, and as such many serious infections may go unnoticed.

One potential risk associated with PBM is inflammation or rejection. PBM is a human-derived biomaterial, and therefore may provoke an immune response in rats. However, the PBM patches did not significantly increase inflammation relative to the Oasis and control treatments. Placental-derived ECM has known anti-inflammatory and antimicrobial properties; this may have offset any inflammation caused by the implantation of a xenogenic material. This has implications for the treatment of humans. Aedicell is human-derived; as a result, human patients should see less inflammation and fewer complications than rats resulting from this treatment.

The promising results of this study show that PBM and other placental-derived therapies are effective wound treatments and further research should be pursued. Further studies on PBM should include large sample sizes, which may be better able to achieve statistical significance. Further studies may also quantify angiogenesis, extracellular matrix production, and fibroblast infiltration in vivo, all of which have implications in wound healing. Finally, the effectiveness of placental treatments should be tested in healthy as well as diabetic animals. Diabetic animals have reduced wound healing; as such, they provide a challenging model for wound healing. The effectiveness of placental patches may therefore be enhanced when implanted in healthy organisms.

Conflicts of Interest

Daniel A Grande holds stock options in Aedicell.

References

1. Center for Disease Control. National Diabetes Statistics Report, 2014. http://www.cdc.gov/diabetes/pubs/statsreport14/national-diabetes-report-web.pdf
2. Center for Disease Control. Diabetes Fact Sheet. http://www.cdc.gov/features/diabetesfactsheet/
3. Schilling, JA. Wound healing. Surg Clin North Am. 1976 Aug; 56(4): 859-74.
4. **The Molecular and Cellular Biology of Wound Repair By Richard Clark**
5. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev. 2003 Jul; 83(3), 835–70.
6. Gottrup F. Oxygen in wound healing and infection. World J Surg. 2004 Mar; 28(3): 312-5.
7. Lerman OZ, Galiano RD, Armour M, Levine JP, Gurtner GC. Cellular dysfunction in the diabetic fibroblast: impairment in migration, vascular endothelial growth factor production, and response to hypoxia. Am J Pathol. 2003 Jan; 162(1): 303-12.
8. Knighton DR, Fiegel V. Growth factors and repair of diabetic wounds. In: Levin ME, O'neal LW, Bowker JH, editors. The Diabetic Foot, 5th edn. St. Louis: Mosby Year Book, 1993: 247–57.
9. Esser JS, Rahner S, Deckler M, Bode C, Patterson C, Moser M. Fibroblast growth factor signaling pathway in endothelial cells is activated by BMPER to promote angiogenesis. Arterioscler Thromb Vasc Biol. 2014 Dec 11. *In press.*

10. Hoeben A, Laduyt B, Highley MS, Wildiers H, Ban Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. Pharmacol Rev. 2004 Dec; 56(4): 549-80.
11. Koob TH, Lim, JJ, Massee, M, Zabek, N, Rennert, R, Gurtner G, Li, WW. Angiogenic properties of dehydrated human amnion/chorion allografts: therapeutic potential for soft tissue repair and regeneration. Vasc Cell. 2014 May 1; 6:10.
12. Rice JB, Desai U, Cummings AK, Birnbaum HG, Skornicki M, Parsons NB. Burden of diabetic foot ulcers for medicare and private insurers. Diabetes Care. 2014 Mar; 37(3): 651-8.
13. Phillips T, Tanton B, Provan A, Lew R. A study on the impact of leg ulcers on quality of life: financial, social, and psychologic implications. J Am Acad Dermatol. 1994 Jul; 31(1): 49-53.
14. Arocho A, Chen B, Ladanyi M, Pan Q. Validation of the 2-DeltaDeltaCT calculation as an alternate method of data analysis for quantitative PCR of BCR-ABL P210 transcripts. Diagn Mol Pathol. 2006 Mar; 15(1): 56-61.
15. Koob TJ, Rennert R Zabek N, Massee M, Lim JJ, Temenoff JS, Li WW, Gurtner G. Biological properties of dehydrated human amnion/chorion composite graft: implications for chronic wound healing. Int Wound J. 2013 Oct; 10(5): 493-500.
16. Koob TJ, Lim JJ, Zabek N, Massee M. Cytokines in single layer amnion allografts compared to multilayer chorion allografts for wound healing. J Biomed Mater Res B Appl Biomater. 2014 Aug 30. *In press.*
17. Cook EA, Cook JJ, Badri H, Mostafa J. Bioengineered alternative tissues. Clin Podiatr Med Surg. 2014 Jan; 31(1): 89-101.

