Vitamin D Effects on Gingival Fibroblast Gene Expression

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Title: Vitamin D Effects on Gingival Fibroblast Gene Expression

Summary: Vitamin D promotes collagen and bone formation but has a mixed effect on pro- and anti-inflammatory cytokines in gingival fibroblasts.

Key Words: Vitamin D, Fibroblasts, Inflammation, Cytokines.

Vitamin D Effects on Gingival Fibroblast Gene Expression

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Abstract

Introduction: Periodontal disease is an inflammatory process associated with many genetic and environmental factors, including vitamin D deficiency. Vitamin D3's active form, 1,25-dihydroxyvitamin D_3 (1,25D3), exhibits potent anti-inflammatory activity. We hypothesize that 1,25D3 can inhibit the pro-inflammatory response to lipopolysaccharide stimulation in gingival fibroblasts.

Methods: Human gingival fibroblasts were stimulated with either E. coli (10 μg/ml) or P. gingivalis (1 μg/ml) lipopolysaccharide for a period of 24 hours. Cells were simultaneously treated with 1,25-dihydroxyvitamin D_3 (100 nM) also for a 24-hour period. RNA was extracted from the cells, and gene expression was quantified and qualified using RT-QPCR.

Results: After 24-hour exposures to LPS and vitamin D, the fibroblasts were observed to have a survival rate of 100%. Vitamin D exposure for 24 hours resulted in an increase of collagen-3, collagen-5, collagen-6, and collagen-10 expression. Vitamin D also yielded an increase in periostin, osteomodulin, and alkaline phosphatase expression. G-CSF, TNFα, and IL-10 expression were all observed to increase when cells were exposed to vitamin D. Only GM-CSF was noted to exhibit a decrease in expression after vitamin D exposure.

Conclusion: Vitamin D was not observed to be cytotoxic at any concentration utilized in this study. Vitamin D exposure displayed beneficial effects on fibroblast transcript expression involved with bone and collagen formation, and mixed effects on pro- and anti-inflammatory cytokines.

Introduction:

Periodontitis is an inflammatory process that destroys the supporting tissues around the teeth that could ultimately result in tooth loss. Periodontitis is a local chronic infectious disease triggered by a mixed, predominantly anaerobic, dysbiotic subgingival bacterial biofilm (Hajishengallis 2017 and Kornman 1997). The destruction of the periodontium is caused by the host immune response to bacterial insult (Hajishengallis 2017 and Page 1976). Periodontitis is the 6th most prevalent disease globally and is the leading cause of tooth loss in adults. As periodontitis progresses it increases the chances that patients will experience tooth loss, nutritional deficiencies, and other systemic problems (Tonetti, 2017).

Vitamin D is a group of fat-soluble steroids that vital in the homeostasis of calcium, phosphorus, and magnesium in the human body. Vitamin D is primarily obtained from ultraviolet ray exposure, but can also be obtained through supplementation and diet. Currently, the recommended daily amount of Vitamin D is 600 IU. A patient is considered vitamin D deficient if vitamin D blood levels are less than 20 nanograms per milliliter. Vitamin D deficiency has been observed to increase the risk of developing osteoporosis, diabetes, and periodontitis. Research suggests a significant association of periodontal health with vitamin D and calcium intake. Vitamin D has anti-inflammatory and anti-bacterial activities, and also enhances macrophage chemotaxis and phagocytosis (Menzel, 2019) (Stein, 2014). Vitamin D also inhibits osteoclastogenic cytokines, IL-6 and IL-8, *Porphyromonas gingivalis*-induced IL-8 secretion, TNF-α and NF-kB levels (Gao, 2018). These cytokines are responsible for immune cell chemotaxis and bone loss in periodontitis (Garlet, 2010). These anti-inflammatory properties contribute to the beneficial effects of vitamin D on oral health when used with conventional periodontal therapies. Dietary supplementation with vitamin D and calcium may improve periodontal health, and a recent review concluded that vitamin D plays a protective role, by regulating innate and adaptive immune responses (Chen, 2013) (Garcia, 2011) (Miley, 2009) (Perayil, 2015). Since Vitamin D is instrumental in maintaining bone homeostasis and host immunity, there is reason to suspect that a supplementation of Vitamin D could positively impact the health of the periodontium (Prietl, 2013) (White, 2008). There is evidence that suggests that

Vitamin D may regulate the production of inflammatory mediators from gingival fibroblasts (Elenkova, 2019) (Krishnan, 2011). Currently, there is a lack of research analyzing the effect vitamin D has on human gingival fibroblast (HGF) expression. HGFs are stimulated by lipopolysaccharide to manufacture a myriad of inflammatory cytokines. Therefore, HGFs are instrumental in the inflammation and host destruction observed in periodontitis. Vitamin D has been reported to reduce the local production of inflammatory cytokines, so it has a protective role during the inflammatory process that is periodontitis. Human gingival fibroblasts are the most numerous cell type in the periodontium, and are therefore the primary focus of this study (Bhatavadekar, 2009). The aim of this study is to analyze the effects of Vitamin D on the gene expression of pro-inflammatory and anti-inflammatory cytokines, collagens, and bone-forming transcripts by gingival fibroblasts.

Materials and Methods:

Cells: Gingival fibroblast cell lines established previously from healthy gingiva obtained from one 23-year-old male patient with no systemic diseases, who underwent oral surgery at LSU School of Dentistry, were used. In all cases, tissues were obtained from subjects following informed consent as prescribed in an approved IRB protocol. The cells were washed and maintained in minimum essential medium alpha (MEMα) containing 10% fetal calf serum (FCS) and 200 units/mL of penicillin and 200 μg/mL streptomycin (GIBCO Grand Island, NY) to reduce bacterial contamination. This media will also be used in the tissue culture assays. All gingival fibroblasts used in this study were between the 8th and 12th passages.

