In vitro Transcript Expression by Gingival Fibroblasts on Barrier Membranes

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Short Title: Gingival fibroblast transcript expression on membranes

Summary: Three membranes were found to differentially support gingival fibroblast expression of growth factors and cytokines that promote wound healing (GDF5, FGF2, IL-1β, IL-6, IL-8, and TNFα).

Abstract

Background: The ideal barrier membrane for periodontal surgeries should be epithelial cell-occlusive, non-reactive, maintain space, promote healing, and easy to handle. Collagen membranes are widely used for regenerative procedures, but success depends on primary closure during healing to prevent infection or rapid resorption of the material. Amnion chorion membranes, from human placenta, are more recently developed and marketed as having enhanced healing potential for periodontal procedures. Three different barrier membranes were compared for their ability to support cell attachment, differentiation and wound healing.

Methods: Three membranes were examined: Bio-Gide™ (BG, collagen), BioMend-Extend™(BM, crosslinked collagen), and BioXclude™(AC, amnion chorion). Gingival fibroblasts (GFs) were allowed to attach to the membranes for 24 hours in minimal essential media containing 10% fetal bovine serum. After attachment, membranes and GFs were transferred to fresh media, and allowed to grow for an additional 48 hours. RNA was isolated from these cells, and transcripts were examined via RT-PCR.

Results: Exposure of GFs to the membranes resulted in reduced expression of growth factor transcripts. However, BM significantly enhanced the expression of GDF5 and FGF2. Exposure of GFs to BM, BG and AC resulted in differential increase in expression of cytokine transcripts. IL-6 and IL-8 were significantly increased with AC, while TNFα was significantly increased with BM. Collagen transcripts were significantly reduced for all membranes.

Conclusion: While all three membranes are designed to block epithelial invasion, they also support the survival of gingival fibroblasts. These membranes differentially support GF expression of growth factors and cytokines that promote wound healing (GDF5, FGF2, IL-1β, IL-6, IL-8, and TNFα).

Key Words: Wound healing, fibroblasts, cell differentiation, cytokines, collagen

Introduction:

The search for an 'ideal' barrier membrane for periodontal surgeries is ongoing. The ideal membrane would be epithelial cell-occlusive, non-reactive, maintain space, promote healing, and be easy to handle[1]. A wide variety of membranes are available for use during periodontal surgery and may be chosen by the clinician based on preferred properties. Bioabsorbable membranes, especially natural membranes, avoid the need for a second surgical procedure and have the potential to stabilize the fibrin clot, improving wound healing[2,3]. New membranes, such as amnion chorion membranes (ACMs), have been promoted as able to improve healing following periodontal surgeries and clinical outcomes [4-7].

Human ACMs have been used in wound healing for chronic wounds in medicine, including ophthalmology[8]. Evidence has shown that ACMs release a large number of growth factors that promote healing, as well as provide a laminin-rich scaffold for tissue attachment[9,10]. In an in vitro study, Koob et al. made an elution of growth factors and found quantifiable levels of platelet-derived growth factor AA (PDGF-AA), PDGF-BB, transforming growth factor a (TGFα), TGFβ1, basic fibroblast growth factor (bFGF), as well as interleukins (ILs) -4,-6,-8, and -10 from processed dehydrated human amnion chorion allografts (dHACM)[9]. Furthermore, this elution from dHACM was able to promote proliferation and migration of human mesenchymal stem cells[9]. Clinically, case reports using ACMs show better flap attachment and wound closure even with an exposed membrane[11,4]. In a histologic report, when ACM and freeze-dried bone allograft were used for socket grafting, average new bone formation was 54.5% at 12 weeks [12]. While evidence is strong for the wound healing potential of ACMs, the membrane itself is thin and lacks rigidity that limits its use for space maintenance.

Collagen membranes are widely used in periodontal procedures with good success[13]. Collagen membranes provide a scaffold of predominantly type I and III collagens, thought to be advantageous as these are major components of periodontal connective tissue[14]. The success

of collagen membranes often depends on primary closure to prevent infection and rapid resorption of the material[15]. Additional modifications to collagen membranes include cross-linking which can slow the rate of resorption during healing and change its handling properties[13,16]. However, some in vitro studies demonstrate that cross-linking with glutaraldehyde may leave cytotoxic residue and may affect cell attachment[3,14]. Even so, collagen membranes have generally been shown to be clinically successful through both animal and human controlled studies in aiding in regeneration and wound healing[3].

