The Effect of Oil Pulling on Inflammatory Factors in Gingival Fibroblasts In vitro.

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Word Count: 3047

Figures/Tables: 5

References: 34

Title: The Effect of Oil Pulling on Inflammatory Factors in Gingival Fibroblasts In vitro.

Summary: Oil pulling induces inflammation on gingival fibroblasts.

Key Words: Plant Oils, Coconut Oil, Oil pulling, Transcripts, Inflammation, Cytotoxicity.

The Effect of Oil Pulling on Inflammatory Factors in Gingival Fibroblasts In vitro.

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Abstract

Introduction: The rise of health advice from online sources and social media have increased patients access to unconventional health practices from around the world. For oral diseases such as periodontitis, ancient methods of treatment such as oil pulling have been discussed for over 3000 years. Currently, there is a lack of well-studied information on the benefits of oil pulling; therefore, the purpose of this study is to examine the anti-inflammatory effects of oil pulling on gingival fibroblasts.

Methods: Fibroblasts were exposed to 100% coconut, sesame, mineral, and vegetable oils for times ranging from 3 minutes to 24 hours and incubated for 24 hours. Cell survival was assayed using calcein-acetyoxymethyl ester for 1 hour, rinsed, and read using a microplate reader. Fibroblasts exposed to 100% oils for 1 hour were analyzed for expression of inflammatory cytokine, matrixmetalloproteinase, and collagen and bone forming transcripts via RT-PCR.

Results: After 3 minutes exposure, gingival fibroblasts displayed nearly 100% survival in coconut oil, compared to only 41% in sesame oil. Coconut oil exposure for 1 hour increased IL-8 expression, while exposure to mineral and vegetable oil increased both IL-6 and IL-8. Coconut oil (as well as mineral and vegetable oils) also decrease alkaline phosphatase and osteomodulin expression, while increasing MMP1 and 3 expression.

Conclusion: Sesame oil was significantly more cytotoxic than coconut oil. However, coconut oil had no specific beneficial effects on gingival transcript expression involved with inflammation, or bone and soft tissue formation.

Introduction:

Periodontal disease is a multifactorial disease of the hard and soft tissues that surround the dentition. It is the 6th most prevalent disease worldwide and is the leading cause of tooth loss in adults. Periodontal disease can increase a patient's risk for edentulism and nutritional deficiencies as well as contributing to other systemic problems. (Tonetti, 2017) Periodontal disease is often unknown to the general population until dental problems arise which lead them to a dental office for diagnosis. (Jin, 2015) After being diagnosed with a disease such as periodontitis, patients often turn to the internet to find more information on etiology, treatment, and prognosis of the disease. In 2009 approximately 61% of adults look online for health information. 41% of those patients relied on information written by a non-healthcare provider from the internet. (Fox, 2009) Since 2009, the number of patients looking for answers to dental issues such as periodontitis on the internet has increased, and social media has given everyone the platform to share opinions, regardless of their level of expertise. One such topic that has become increasingly popular on the internet is oil pulling. Proponents of oil pulling claim that it can reverse decay, whiten teeth, prevent gum disease, and provide other dental and systemic benefits.

Oil pulling can be traced back to the Ayurveda, a holistic system of medicine which evolved in India 3000-5000 years ago but is now practiced in other parts of the world as a form of supplementary medicine. Dentistry was not a specialized branch of Ayurveda, but it was a system of surgery and was able to treat issues such as deformities, plaque, and infections in the oral cavity. Oil pulling is discussed in the text Charaka Samhita and claims to cure systemic diseases from headaches to diabetes. It has been used as a traditional Indian folk remedy to treat decay, bad breath, bleeding gums, dry throat, cracked lips, while strengthening teeth, gingiva, and the jaws. (Singh, 2011) Specific protocols for oil pulling vary greatly on the type of oil, amount of oil used, time spent pulling as well as hygiene before and after the procedure. Time frames anywhere from 3-20 minutes have been described (Gbingie 2016, Hebbar 2010, Singh 2011, Tomar 2014), on an empty stomach after normal hygiene has been completed three times per day. It is hypothesized that oil pulling acts by drawing toxins out of the blood