Vitamin D: 1,25-dihydroxycholecalciferol (1,25D3) was solubilized in ethanol to produce 100 μM stock solutions and stored at −80°C. Vitamin D stock solution was then mixed with MEMα to achieve 0.1, 1, 10, and 100 nM vitamin D concentrations.

Cell Survival (Vitamin D): Fibroblasts were plated onto 48-well plates at 4,000 cells/well. Vitamin D solutions were added in varying concentrations (0.1, 1, 10, and 100 nM). Cells were incubated at 37°C for 24 hours. Vitamin D solutions were removed via aspiration and replaced with MEMα. Cells were incubated for up to 24 hours prior to assess cell survival. Cell survival was assayed using calcein-acetyoxymethyl ester (Molecular Probes, Eugene, Or) for 1 hour; cells were rinsed in PBS and fluorescence recorded using a Bio Tek Synergy2 fluorescent multi-well plate read appropriately for 480nm excitation and 520nm emission. Effective concentration of vitamin D was determined to be 100 nM.

E. coli and P. gingivalis LPS: Stock solution of 1 mg/ml of E. coli LPS was mixed with MEMα to achieve 1, 2, 5, and 10 μg/ml concentrations of E. coli LPS. Stock solution of 1 mg/ml of P. gingivalis LPS was mixed with MEMα to achieve 1, 2, 5, and 10 μg/ml concentrations of P. gingivalis LPS. LPS was used to stimulate gingival fibroblasts.

Cell Survival (LPS): Fibroblasts were plated onto 48-well plates at 4,000 cells/well. E. coli and P. gingivalis LPS solutions were added to wells in varying concentrations (1, 2, 5, and 10 μg/ml). Cells were incubated at 37°C for 24 hours. LPS solutions were removed via aspiration and replaced with MEMα. Cells were incubated for up to 24 hours prior to assess cell survival. Cell survival was assayed using calcein-acetyoxymethyl ester (Molecular Probes, Eugene, Or) for 1 hour; cells were rinsed in PBS and fluorescence recorded using a Bio Tek Synergy2 fluorescent multi-well plate read appropriately for 480nm excitation and 520nm emission. Effective concentration of E. coli LPS was determined to be 10 μg/ml, and for P. gingivalis LPS it was determined to be 1 μg/ml.

RNA Isolation: Fibroblasts (passage 15) were separated into the following 6 treatment groups: control (MEMα), E. coli LPS (10 μg/ml), P. gingivalis LPS (1 μg/ml), Vitamin D (100 nM), Vitamin D + E. Coli LPS, and Vitamin D + P. gingivalis LPS. Exposures of 24 hours were done for each group. Solutions was removed and cells were incubated in MEMα for 48 hours at 37°C. Cells were harvested by trypsinization, spun at 3000RPM for 5 minutes and the supernatant removed. Samples were frozen at -80°C. RNA was isolated using Qiagen Mini-Prep procedure (following the manufacturer's instructions). These RNA samples were analyzed for specific gene expression by RT-PCR.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis: Differential transcript expression was examined by reverse transcriptase - polymerase chain reaction (RT-PCR). Briefly, RNA was extracted from tissue and cell samples using Qiagen kits and gene expression determined by RT-PCR. cDNAs was made using MMLV-RT and the Retroscript Kit (Invitrogen) as per the manufacturer's instructions. Briefly, 1 μg 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+}. Various primers for specific transcripts are described in Table 1. PCR reactions was 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. The primers for these studies were derived from the published DNA sequences of human genes. Duplicate samples will be examined for each transcript, and RT-PCR analysis will be repeated to ensure accuracy. Statistical test will be used to determine the amount of change seen before and after cells were exposed to vitamin D.

Statistics: Paired t-tests evaluated differences between samples. Significance was defined as $P<0.05$.

Results:

Effects of Vitamin D and LPS Exposure on Cell Survival: Experiments assessing the cytotoxicity of vitamin D and LPS were conducted prior to experiments involving gene expression. Gingival fibroblasts were exposed concentration of vitamin D up to 100 nM for a period of 24 hours. Gingival fibroblasts were exposed separately to both E. coli and P. gingivalis LPS at concentrations up to 10 µg/ml for a period of 24 hours. Gingival fibroblasts that were exposed to a maximum of 100 nM of vitamin D displayed no statistically significant change in the percentage of cell survival (Figure 1). Gingival fibroblasts that were exposed to a maximum of 10 µg/ml of LPS displayed no statistically significant change in the percentage of cell survival (Figure 2). Gingival fibroblasts exposed to vitamin D and LPS displayed survival rates of 100% throughout the 24-hour exposure. Therefore, it was determined that these concentrations of vitamin D and LPS were not cytotoxic to gingival fibroblasts. For gene expression experiments, cells will be exposed to 10 µg/ml of E. coli LPS, 1 µg/ml of P. gingivalis LPS, and 100 nM of vitamin D.

Effects of LPS and Vitamin D Exposure on Transcript Expression: Vitamin D, E. coli LPS, and P. gingivalis LPS treated gingival fibroblasts were examined for changes in gene expression for certain collagen, bone, cytokine, interleukin, and matrix metalloproteinase transcripts.
Collagen transcripts: Collagen-1, 3, 5, 6, 8, and 10 were all expressed following exposure to LPS, vitamin D, and vitamin D plus LPS. Collagen-1 and 8 displayed no change in expression for all exposure groups. A statistically significant increase in the expression of collagens 3, 6, and 10 was observed in cells exposed to P. gingivalis LPS alone. A statistically significant increase in the expression of collagens 3, 5, 6, and 10 was observed in cells exposed to vitamin D alone, vitamin D plus E. coli LPS, and vitamin D plus P. gingivalis LPS. (Figures 3A and 3B)
Bone-forming transcripts: Osteocalcin and RANKL were not expressed following exposure to LPS, vitamin D, and vitamin D plus LPS. Alkaline phosphatase, osteoprotegerin, periostin, osteomodulin, and osteonidogen were all expressed following exposure to LPS, vitamin D, and vitamin D plus LPS. A statistically significant increase in the expression of alkaline phosphatase, periostin, and osteomodulin was observed in cells exposed to P. gingivalis LPS alone, vitamin D

alone, vitamin D plus E. coli LPS, and vitamin D plus P. gingivalis LPS. (Figures 4A, 4B, 5A, and 5B)