18. Zelen CM, Gould L, Serena TE, Carter MJ, Keller J, Li, WW. A prospective, randomised, controlled, mulit-center comparative effectiveness study of healing using dehydrated human amnion/chorion membrane allograft, bioengineered skin substitute or standard of care for treatment of chronic lower extremity diabetic ulcers. Int Wound J. 2014 Nov 26. *In press*

Nonstandard Abbreviations

Placental biomaterial: PBM

Figure Legends

Figure 1: Cell proliferation as measured by tritiated proline uptake normalized by DNA content. Proline uptake was substantially increased by the placental biomaterial treatment relative to the controls ($p=0.017$).

Figure 2: Cell proliferation as measured by tritiated thymidine uptake normalized by DNA content. Thymidine uptake was substantially increased by the placental biomaterial treatment relative to the controls ($p=0.016$).

Figure 3: The above figure shows rats receiving PBM and SIS treatments after one week. The PBM- and Oasis-treated defects are similar in size, but are substantially smaller than untreated controls. More extensive epithelialization is evident in the PBM-covered lesions. The arrows indicate the PBM- and SIS-treated lesions.

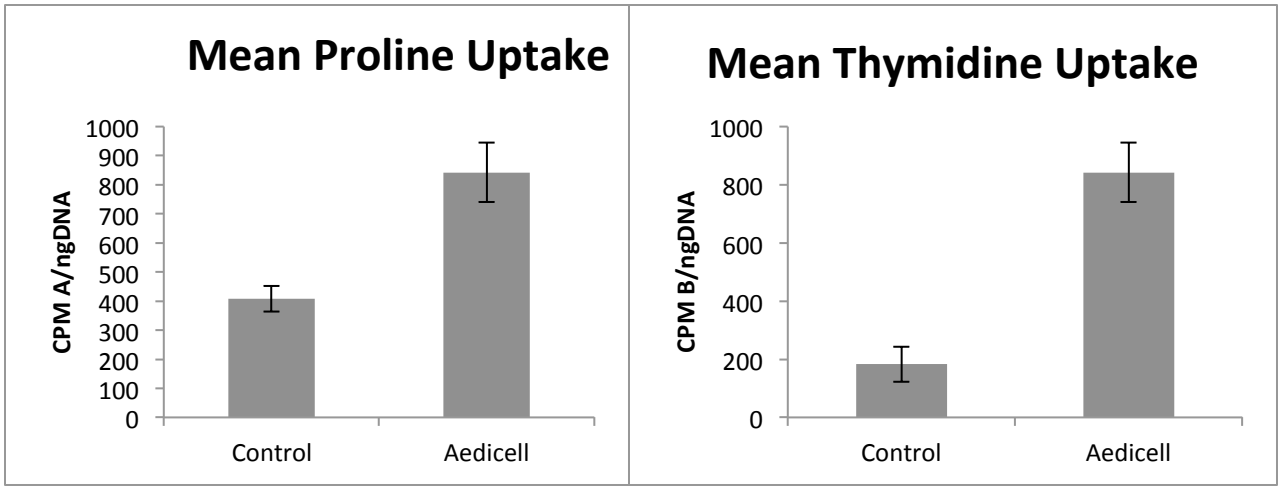
Figure 4: The above figure shows the extent of healing after two weeks. Both the PBM- and SIS-defects have decreased in size, and the PBM defect has re-epithelialized. The arrows indicate the PBM- and SIS-treated lesions.

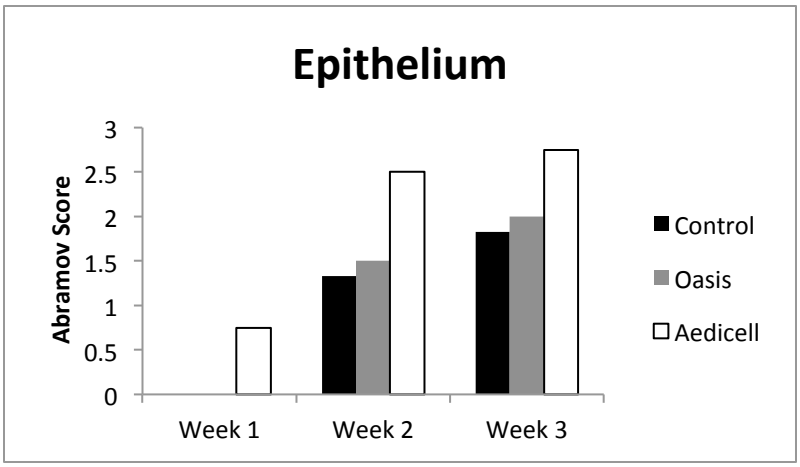
Figure 5: By week three, the PBM-treated lesions have completely healed, and the SIS lesions have been re-epithelialized. The arrows indicate the PBM- and SIS-treated lesions.