through the oral mucosa and tongue or by damaging the cell walls of microorganisms directly. (Gbingie 2016, Hebbar 2010, Shanbhag 2016) A study on oil pulling found a statistically significant difference in plaque scores and gingival scores after 15 days of oil pulling. However, it was mentioned that due to the study design that the Hawthorne Effect could have influenced the results of the study. (Amith, 2007) Sesame seed oil, a probiotic mouthwash, and chlorhexidine were compared and found reduced plaque and gingival scores by the end of the 30-day study but no difference between any of the groups studied. (Kandaswamy, 2018) Several systematic reviews have been done on the practice of oil pulling, and their results have all been very similar. Limited available research was found on the effects of oil pulling with some possible promising benefits on the oral cavity but overall, not a replacement for dental therapy. (Gbingie 2016, Lakshmi 2013, Mythri 2017, Shanbhag 2016) All reviews of this topic have unanimously stated that the mechanism for a beneficial effect of oil pulling remains unknown, and there is a need for more studies to demonstrate its effect on the oral cavity. Many of the clinical studies on oil pulling focus on young patients with an experimental design that does not isolate oil pulling benefits from those achieved by oral hygiene instruction and patient education.

There is currently a lack of evidence showing the cellular effects of oil pulling in dental therapy. The aim of this study is to examine and compare the cytotoxic effects and anti-inflammatory effects of coconut oil, mineral oil, vegetable oil, and sesame oil on human gingival fibroblasts.

Materials and Methods:

Cells: Gingival fibroblasts isolated and established previously from a patient with healthy gingiva who underwent oral surgery at the Louisiana State University School of Dentistry, were used. The cells were washed and maintained in minimum essential medium alpha (MEMα) containing 10% fetal calf serum (FCS), 200 units/mL of penicillin and 200 µg/mL streptomycin (GIBCO Grand Island, NY) to reduce bacterial contamination. This media was also used in the tissue culture assays. All gingival fibroblasts used in this study were between the 8th and 12th passages.

Oils: 100% organic, virgin, coconut oil (Nutiva, Richmond, CA); pure sesame oil (Kadoya, Shinagawa-ku, Japan); 100% mineral oil, white, light (Lot# 6358N32599, Mallinckrodt, Paris, KY), and vegetable oil (Crisco, Orrville, OH) were tested..

Defining an Oil Pulling Event:

Commonly, an oil pulling event is defined as the use of 1 tablespoon of oil, swished in the mouth for 3-5 minutes. (???) For this study, we found that 1 tablespoon of oil was ~12 mL, and the resulting oil/saliva mixture in the mouth after 3 minutes was 18 mL (~66% oil at the termination of the event). Notably, when using a semi-solid oil (e.g., coconut oil) the solution was not completely liquified in the oral cavity until after 2 minutes.

Cell Survival: Fibroblasts were plated onto 48-well plates at 4,000 cells/well. 100% oils were added to wells for various times. Cell were incubated at 37°C for 3 minutes to 24 hours. Oils were removed by aspiration and replaced with MEMα. Cells were incubated for up to 24 hours prior to assessment for 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.

RNA Isolation: Fibroblasts (passage 7) were exposed to 100% oil for 1 hour. Oil 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) for Cytokines: RT-PCR was completed on gingival fibroblasts to establish a baseline. RT-PCR will be used to assess transcript expression of cytokines, collagens, and transcripts involved with bone formation. cDNAs will be made using MMLV-RT and the Retroscript Kit (Invitrogen) as per the manufacturer's instructions. Briefly, 1g of total RNA will be reverse-transcribed using 100 units of MMLV-RT in 20 μl reactions. 2 μl of each reaction will be used as template in 25 μl PCR, using 50 pmoles of primers, 250 μM of dNTPs, and 1 mM Mg^{2+}. PCR reactions will be 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 will be 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 will be chosen to be within the linear range of detection for all the cytokines examined. Statistical test will be used to determine the amount of change seen before and after cells were exposed to coconut oil.