Cytokine transcripts: MIP-1a and MIP-1b were not expressed following exposure to LPS, vitamin D, and vitamin D plus LPS. TNF-α, MCP-1, G-CSF, and GM-CSF were all expressed following exposure to LPS, vitamin D, and vitamin D plus LPS. A statistically significant increase in the expression of TNF-α was observed in cells exposed to E. coli LPS alone, P. gingivalis LPS alone, vitamin D alone, vitamin D plus E. coli LPS, and vitamin D plus P. gingivalis LPS. A statistically significant increase in the expression of G-CSF was observed in cells exposed to vitamin D alone, vitamin D plus E. coli LPS, and vitamin D plus P. gingivalis LPS. A statistically significant decrease in the expression of GM-CSF was observed in cells exposed to vitamin D alone, vitamin D plus E. coli LPS, and vitamin D plus P. gingivalis LPS. (Figures 6A, 6B, 7A, and 7B)

Interleukin transcripts: IL-1 and IL-4 were not expressed following exposure to LPS, vitamin D, and vitamin D plus LPS. IL-6, IL-8, and IL-10 were all expressed following exposure to LPS, vitamin D, and vitamin D plus LPS. A statistically significant increase in the expression of IL-10 was observed in cells exposed to P. gingivalis LPS alone, vitamin D alone, vitamin D plus E. coli LPS, and vitamin D plus P. gingivalis LPS. (Figures 8A and 8B)

Matrix metalloproteinase transcripts: MMP-1, MMP-2, and MMP-3 were all expressed following exposure to LPS, vitamin D, and vitamin D plus LPS. However, no change in gene expression was observed. (Figure 9)

Figure 1. Effects of Vitamin D on Gingival Fibroblast Cell Survival

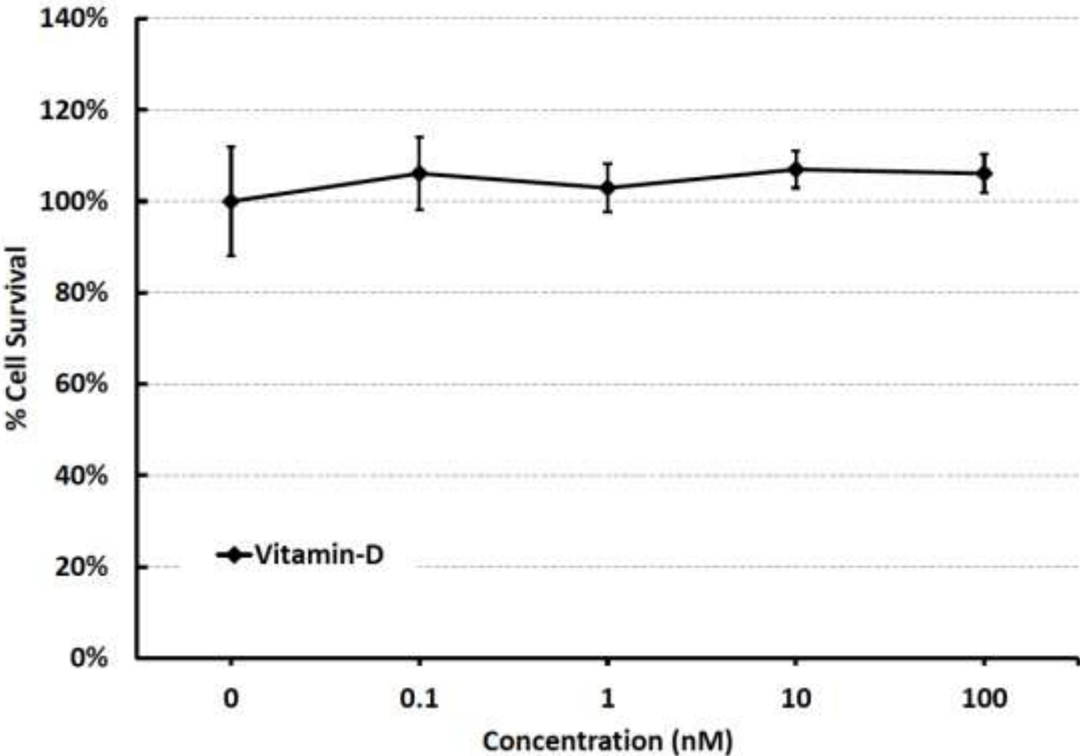


Figure 1: Survival of gingival fibroblasts exposed to various concentrations of vitamin D
Fibroblasts were exposed to 0.1, 1, 10, and 100 nM of vitamin D for a period of 24 hours.