In order to select the best materials for a periodontal procedure, a thorough understanding of wound healing mechanisms and differences between membranes is needed. In vitro studies evaluating the molecular components of amnion chorion membrane (ACM) have shown cell proliferation and migration in the presence of ACM growth factors which were isolated from the membrane[9,10]. However, in order to isolate these growth factors, the membranes were processed beyond their usual clinical form which is only applicable in an in vitro environment. ACMs have also been shown to have a significant amount of laminin-5[9], which is theorized to be involved in cell attachment and migration[18]. Attachment of human gingival fibroblasts has been demonstrated on both collagen and laminin as extracellular matrix proteins[19]. A comparative study of different cross-linked and non-cross-linked collagen membranes confirmed PDL fibroblast attachment to intact collagen membranes[14]. However, limited data exists on how cells respond to these membranes. Additionally, little to no evidence exists comparing the in vitro properties of ACM with other known bioabsorbable membranes. The key question that arose was whether all membranes promote the same response within gingival fibroblasts.

Therefore, the purpose of this in vitro study was to compare transcript expression of specific genes by gingival fibroblasts when exposed to three different intact bioabsorbable barrier membranes in order to evaluate their ability to support cell attachment, differentiation and wound healing.

Materials and Methods

Cells:

Gingival tissue was obtained from a healthy 23-year-old male patient requiring premolar extraction during orthodontic treatment)[20,21]. Gingival fibroblasts (GFs) were isolated from excised pieces of gingiva, as previously described[20]. Cells were maintained in Minimal Essential Media alpha (MEMα[§]) containing 10% fetal calf serum (FCS) and 200 units/ml of penicillin and 200 µg/ml streptomycin. GFs between 8th and 15th passage were used in this study.

Membranes:

Three bioabsorbable barrier membranes were chosen for the study: a cross-linked bovine collagen type I membrane[‖] (BM), a non-cross-linked porcine collagen type I and III membrane[¶] (BG) and an allograft amnion chorion membrane[#] (AC). All membrane samples were trimmed with a sterile blade to 10x10mm.

Cell exposure to membranes:

After trimming membranes, BM, BG and AC were rehydrated in MEMα medium. Cells in the 8th and 15th passage were used. After GFs reached confluence, they were removed from tissue culture bottles via trypsinization. Prepared GFs were then placed on membranes in agarose-coated wells, to prevent sticking of cells to the base of the culture wells. GFs grown on plastic well surfaces were used as controls. GFs were allowed to attach to the membranes for 24 hours. After attachment, membranes and GFs were transferred to fresh media, and allowed

§ Invitrogen, GIBCO, Grand Island, NY
‖ BioMend Ext™, Zimmer Dental, Carlsbad, CA, USA
¶ BioGide™, Geistlich Pharma AG, Wolhusen, Switzerland
# BioXclude™, Snoasis Medical, Denver, CO, USA

to grow for an additional 48 hours. Membranes were washed with MEMα and frozen at -80° C to facilitate cell lysis.

Transcript Analysis:

Membranes were vortexed vigorously for 15 minutes in an RNA extraction buffer. RT-PCR was used to assess transcript expression. cDNAs were made using MMLV-RT and the Retroscript Kits[§] per the manufacturer's instructions. Briefly, 1 gram of total RNA was reverse-transcribed using 100 units of MMLV-RT in 20 μl reactions. 2 μl of each reaction was used as template in 25 μl polymerase chain reactions (PCR), using 50 pmoles of primers, 250 μM of dNTPs, and 1 mM Mg^{2+}. PCR reactions were performed using the following protocol: 93°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute for 25 cycles. The number of cycles was chosen by creating a standard curve using serial dilutions of DNA template known to include the genes in question and determining the PCR conditions that revealed 2-fold differences in gene expression over three (3) orders of magnitude. Twenty-five (25) cycles of PCR were chosen to be within the linear range of detection for all the genes examined. Genes examined included ribosomal protein S15 (control); Collagens I, III, V, VI, and VIII; pro-inflammatory cytokines, IL-6, IL-8, and tissue necrosis factor-α (TNF-α); and growth factors, TGFβ-1, bone morphogenic protein-2 (BMP-2), BMP-4, growth differentiation factor 5 (GDF5), FGF2, FGF5, and connective tissue growth factor (CTGF). These particular genes were selected due to their key roles in the regulation of gingival wound healing (Table 1). Quantification of transcript expression was carried out using ImageJ software. Statistical analysis included ANOVA and multiple paired t-tests. Statistical difference was set at $P < 0.05$.