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

Results:

Effects of Oil Exposure on Cell Survival: The end of a 3-minute oil pulling event results in a diluted oil by 33%. . Initial experiments examining oil cytotoxicity at concentrations below 10%-100% revealed no noticeable cell death after a 24-hour exposure (data not shown). Therefore, in order to reduce variability, all subsequent experiments were preformed using 100% oils. Gingival fibroblasts exposed to 100% oils displayed differential cell survival (Figure 1). Fibroblasts exposed to mineral, coconut, and vegetable oils showed high survival rates throughout a 24 hour exposure time with significant differences between the oils first starting at 30 minutes to 1 hour. Sesame oil, however, showed a significant decrease in cell survival at 3 minutes that continued to decrease throughout the experimental window. After 24 hours exposure, gingival fibroblast displayed 76% survival in mineral oil, 47% survival in coconut oil, 23% in vegetable oil, and 3% survival in sesame oil. Thus, of the commonly available oils to most consumers, coconut oil was the least toxic to cells.

Effects of Oil Exposure on Transcript Expression:

Coconut, mineral or vegetable oils were tested for changes in expression for specific cytokines, osteogenic transcripts and collagens

Cytokines: IL-1B and TNF-α were not expressed following exposure to coconut, mineral or vegetable oils. IL-6 had no change in expression when cells were exposed to coconut oil, but a statistically significant increase in IL-6 expression was seen for cells exposed to both mineral and vegetable oils. IL-8 had an increase in expression for cells exposed to coconut oil, mineral oil, and vegetable oil. (Figures 2a and 2b)

Osteogenic Transcripts: Alkaline Phosphatase and Osteomodulin expression was significantly decreased when exposed to coconut oil, mineral oil, and vegetable oil. Osteoprotegerin was expressed, but there was no change in gene expression for cells exposed to all oils tested. RANKL was not expressed following exposure to coconut, mineral or vegetable oils. Osteonidogen expression was unchanged in cells exposed to coconut oil, but a significant increase in expression was seen in cells exposed to mineral and vegetable oil. Periostin

expression was not significantly increased for coconut oil, but was significantly increased for mineral and vegetable oil. (Figures 3a and 3b)

Collagens: Collagen 1 and Collagen 5 expression was unchanged when exposed to all oils tested. Collagen 3 expression was unchanged in cells exposed to coconut oil and vegetable oil, but was significantly increased for cells exposed to mineral oil. Collagen 6 and collagen 10 expression were significantly increased in cells exposed to all oils tested. Collagen 8 expression was unchanged in cells exposed to coconut oil, but was increased in cells exposed to mineral and vegetable oil.

MMPs: MMP1 and 3 expression was significantly increased for all oils tested; however, MMP2 expression did not exhibit significant differences after exposure to any oil.