Figure 2. Effects of E. coli and P. gingivalis LPS on Gingival Cell Survival

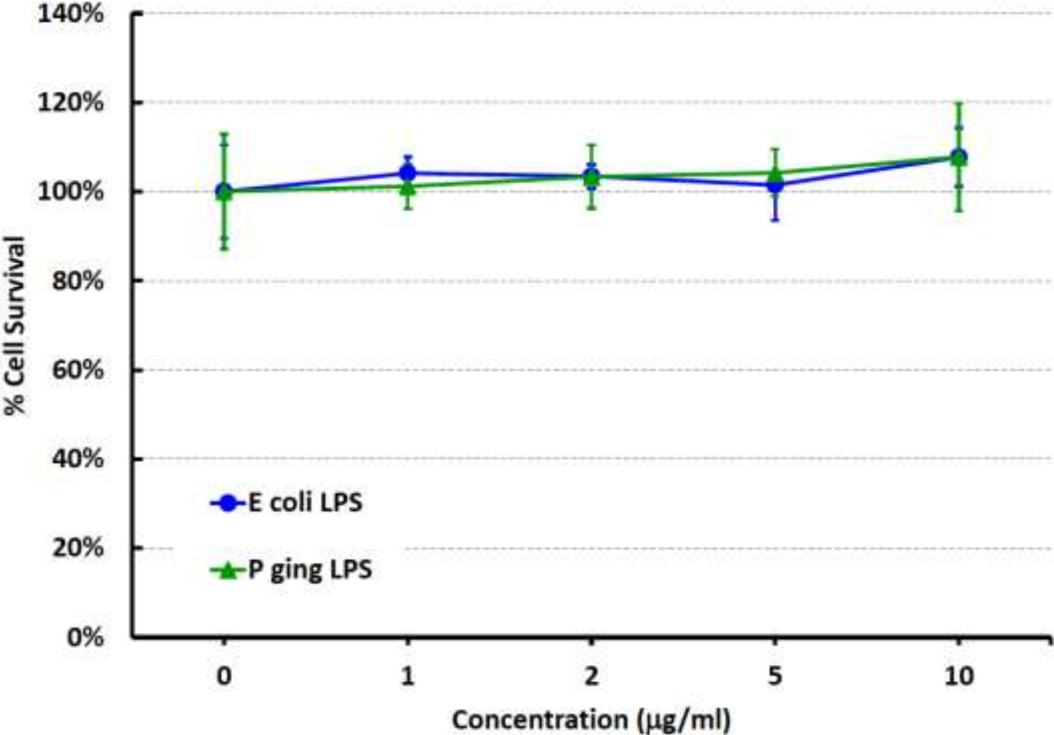
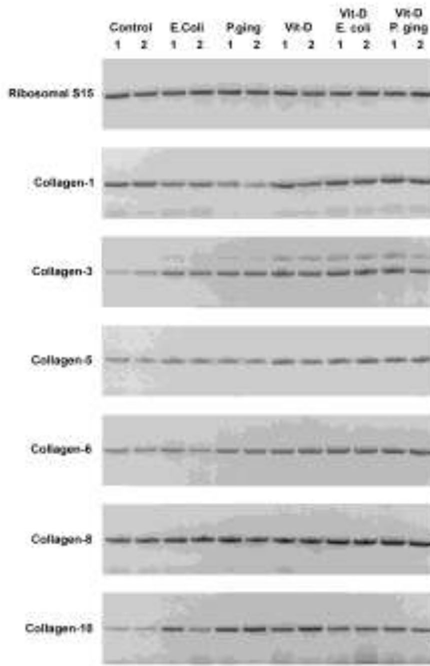
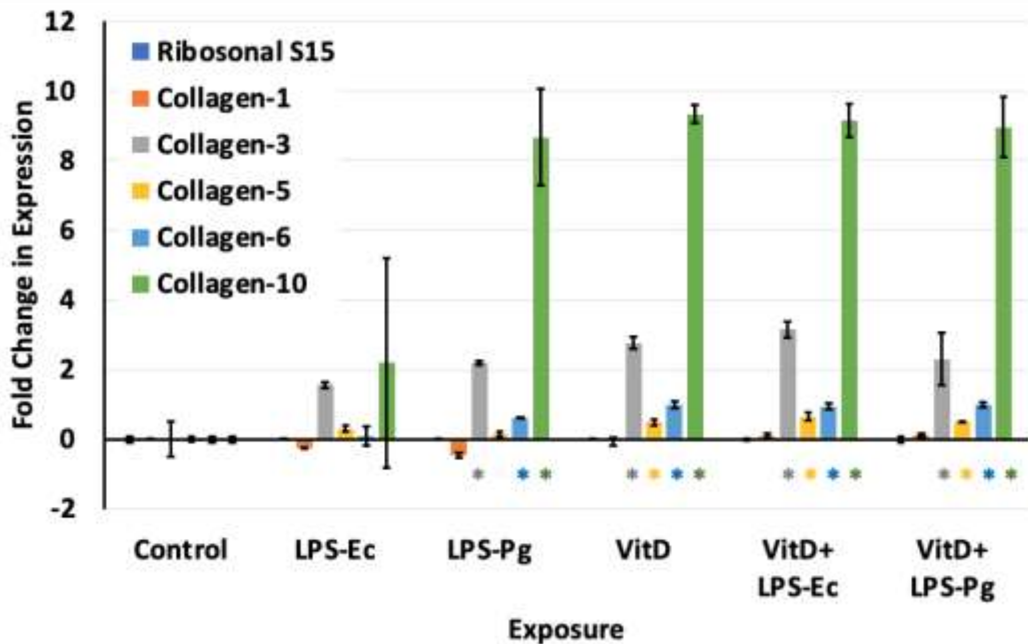


Figure 2: Survival of gingival fibroblasts exposed to various concentrations of E. coli and P. gingivalis LPS. Fibroblasts were exposed to 1, 2, 5, and 10 μg/ml of E. coli and P. gingivalis LPS for a period of 24 hours.

Figure 3.

A.



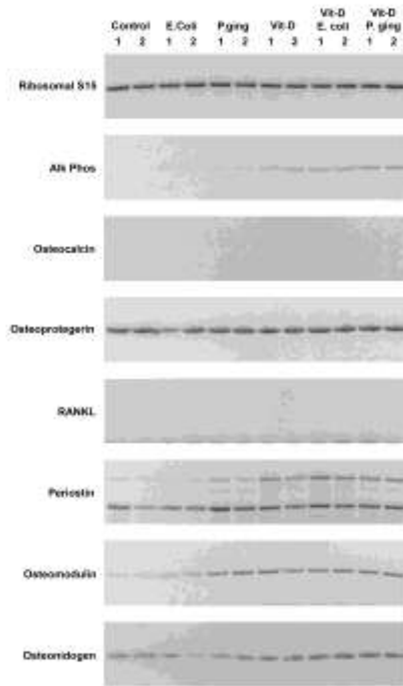


B.