Results

GFs remained viable on all three barrier membranes throughout the 72hr incubation period. The control GFs typically produced robust levels of collagen and growth factors transcripts assessed. In contrast, exposure to the membranes tended to reduce transcript expression for collagens (Figure 1A) and growth factors (Figures 2A when compared to cells grown on standard tissue culture polystyrene. Exposure of GFs to all three membranes resulted in decreased expression of Collagens I, III, V, VI and VIII (Figure 1). No significant differences were observed between the three membranes examined. The greatest reduction was seen for Collagens III, V, and VI transcripts on all membranes (Figure 1B). Similarly, exposure of GFs to these membranes tended to reduce their expression of growth factors (Figure 2). The only exception to this general trend was the significant increase in expression of GDF5 and FGF2 by cells grown on BM membranes (Figure 2B).

In contrast, GFs tended to express barely detectable levels of several cytokines (IL-6, IL-8 and TNFα) when grown on tissue culture polystyrene (Figure 3A). However, GFs grown on AC membranes expressed significantly higher levels of IL-6 and IL-8, while those grown on BM membranes expressed significantly higher levels of TNFα (Figure 3B). No increase of any cytokines was seen with GFs grown on BG membranes. Thus, exposure to these membranes resulted in membrane specific alterations in a select subset of the transcripts examined.

Discussion

Bioabsorbable barrier membranes are thought to promote wound healing and have high tissue compatibility. This study was designed to assess the survival of gingival fibroblasts on intact membranes and evaluate transcript production in response to three different membranes. As shown in previous studies[14], it is expected that the number of cells in a control environment will be greater than those cells able to attach to intact bioabsorbable membranes. Additionally, in the control environment the cells are in an active state of cell division so as expected, we saw

that GFs on standard tissue culture polystyrene were actively producing collagens and growth factors. All three bioabsorbable membranes supported cell attachment and GFs survival on the intact membranes during the incubation period. As an important first step in wound healing is stabilization of the clot, followed by recruitment of cells for wound closure, survival of GF on these membranes is of utmost importance.

We observed that there was an overall reduction of collagen transcripts expressed by the GFs exposed to the membranes. A plausible explanation might be the GFs interaction with the surrounding environment. As GFs attach to the membranes, they become embedded in the extracellular matrix created by the membrane. This may result in downregulation of production of those transcripts for proteins if they are already present in the surrounding matrix. All membranes that we tested showed a decreased expression of growth factors, with the exception of the GFs attached to BM. These GFs showed an increased expression of GDF-5 and FGF-2, both of which are involved in fibroblast proliferation and angiogenesis[22]. BG had little to no increase in activation of pro-inflammatory cytokines, which could be attributed to it being a non-cross-linked membrane[14]. BM, a cross-linked collagen membrane, induced an increase in TNF-α, which is both a pro and anti-inflammatory cytokine which can activate growth factor expression in fibroblasts[22]. As other studies have shown that IL6, IL8 and TNF-α have a detrimental effect on wound healing by increasing GF apoptosis, it is unclear if an increase in expression of any of these cytokines would have a negative effect clinically[23]. GF grown on AC showed the greatest activation of IL-6 and IL-8. As with TNF-α, it is unclear what effect upregulation of these pro-inflammatory cytokines will have, as their roles in wound healing are much more complex. IL-6 and IL-8 are important regulators of the inflammatory process and recruiters of growth factors crucial for angiogenesis[9]. Previous studies looking at the effect of growth factor elutions from dHACMs, including interleukins, reported increased proliferation and

migration of fibroblasts[9]. Increased recruitment of inflammatory factors may play a role in rapid proliferation and migration of cells which could mean faster wound closure clinically.