Figure 1. Effects of Oil on Gingival Fibroblast Cell Survival

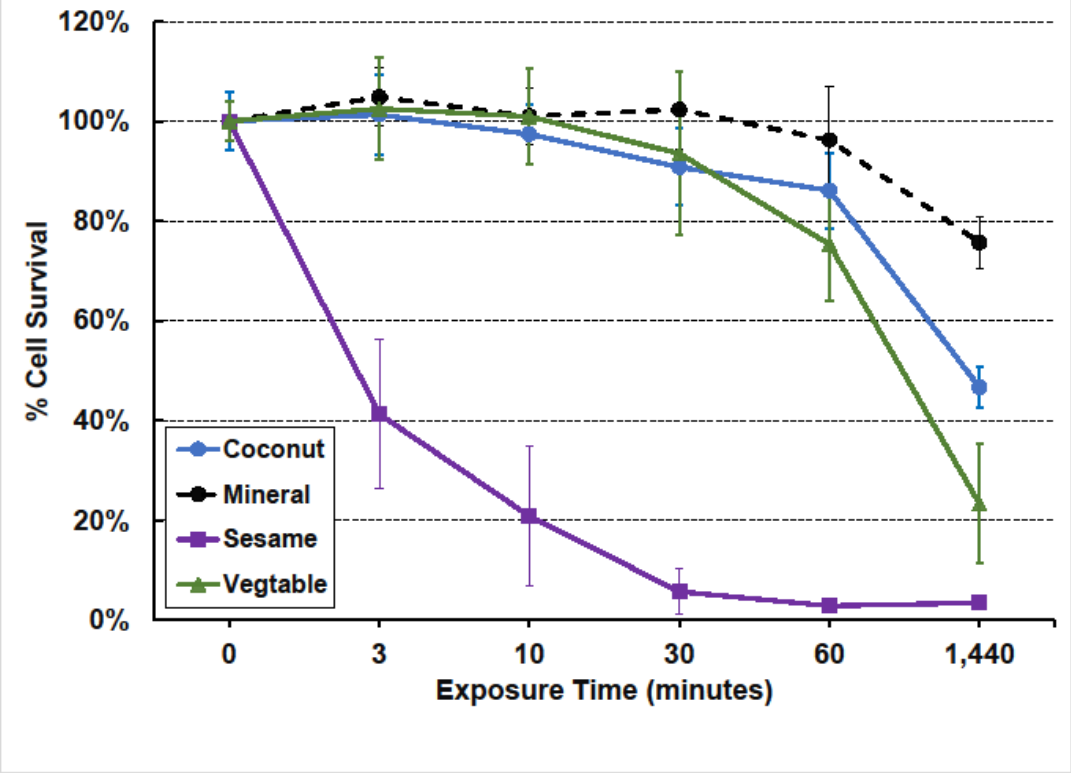

Figure 1: Survival of gingival fibroblasts exposed to 100% Oils for various times. Oils examined were coconut oil, mineral oil, sesame oil, and vegetable oil. Cell were exposed for 0, 3, 10, 30,

60 and 1440 minutes. Symbols represent the mean and standard deviation of 8 samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

Figure 2

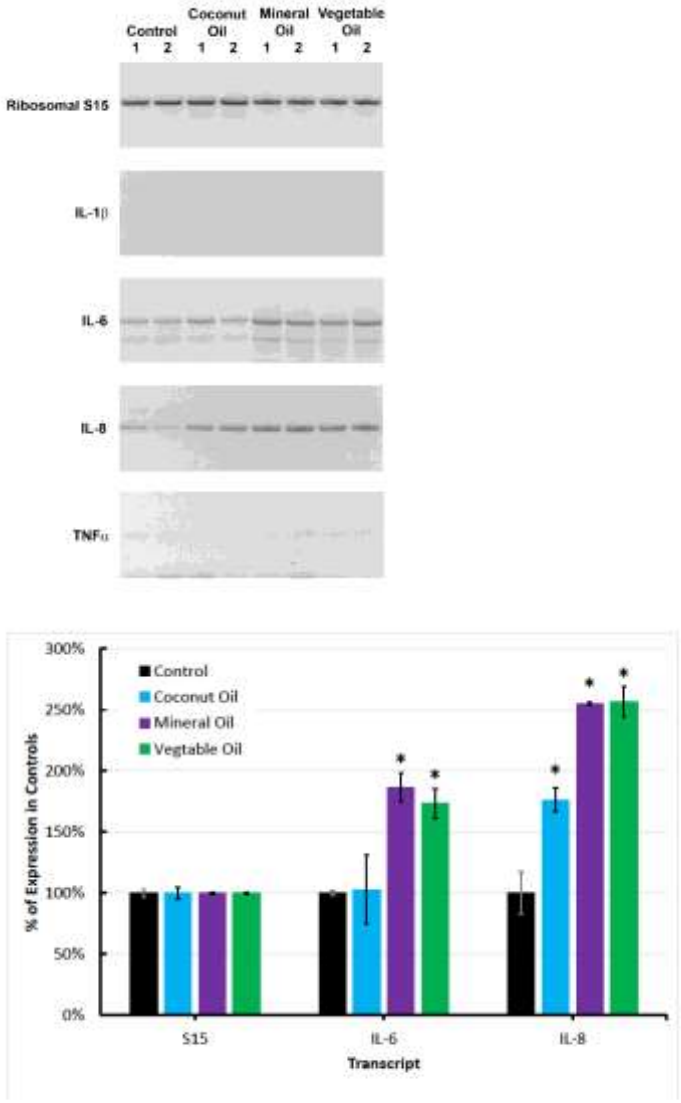

Figure 2: Transcript expression analysis for cytokines using RT-PCR (A). Gingival fibroblasts were exposed to coconut oil, mineral oil, and vegetable oil for 24 hours. (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