Figure 3: Gingival Fibroblast Collagen Transcript Expression.
Gingival fibroblasts were exposed to LPS, vitamin D, and vitamin D plus LPS for 24 hours. Collagen transcripts analyzed using RT-PCR (A). (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

Figure 4.

A.



B.

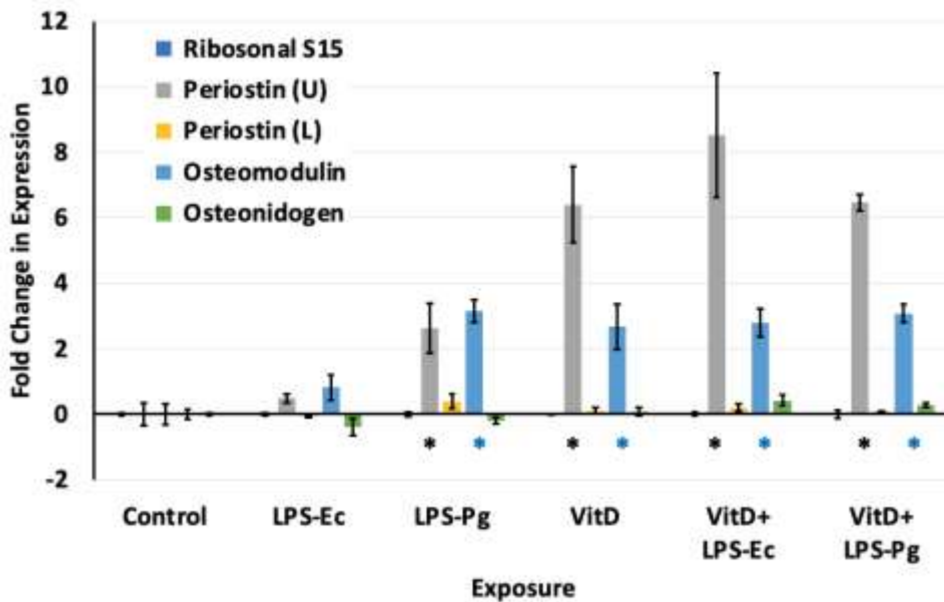
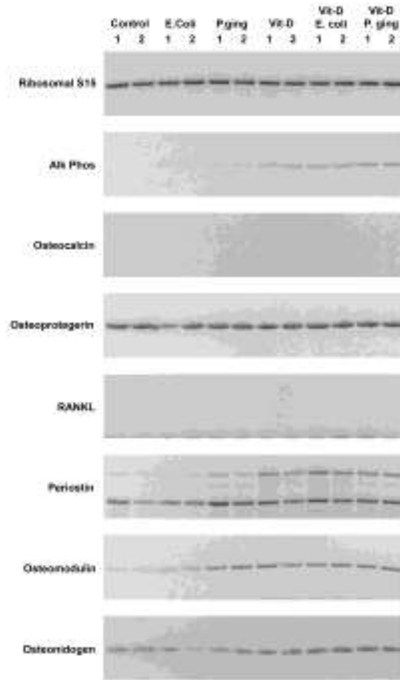


Figure 4: Gingival Fibroblast Bone Transcript Expression
Gingival fibroblasts were exposed to LPS, vitamin D, and vitamin D plus LPS for 24 hours. Transcript expression analysis for bone formation using RT-PCR (A). (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

Figure 5.

A.



B.

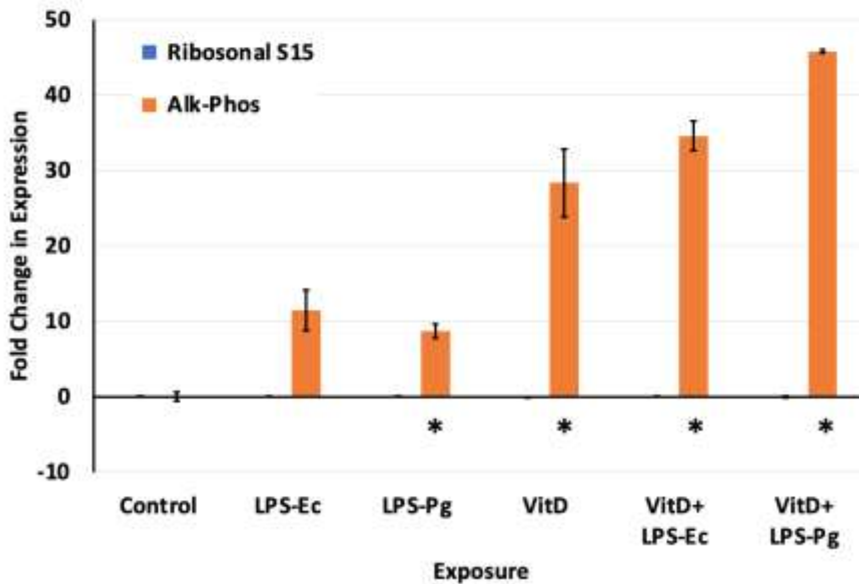
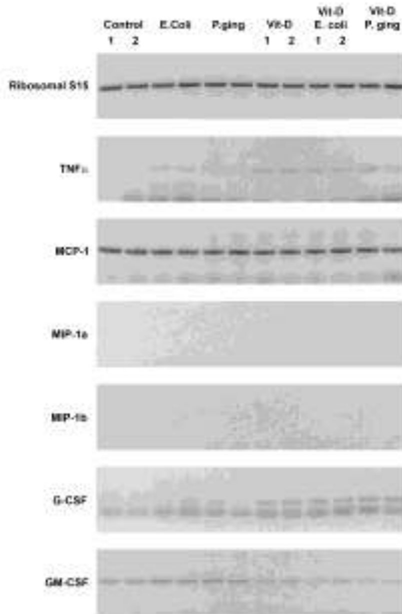


Figure 5: Gingival Fibroblast Bone Transcript Expression
Gingival fibroblasts were exposed to LPS, vitamin D, and vitamin D plus LPS for 24 hours. Transcript expression analysis for bone formation using RT-PCR (A). (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls (P<0.05).