In conclusion, the current study supports that survival and attachment of gingival fibroblasts on three commonly used commercially available intact membranes is viable, although the included membranes were noted to differentially promote transcript expression of cytokines and growth factors important to wound healing (GDF5, FGF2, IL-1β, IL-6, IL-8, and TNFα). The present study was limited in the number of resorbable membranes analyzed along with the limited number of transcripts assessed. Studies of dHACMs and wound healing have shown that many more factors could potentially be affected by the different membranes. Future research is required focusing on the expression of these factors along with evaluation of cell attachment and migration in relation to these membranes. Additionally, rapid wound closure has been promoted as an advantage with the use of ACMs and this would be best evaluated by observation of cell migration on the surface of intact membranes.

Footnotes

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§ Invitrogen, GIBCO, Grand Island, NY

‖ BioMend Ext™, Zimmer Dental, Carlsbad, CA, USA

¶ BioGide™, Geistlich Pharma AG, Wolhusen, Switzerland

BioXclude™, Snoasis Medical, Denver, CO, USA

References

1. Gottlow J. Guided tissue regeneration using bioresorbable and non-resorbably devices: initial healing and long-term results. *J Periodontol* 1993; 64:1157-1165.
2. Dimitrou R, Mataliotakis GI, Calori GM, Giannoudis PV. The role of barrier membranes for guided bone regeneration and restoration of large bone defects: current experimental and clinical evidence. *BMC Medicine* 2012; 10:81.
3. Bunyaratavej P, Wang HL. Collagen membranes: a review. *J Periodontol* 2001; 72: 215-229.
4. Holtzclaw D, Toscano N. Amnion chorion allograft barrier: indications and techniques update. *J Implant Adv Clin Dent* 2012; 4(2): 25-38.
5. Holtzclaw D, Toscano N. BioXclude™ placental allograft tissue membrane used in combination with bone allograft for site preservation: a case series. *J Implant Adv Clin Dent* 2011; 3(3): 35-50.
6. Holtzclaw D, Toscano NJ. Amnion-chorion allograft barrier used for guided tissue regeneration treatment of periodontal intrabony defects: a retrospective observational report. *Clin Adv Periodontics* 2013; 3(3): 131-137.
7. Rosen PS. Comprehensive periodontal regenerative care: combination therapy involving bone allograft, a biologic, a barrier, and a subepithelial connective tissue graft to correct hard- and soft- tissue deformities. *Clin Adv Peridontics* 2011; 1(1): 154-159.
8. Kesting MR, Wolff KD, Nobis CP, Rohleder NH. Amniotic membranes in oral and maxillofacial surgery. *Oral Maxillofac Surg* 2014; 18: 153-164.
9. Koob TJ, Rennert R, Zabek N, et al. Biological properties of dehydrated human amnion/chorion composite graft: implications for chronic wound healing. *Int Wound J* 2013; 10: 493-500.

10. Koob TJ, Lim JL, Massee M, et al. Angiogenic properties of dehydrated human amnion/chorion allografts: therapeutic potential for soft tissue repair and regeneration. *Vascular Cell* 2014; 6:10.
11. Hotlzclaw D, Hinze HF, Toscano N. Gingival flap attachment healing with amnion-chorion allograft membrane: a controlled, split mouth case report replication of the classic 1968 Hiatt study. *J Implant Adv Clin Dent* 2012; 4(5): 19-25.
12. Wallace S, Cobb C. Histological and computed tomography analysis of amnion chorion membrane in guided bone regeneration in socket augmentation. *J Implant Adv Clin Dent* 2011; 3(6): 61-72.
13. Malmquist JP, Scantlebury TV, Triplett RG. Osteo Science Foundation Collagen White Paper, 2019.
14. Rothamel D, Schwarz F, Sculean A, Herten M, Scherbaum W, Becker J. Biocompatibility of various collagen membranes in cultures of human PDL fibroblasts and human osteoblast-like cells. *Clin Oral Implants Res* 2004; 15: 443-449.
15. Pitaru S, Tal H, Soldinger M, Azar-Avidan O, Noff M. Collagen membranes prevent apical migration of epithelium during periodontal wound healing. *J Periodontal Res* 1987; 22:331-333.
16. Tal H, Kozlovsky A, Artzi Z, Nemcovsky CE, Moses O. Long-term bio-degradation of cross-linked and non cross-linked collagen barriers in human guided bone regeneration. *Clin Oral Implants Res* 2008; 19:295-302.
17. Behring J, Junker R, Walboomers XF, Chessnut B, Jansen JA. Toward guided tissue and bone regeneration: morphology, attachment, proliferation, and migration of cells cultured on collagen barrier membranes. A systematic review. *Odontology* 2008; 96:1-11.
18. Terranova VP, Rohrbach DH, Martin GR. Role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen. *Cell* 1980; 22:719-726.