Figure 3

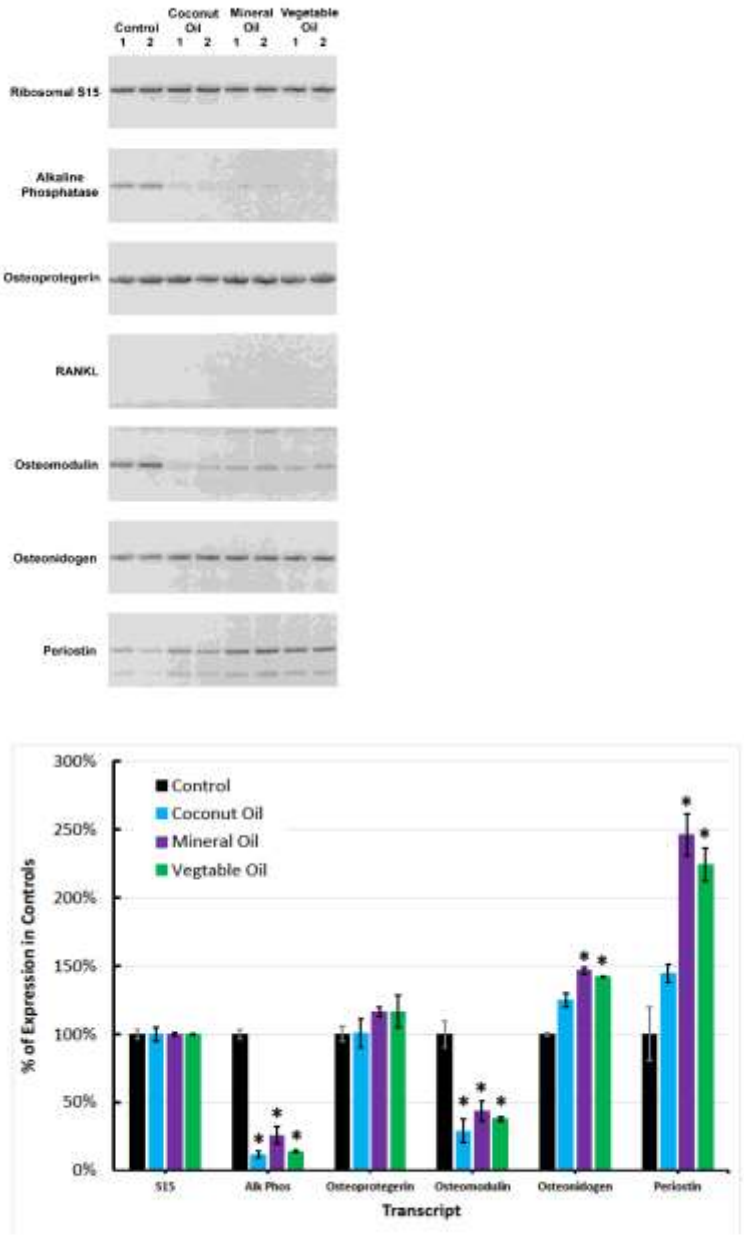

Figure 3: Transcript expression analysis for bone formation using RT-PCR (A). Gingival fibroblasts were exposed to coconut oil, mineral oil, and vegetable oil for 24 hours. (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