Figure 6.

A.



B.

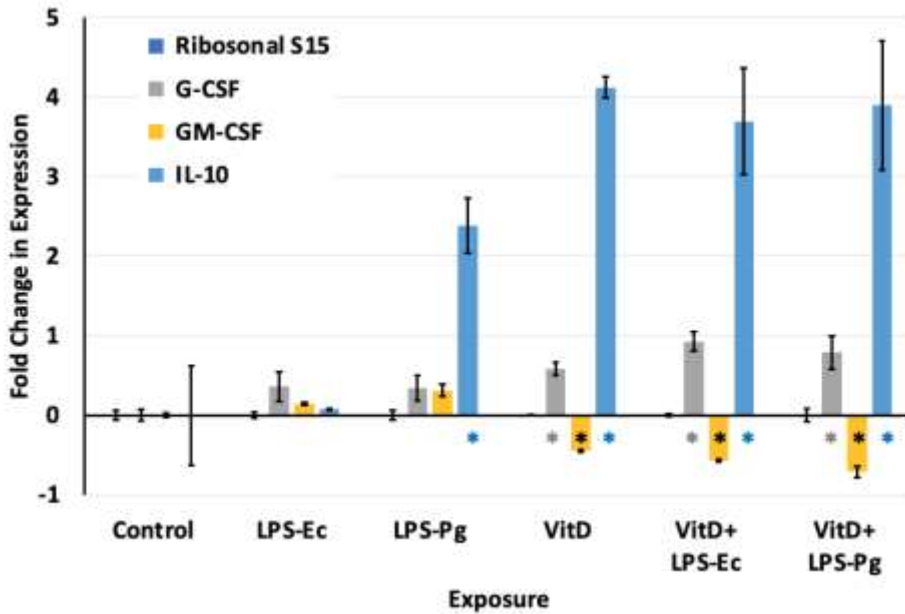
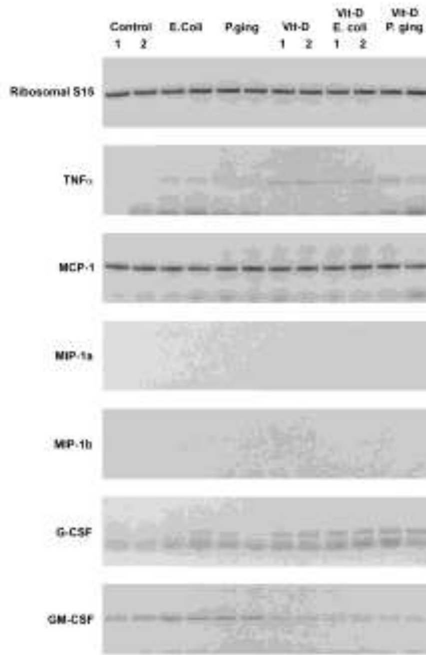


Figure 6: Gingival Fibroblast Cytokine Transcript Expression
Gingival fibroblasts were exposed to LPS, vitamin D, and vitamin D plus LPS for 24 hours. Transcript expression analysis for cytokine formation using RT-PCR (A). (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls (P<0.05).

Figure 7.

A.



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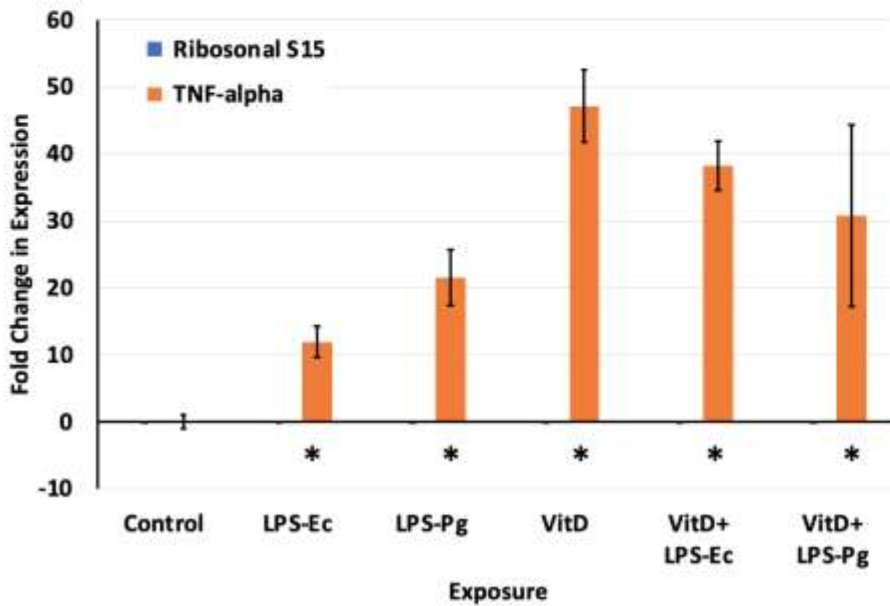
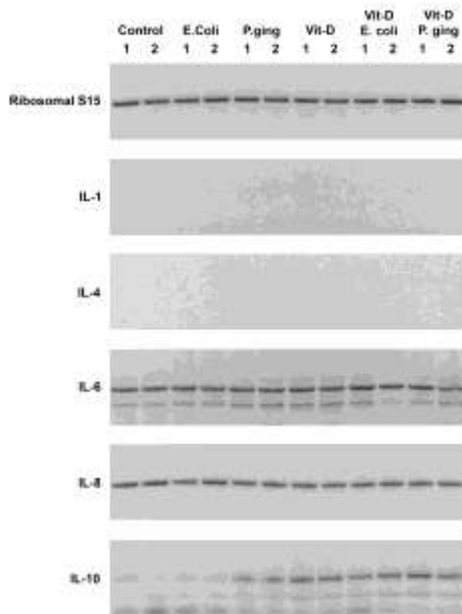


Figure 7: Gingival Fibroblast TNF-alpha Transcript Expression
Gingival fibroblasts were exposed to LPS, vitamin D, and vitamin D plus LPS for 24 hours. Transcript expression analysis for TNF-alpha formation using RT-PCR (A). (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

Figure 8.