19. Lallier TE, Miner QW, Sonnier J, Spencer A. A simple cell motility assay demonstrates differential motility of human periodontal ligament fibroblasts, gingival fibroblasts and pre-osteoblasts. *Cell Tissue Res* 2007; 328: 339-354.
20. Lallier TE, Spencer A, Fowler MM. Transcript profiling of periodontal fibroblasts and osteoblasts. *J Periodontol* 2005; 76:1044-55.
21. Tsourounakis I, Palaiologou-Gallis AA, Stoute D, Maney P, Lallier TE. Effect of essential oil and chlorhexidine mouthwashes on gingival fibroblast survival and migration. *J Periodontol* 2013; 84: 1211-20.
22. Aukhil, I. Biology of wound healing. *Periodontol 2000* 2000; 22: 44-50.
23. Basso FG, Pansani TN, Turrioni APS, Soares DG, de Souza Costa CA, Hebling J. Tumor necrosis factor-α and interleukin (IL)-1β, IL-6, and IL-8 impair in vitro migration and induce apoptosis of gingival fibroblasts and epithelial cells, delaying wound healing. *J Periodontol* 2016; 87: 990-996.

Figure Legends

Figure 1: Expression of collagen transcripts of GFs exposed to barrier membranes.

Gingival fibroblasts exposed to barrier membranes: Amnion chorion BioXclude™ (AC), collagen BioMend-Extend™ (BM), collagen Bio-Gide™ (BG) for three days. (A) RT-PCR analysis of collagen transcript expression was quantified (B) for comparison. Bars represent the mean and standard deviation of two samples. Asterisks (*) represent statistical differences from control cells grown on standard tissue culture polystyrene.

Figure 2: Differential expression of growth factor transcripts of GFs exposed to barrier membranes.

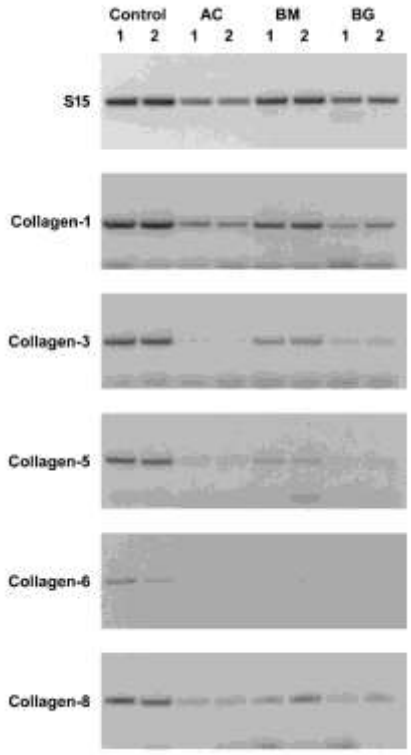
Gingival fibroblasts exposed to barrier membranes: Amnion chorion BioXclude™ (AC), collagen BioMend-Extend™ (BM), collagen Bio-Gide™ (BG) for three days. (A) RT-PCR analysis of collagen transcript expression was quantified (B) for comparison. Bars represent the mean and standard deviation of two samples. Asterisks (*) represent statistical differences from control cells grown on standard tissue culture polystyrene.

Figure 3: Differential expression of pro-inflammatory transcripts of GFs exposed to barrier membranes

Gingival fibroblasts exposed to barrier membranes: Amnion chorion BioXclude™ (AC), collagen BioMend-Extend™ (BM), collagen Bio-Gide™ (BG) for three days. (A) RT-PCR analysis of pro-inflammatory transcripts expression was quantified (B) for comparison. Bars represent the mean and standard deviation of two samples. Asterisks (*) represent statistical differences from control cells grown on standard tissue culture polystyrene.