Figure 4

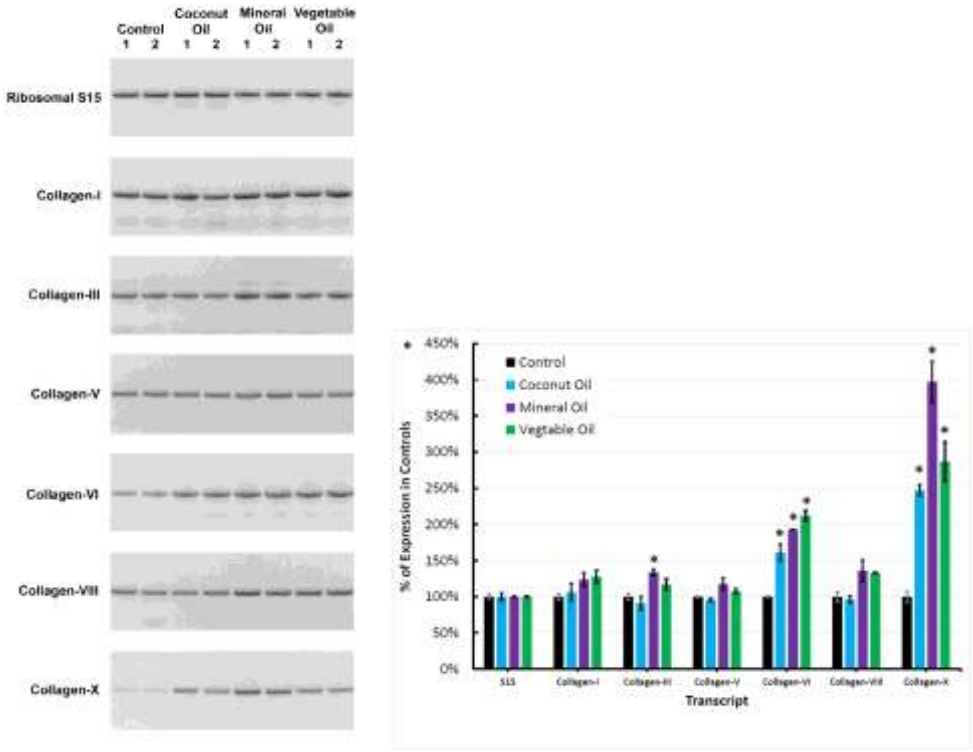

Figure 4: Transcript expression analysis for collagen using RT-PCR (A). Gingival fibroblasts were exposed to coconut oil, mineral oil, and vegetable oil for 24 hours. (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

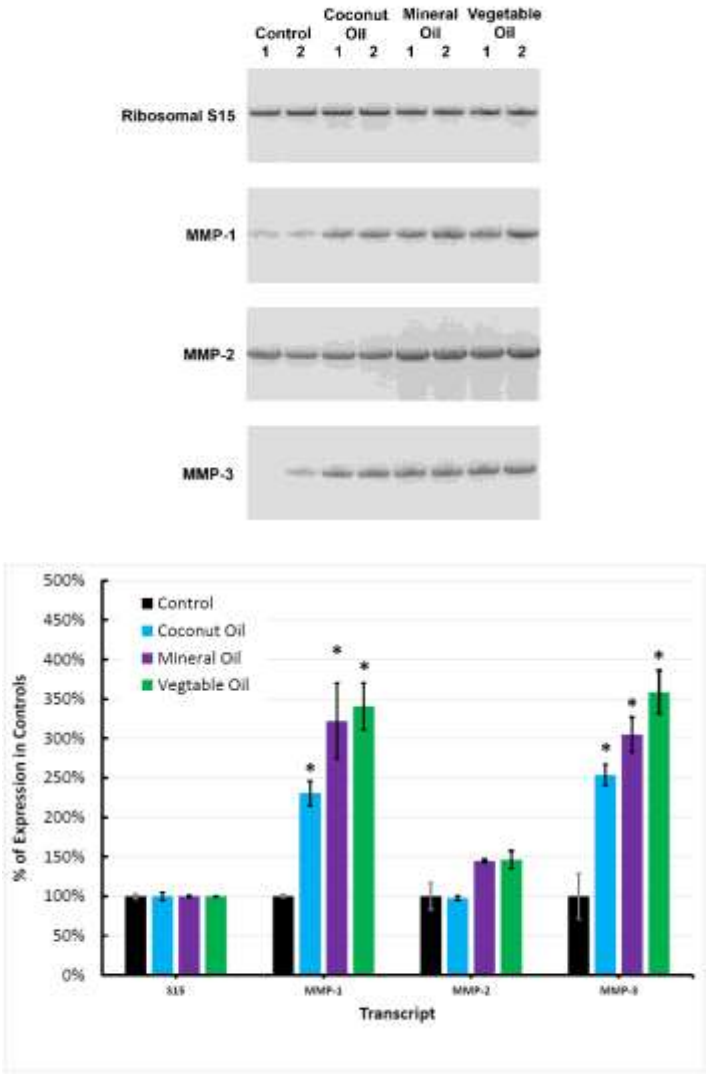

Figure 5 Transcript expression analysis for matrix metalloproteinsase (MMPs) using RT-PCR (A). Gingival fibroblasts were exposed to coconut oil, mineral oil, and vegetable oil for 24 hours. (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks(*) represent statistically significant differences from controls ($P<0.05$).