A.



B.

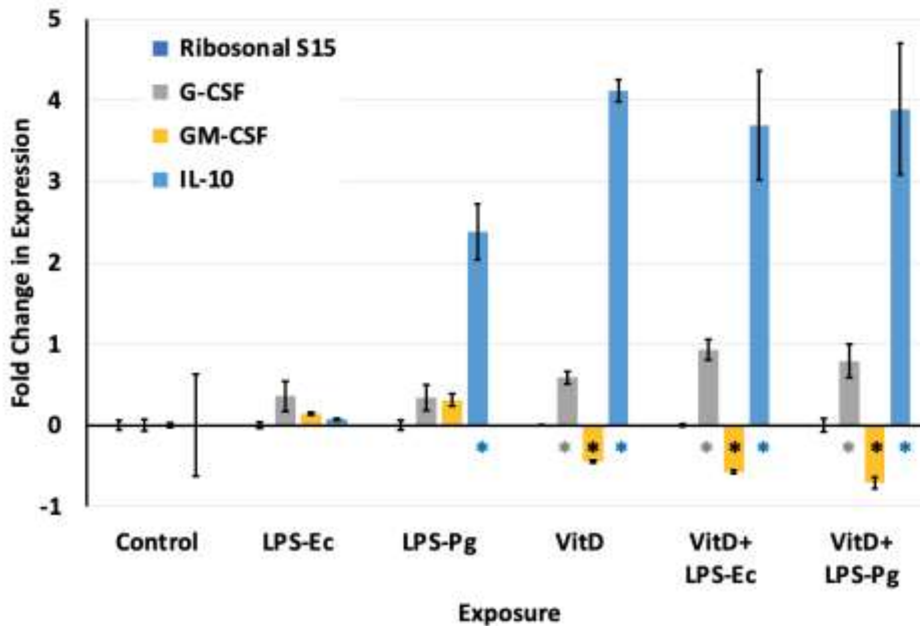


Figure 8: Gingival Fibroblast Interleukin Transcript Expression
Gingival fibroblasts were exposed to LPS, vitamin D, and vitamin D plus LPS for 24 hours. Transcript expression analysis for interleukin formation using RT-PCR (A). (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

Figure 9.

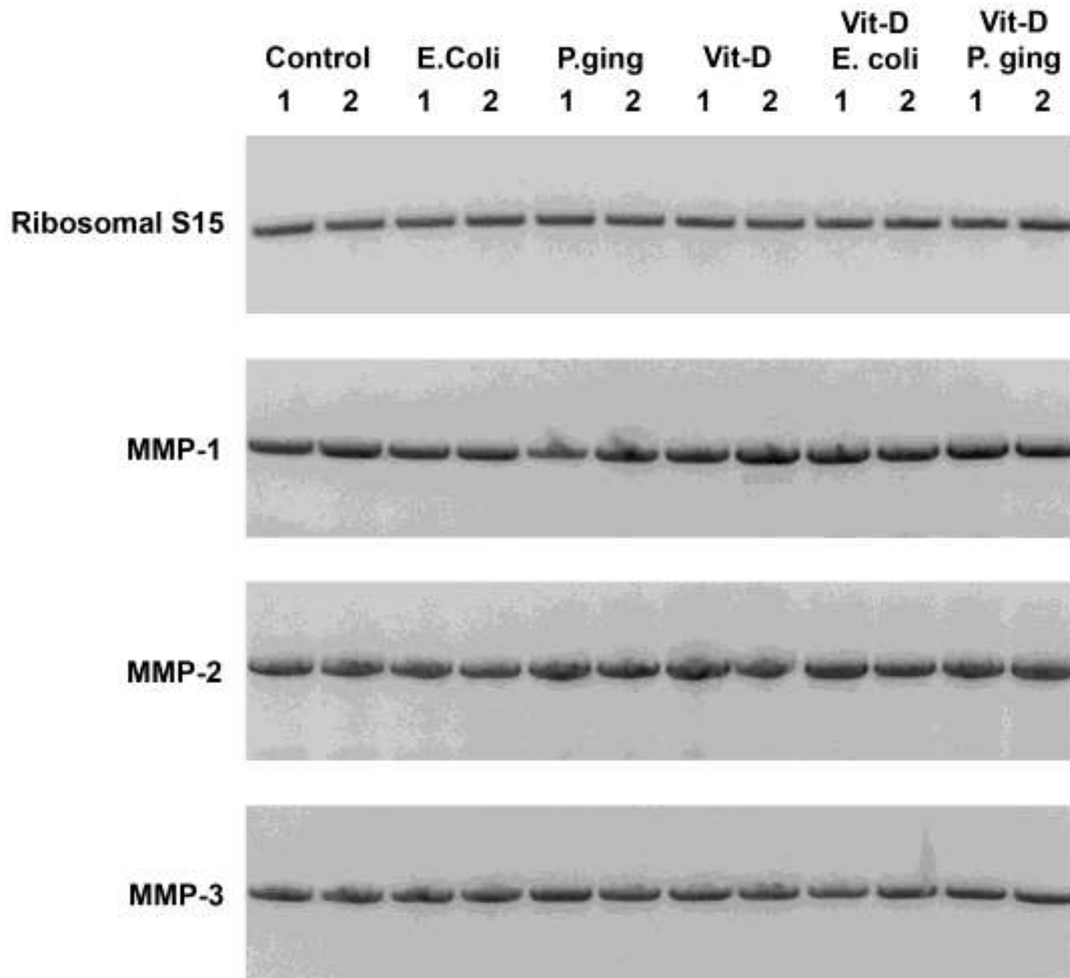


Figure 8: Gingival Fibroblast Interleukin Transcript Expression
Gingival fibroblasts were exposed to LPS, vitamin D, and vitamin D plus LPS for 24 hours. Transcript expression analysis for matrix metalloproteinase formation using RT-PCR.