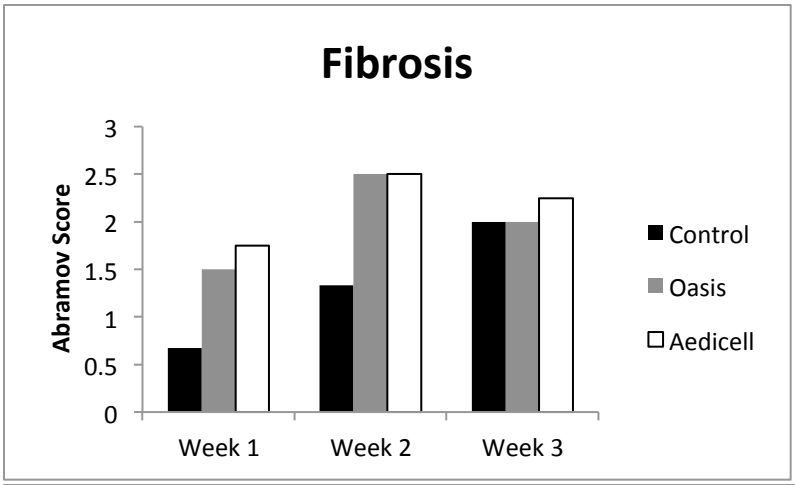
Figure 6: Epithelialization scoring for untreated, PBM-treated, and SIS-treated lesions as graded by a blinded pathologist. No epithelialization was evident for the control and SIS treatments at week one. The PBM-treated lesions showed early and sustained increased levels of epithelialization relative to the SIS and control treatments.

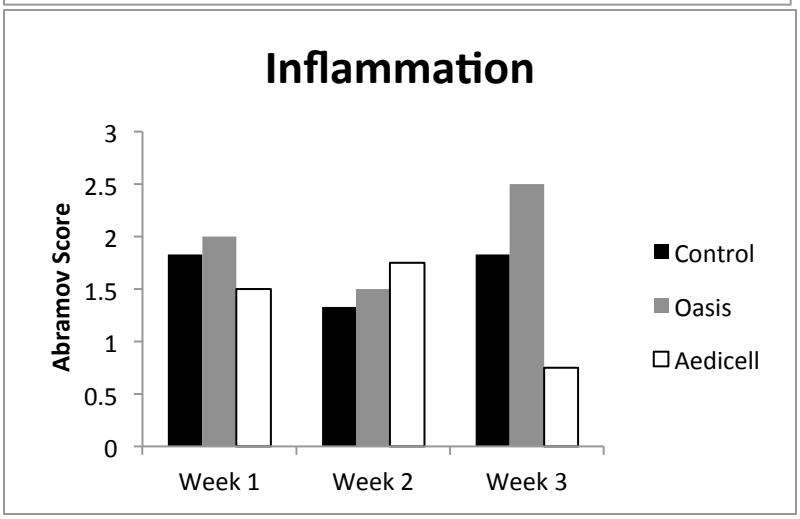
Figure 7: Fibrosis scoring for untreated, PBM-treated, and SIS-treated lesions as graded by a blinded pathologist. PBM treatments significantly accelerated collagen synthesis relative to the untreated controls, resulting in more extensive fibrosis at weeks one and two ($p<0.02$).

Figure 8: Inflammation scoring for untreated, PBM-treated, and SIS-treated lesions as graded by a blinded pathologist. There were no significant differences in inflammation between the three groups.




Epithelium
Abramov Score
3
2.5
2
1.5
1
0.5
0
Week 1
Week 2
Week 3
Control
Oasis
Aedicell


Fibrosis
Abramov Score
3
2.5
2
1.5
1
0.5
0
Week 1
Week 2
Week 3
Control
Oasis
Aedicell


Inflammation
Abramov Score
3
2.5
2
1.5
1
0.5
0
Week 1
Week 2
Week 3
Control
Oasis
Aedicell