Discussion

Summary of Data

Our study found that a 3-minute exposure of sesame oil to gingival fibroblasts had a significant decrease in cell survival and was significantly more cytotoxic than the other oils examined. For transcript expression, cell exposure to oils had a differential and specific effect that increased expression of pro-inflammatory cytokines, and downregulating transcripts responsible for osteoclasts and regulators of bone formation. Increased expression for collagens was limited to those primarily associated with basement membrane and the dominant types found in periodontal tissues were unchanged. . Additionally, there was an increase in collagenase expression.

Defining an oil pulling event

The procedure of oil pulling has been described by different authors with varying instructions. However, in general, oil pulling can be described as taking about a tablespoon of oil into the mouth, and swishing anywhere from 3-20 minutes while pulling the oil between the teeth and around the mouth.(Lakshmi 2013, Shanbhag 2016, Singh 2011) An oil pulling event can be done three times per day and should be done in the sitting position with the chin up and on an empty stomach before drinking any liquids. (Shanbhag 2016) After the event has been completed, the oil will become thin and milky, which should be expectorated and rinsed with warm salt water. (Hebbar 2010, Tomar 2014) In our study, we standardized an oil pulling event as a tablespoon (~12mL) of oil, swished in the mouth for 3 minutes. Over 2 minutes were needed to completely emulsify coconut oil, and at the end of the oil pulling event a dilution of 33% was recorded.

Cell Survival

Oil pulling can be performed with many different oils but coconut and sesame oils are two very commonly available products in households across the world. Sesame oil contains a high concentration of polysaturated fatty acids and contains lignans such as sesamin,

sesamolin, and sesaminol that act as antioxidants to reduce free radial injury. In animal models, they have shown to increasing plasma gamma-tocopherol which enhances the activity of vitamin E. (Cooney 2001, Hebbar 2010, Tomar 2014) The composition of coconut oil is a medium chain fatty acid, whereas most other oils are composed of long chains. Coconut oil contains 92-96% saturated acids, 46-48% of which is lauric acid. (Pehowick 2000, Marina 2009)

Sesame oil had the highest cytotoxic effect on cells of oils in our study, with coconut oil showing a cell survival rate lower than that of mineral oils but higher than vegetable oil. Our study found that a 3-minute exposure of gingival fibroblasts to sesame oil significantly decreased cell survival when compared to coconut, mineral, and vegetable oils. A significant change between coconut, mineral, and vegetable oils were only apparent after exposure for 1-hour, with the highest cell survival occurring with mineral oils. Our findings are contradictory to previous studies which showed a beneficial effect of sesame oil on periodontal conditions (Asokan et al 2009, Kaliamoorthy et al 2018, Kandaswamy et al 2018). However, our study trends were more supportive of a study that reported greater gingival health when oil pulling with coconut oil compared to sesame oil. (Kaliamoorthy 2018)

Cytokine Transcription

Coconut oil has been reported to have potential anti-inflammatory properties. Previous research has shown that coconut oil is protective against oxidative stress from physiological oxidants (Nevin 2007), inhibits both T and B cell function at high levels which may increase IL-2 production (Witcher 1996), and reduces some stages of acute and chronic phases of the inflammatory pathway when used in high doses. (Intahphuak) In immunosuppressed mice fed with coconut protein, there was an increase RBC, WBC, platelets, neutrophils, monocytes, and eosinophils compared to immunosuppressed mice who were fed a normal diet. (Viglia 2008) Human studies on oil pulling involving sesame oil (Kaliamoorthy 2018, Kandaswamy 2018), sunflower oil, (Amith 2007) and coconut oil (Kaliamoorthy 2018, Peedikayil 2015) saw a reduction in both plaque and gingival indices. However, since these studies required the subjects to brush their teeth prior to oil pulling, their improved gingival indices may be attributed to improvement in their oral hygiene regardless of the oil pulling procedure.