Discussion

Summary of Data

This study observed that a 24-hour exposure of E. coli LPS 10 µg/ml, P. gingivalis LPS 10 µg/ml, and vitamin D 100 nM to gingival fibroblasts had no impact on cell survival. When analyzing transcript expression, gingival fibroblasts exposed to vitamin D displayed a significant increase in collagen and bone-forming transcripts. However, vitamin D exposure resulted in a mixed effect on pro-inflammatory and anti-inflammatory cytokine expression.

Cell Survival

Vitamin D, an important fat-soluble steroid, plays a vital role in calcium homeostasis and the immune system (Stein 2014). In the present study, 24-hour exposure of gingival fibroblasts to vitamin D concentrations less than or equal to 100 nM did not result in a decrease in cell viability. This result was in agreement with two previous reports on gingival epithelial cells (Menzel et al. and Elenkova et al Cytotoxic effects of vitamin D may depend on concentration, time of exposure, and the type of cell. Clinical and epidemiological studies have reported that vitamin D is important for bone homeostasis and immune system regulations, but there is currently little information on the effects of vitamin D on the in vitro properties of gingival fibroblasts (Garcia 2011, Bhatavadekar 2009, Perayil 2015, & Miley 2009).

Collagen-Forming Transcripts

Collagen fibers are components of the extracellular matrix, which support tissues and cell structures in various connective tissues throughout the periodontium (Page 1976). This study analyzed the following collagens: Collagen-1, 3, 5, 6, 8, and 10. The periodontium is primarily made up of Collagen 1, 3, and 5, the remaining collagens are present in the periodontium, but are minor collagens (Fujita 2018). Gingival fibroblasts in this study displayed a significant increase in Collagen-3, 5, 6, and 10 when exposed to vitamin D. The increase in major collagen types 3 and 5 may suggest that vitamin D enhances the state of the periodontal ligament. The increase in collagen 6 may be indicative of an increase in oxytalan fibers and in

the maintenance of the integrity and elasticity of the extracellular matrix. Collagen 10 increase may suggest that cartilage formation is being promoted, which could directly result in bone formation (Fujita 2018).

Bone-Forming Transcripts

Periodontitis is a disease that results in the destruction of the periodontium, and particularly the alveolar bone that supports the teeth (Page 1976 & Kornman 1997). Vitamin D has known impacts on bone homeostasis (Gao 2018 & Stein 2014). Therefore, the effects of vitamin D exposure on bone-forming transcripts were analyzed. A significant increase in alkaline phosphatase, periostin, and osteomodulin was observed in gingival fibroblasts that were exposed to vitamin D. The increase in alkaline phosphatase was nearly 30-fold, suggesting new bone production to be a likely occurrence. The increase in periostin suggest that vitamin D can promote epithelial cell adhesion and migration, which is vital for tissue regeneration (Christako 2013). Osteomodulin displays hydroxyapatite-binding capacity and is involved in governing the shape of collagen fibrils, which positively affected by vitamin D. All cell in this study expressed osteoprotegerin at relatively similar levels suggesting LPS and vitamin D did not promote bone resorption. Overall, vitamin D appeared to promote the formation of new bone.

Matrix Metalloproteinase Transcripts

The breakdown of collagen is directly attributed to the enzymatic activity of matrix metalloproteinases (Hajishengallis 2017). This study observed for all exposure there was no change in the expression of any matrix metalloproteinases analyzed. The exposure of gingival fibroblasts to vitamin D or LPS did not increase or decrease the collagen destruction attributed to matrix metalloproteinases.

Cytokine Transcription

Vitamin D has previously been reported to possess anti-inflammatory properties (Jeffery 2009). Specifically, vitamin D has been shown to decrease the production of pro-inflammatory cytokines (IL-1, IL-6, and Il-8) by gingival fibroblasts (Menzel 2019, Nakashyan 2017, & Elenkova 2019). In a study by Menzel et al., the topical application of vitamin D in vivo was observed to inhibit a localized inflammatory response. This supports the hypothesis that the presence of vitamin D leads to an anti-inflammatory state. This current study found that all cells expressed

IL-6, IL-8, and MCP-1 at constant levels independent of exposure to LPS and/or vitamin D. This finding contradicted several studies that reported a reduction of IL-6 and IL-8 by cells exposed to vitamin D (Menzel 2019, Nakashyan 2017, & Elenkova 2019). A significant increase in IL-10 production was observed in all cells exposed to vitamin D this may be indicative of vitamin D's anti-inflammatory properties. IL-10 is an anti-inflammatory cytokine that is involved in downregulating the expression of T helper cells cytokines and can block macrophage stimulation (Garlet 2010). A significant reduction in the production of GM-CSF was also observed in all cells exposed to vitamin D. This suggests that vitamin D exposure resulted a reduction in the production of a pro-inflammatory cytokine (GM-CSF). GM-CSF has been observed to directly stimulate stem cells to produce granulocytes and monocytes (Jeffery 2009). However, a significant increase of TNF-α and G-CSF production was observed by cells exposed to vitamin D. Both cytokines are pro-inflammatory, TNF-α is produced by macrophages during episodes of acute inflammation and results in apoptosis (White 2008) and G-CSF stimulates the bone marrow to produce more leukocytes (Garlet 2010). This finding seems to suggest that vitamin D may also promote a pro-inflammatory state.

Conclusion: Vitamin D positively impacts collagen and bone formation expression in gingival fibroblasts. However, vitamin D has a mixed effect on the expression of pro- and anti-inflammatory cytokines by gingival fibroblasts.

Conflicts of Interest: There are no conflicts of interest to report for this study.

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