Figure 1

A


Control
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Collagen-1
Collagen-3
Collagen-5
Collagen-6
Collagen-8

B

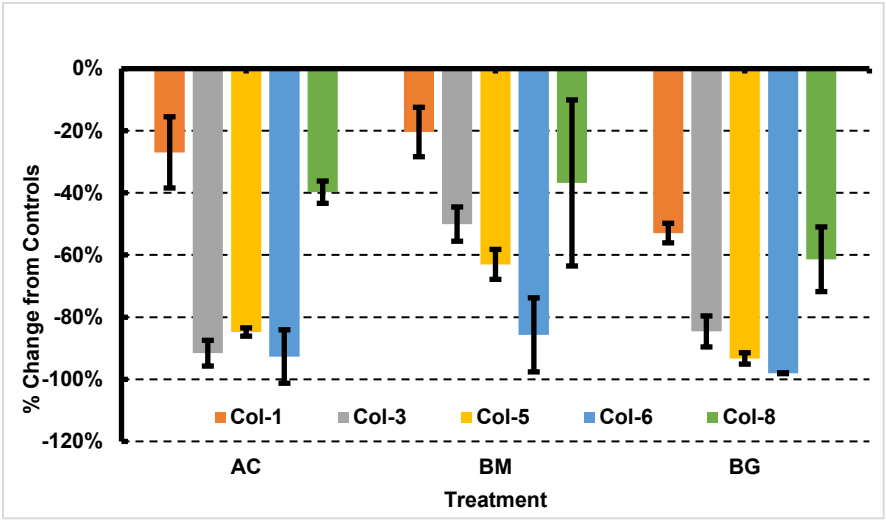
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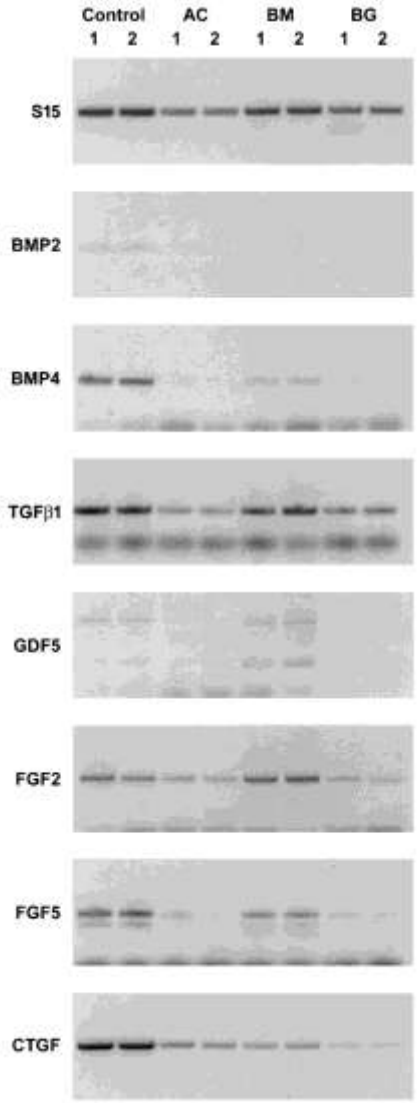
Figure 2

A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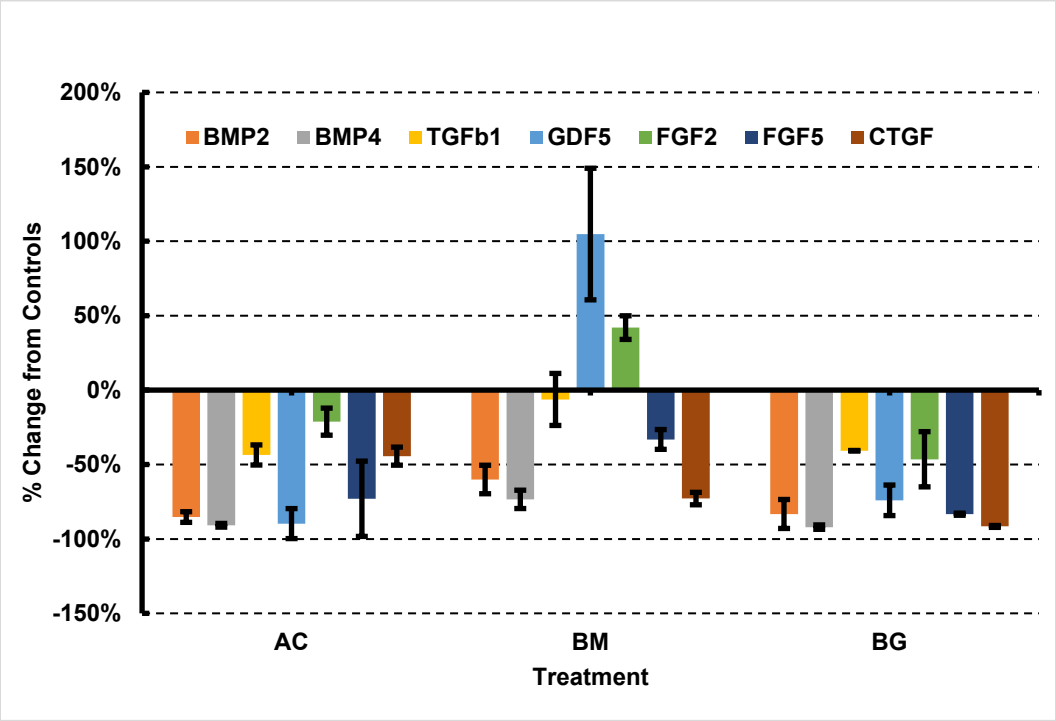
BMP4
TGFβ1
GDF5
FGF2
FGF5
CTGF