Our study found that all oils stimulated the expression of IL-8, a chemoattractant cytokine produced by tissues and cells has a specific target for activating neutrophils in inflammatory regions. However IL-6 expression was unaltered after exposure to coconut oil but increased expression when exposed to vegetable and mineral oils. IL-6 is a proinflammatory cytokine involved in all aspects of the immune response including synthesis of acute phase proteins, neutrophil infiltration, and shaping of the T cell response against viral, parasitic, fungal, and bacterial infections. (Rose-John 2017). Our results involving cytokine expression do not agree with other studies that concluded that oil pulling improved gingival indices by reducing cytokine expression.

Bone Forming Transcripts

Since periodontitis involves the destruction of alveolar bone and other supporting structures, the effects of oil pulling on the expression of several transcripts associated with bone remodeling were examined.

Our study found a decrease in Alkaline Phosphatase and Osteomodulin after exposure to all oils, suggesting a potential decreased in production of new bone. There was no reduction in the expression of Osteoprotegerin nor increase in the expression of RANKL when exposed to any oil. This suggests that these cells are predominantly in an osteoclast suppressive state that is not affected by oil exposure. Osteonidogen and Periostin were both increased by exposure to mineral and vegetable oils, but no significance change in expression was detected after exposure to coconut oils. Transcripts highly involved in bone maintenance such as osteoprotegerin, RANKL were either unexpressed or unchanged when exposed to oils. Transcripts involved in minor aspects of bone maintenance such as mineralizing new bone (Lowe 2021, Lin 2021) and stabilizing collagen precursors to bone (Kohfeldt 1998, Naylor 2015) were differentially increased or unchanged for different oils.

Thus, exposure to oils have differential effects on bone remodeling, suggesting that there is an overall down regulation in new bone formation, but may have greater potential to form non-mineralized gingival tissues.

Collagen Forming Transcripts

Collagen is the predominant connective tissue protein in both mineralized and soft connective tissues. The trascripts examined in this study include Collagen 1, 3 and 5 which are dominant collagen types found in the supporting periodontal tissues (Boraschi 2017, Kuivaniemi 2019, Wenstrup 2004). The other collagen types examined are involved in basement membrane maintenance (Sun 2016), angiogenesis (Shuttleworth 1997), and establishment of a chondro-ossoeus junction (Ricard-Blum 2011) that are relevant in the normal turnover of periodontal structures. Our study found that exposure to coconut, mineral and vegetable oils significantly increased Collagen 6 and 10. Additionally, mineral oil significantly increased the expression Collagen 3.

Overall there were minimal effects on major collagen types that are associated with periodontal tissues. The increase in collagen 6 that we observed may indicate an increased prevalence for connective tissue attachment of basement membrane of the epithelia, while an increase in collagen 10 observed may indicate a shift promoting endochondral bone formation.

Matrix Metalloproteinase Transcripts

Matrix metalloproteinases are enzymes involved in collagen degradation. Our study found that exposure to all oils significantly increase the expression of MMP-1 and MMP-3 which are heavily involved in collagen degradation of major collagens found in periodontal tissues (Ricard 2011) as well as aiding in cell mediated inflammatory pathways such as periodontitis (Laronha)

Thus, exposure to oils has the potential to promote collagen destruction in gingival tissues. Combined with our observation of little change i the expression of important collagen types following oil exposure, this suggests the potential for a net loss of collagen in these tissues.

Conclusion: Sesame oil had the most cytotoxic effects on gingival fibroblasts after only 3 minutes, while coconut oil was significantly less toxic after exposures of 60 minutes. However,

oil pulling with coconut oil increased a pro-inflammatory cytokine expression (IL-8), while reducing bone promotion transcripts (Alkaline Phosphatase and Osteomodulin), and enhancing collagen destroying enzymes (MMP1 and MMP-3). Thus, overall there were no beneficial effects on gingival fibroblasts in vitro for any of the oils used.

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

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