B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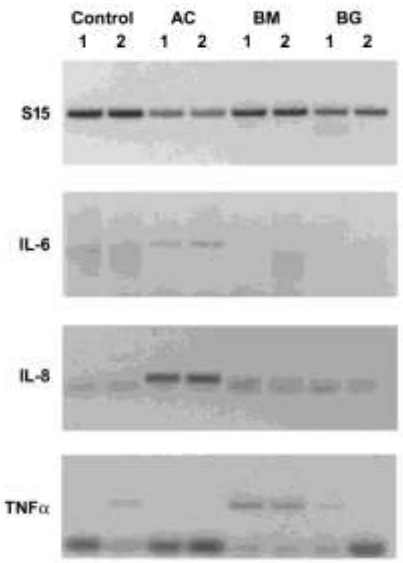
Treatment

Figure 3

A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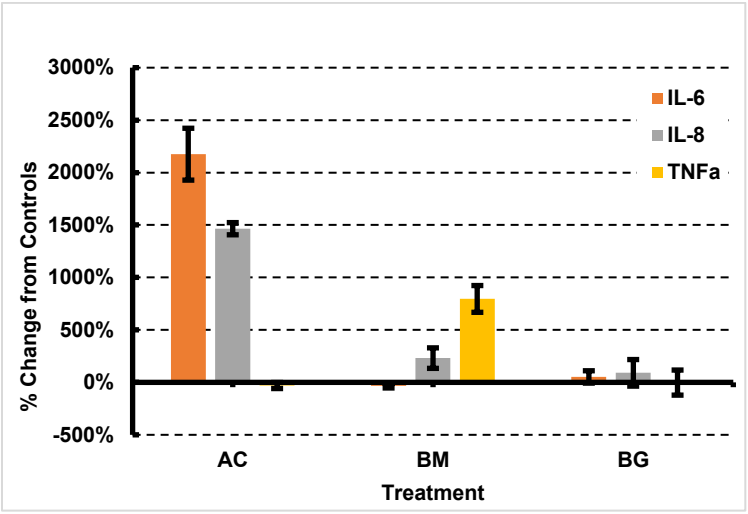
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Tables

Table 1. Functions of genes tested

Gene group:	Functions:
S15	Ribosomal gene, responsible for protein production (control)
Collagen I, III, V, VI, VIII	Variants of collagen transcripts for collagen synthesis
IL-6, IL-8, TNF-α	Pro-inflammatory and anti-inflammatory cytokines with roles for multiple cell types and IL-8 is linked to angiogenesis[6]
TGFβ1	Transforming growth factor; controls proliferation, differentiation and other functions in many cell types
BMP2/BMP4	Bone morphogenic protein; induce formation of bone and cartilage
GDF5	Growth differentiation factor; regulators of cell growth and differentiation
FGF2/FGF5	Fibroblast growth factor; have regulatory, morphological, and endocrine effects
CTGF	Connective tissue growth factor; plays a role in cell adhesion, migration, proliferation, angiogenesis, skeletal development, and tissue wound repair