Comparison of Zinc Based Mouth Rinses to Chlorhexidine and Essential Oil Mouth Rinses

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Title: Zinc-Based Mouth rinses

Summary: Zinc-based rinse, Zinc-C, is a more cytotoxic and greater reduction in cell motility than Chlorhexidine

Key Words: Chlorhexidine, Inflammation, Cytotoxicity, Cell Motility

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Abstract

Background: There are a variety of mouth rinses for consumers to choose from on the market. In particular, zinc-based mouth rinses use a zinc ion solution. One zinc-based mouth rinse, Zinc-C[§], claims to be a substitute for the prescription mouth wash, Chlorhexidine (CHX). Chlorhexidine has been known to cause negative side effects, including tooth staining and taste alteration. Zinc-C[§] claims it does not have such effects. The purpose of this study is to determine the anti-inflammatory effects of Zinc-C by evaluating the change in cytokine expression of gingival fibroblasts when patients are exposed to different types of mouth rinses.

Methods: Gingival fibroblasts were exposed to 5% of each mouth EO*, CHX[‖], Zinc-based mouth rinses, Zinc-A[†], Zinc-B[‡], and Zinc-C[§] for exposure times of 3 mins. Cell survival was assayed using calcein-acetoxymethyl ester for 1 hour, rinsed, and read using a microplate reader. Gingival fibroblasts were then exposed to LPS for 24 hours followed by a 3-minute exposure to mouth rinses and analyzed for expression of inflammatory cytokine, Interleukins, and growth factors via RT-PCR. Cell Motility was then analyzed every 24 hours for 3 days after 3 minutes exposure times.

Results: Zinc-A and Zinc-B were no more toxic than essential oil mouth rinses, whereas Zinc-C was more cytotoxic than CHX. Zinc-C selectively increased BMP-2, GDF-2, and CTGF. By the third day, Zinc-C significantly decreased cell motility with only 62% wound closure.

Conclusion: Zinc mouth rinses displayed similar anti-inflammatory effects to essential oil and chlorhexidine. In addition, Zinc-C was more cytotoxic and inhibited cell motility to a greater extent than any of the other mouth rinse. Therefore, Zinc-C as an alternative to CHX may not be recommended after oral surgery

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Introduction:

Since the late 1800s, consumers have used mouth rinses because of their potential beneficial effects, including dry mouth relief, bad breath reduction, and their antiseptic, anti-inflammatory, anti-fungal, anti-plaque, and fluoridation properties. There are a variety of mouth rinses for consumers to choose from on the market, with each rinse advertised as having certain advantageous effects. In general, mouth rinse users are instructed to swish the mouth rinse around their oral cavity for thirty seconds without swallowing. While mouth rinses are beneficial, they are not considered a replacement for brushing but used in conjunction.

An essential oil (EO) * mouth rinse is sold over the counter and used by patients to treat gingival inflammation and gum disease. The active ingredients in an EO Mouth rinse are menthol, thymol, methyl salicylate, and eucalyptol. Several studies have tested the effectiveness of essential oil mouth rinses. A meta-analysis study found improvements in gingivitis and plaque after 6 months of using EO mouth rinses (1) Like essential oil rinses, Zinc based rinses are commercially available over the counter to patients.

A new brand of zinc-based mouth rinse, with three different types of rinses, became available on the market: Zinc-A†, Zinc-B‡, and Zinc-C§. Zinc ion solution inhibits sulfur production from bacteria in the mouth (2). Sulfur byproduct is correlated to malodor, which is often found in patients with chronic periodontal disease (3).

The Zinc-C rinse is advertised to have anti-plaque and anti-gingivitis properties, as well as reduce the presence of bad breath. Further, Zinc-C claims to be a substitute for the prescription Chlorhexidine‖ (CHX) mouth rinse. CHX is known to cause patients negative side effects including tooth staining and taste alteration (4). Importantly, Zinc-C does not have any of these side effects. Traditionally, Chlorhexidine is available at 0.12% concentration as a prescription mouth rinse that is prescribed for a maximum of 4-6 weeks.

Chlorhexidine mouth rinse is often used by periodontists as an antiseptic before and after surgery (5). Periodontists may also prescribe it to patients to prevent dental

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plaque formation and treat gingivitis (6). While the benefits of chlorhexidine outweigh its drawbacks, the negative side effects include teeth staining and an unpleasant taste, which has been shown to decrease patient compliance (7).

Currently, only a few clinical studies have been published on the effectiveness of Zinc mouth rinses. One study compared Zinc-based mouth rinses to other mouth rinses (2). The study analyzed the patients' gingival index (GI), bleeding on probing (BOP), plaque index (PI), and tooth staining. Although the Zinc rinses, Chlorhexidine (CHX), and placebo rinse all reportedly showed improvements in GI, BOP, PI, and tooth staining. However, the Zinc-C rinse was reported to have less alteration in taste and less tooth staining, and better compliance when compared to chlorhexidine (2). Another study analyzed the reduction of bad breath by several commercial mouth rinses, including Zinc rinses (8). This study found that Zinc rinses were effective in reducing malodor in subjects.

Since Zinc-C is a relatively new mouth rinse on the market, there is insufficient evidence proving the advertised benefits and effectiveness of using this mouth rinse. Additionally, there are no studies published on the effects of zinc-based mouth rinses on gingival fibroblasts compared to other mouth rinses on the market, such as chlorhexidine and essential oil mouth rinses.

Therefore, the purpose of this study was to evaluate, in vitro, the effects of zinc-based mouth rinses, on the cell survival, gene expression, and cell motility of human gingival fibroblasts after exposure, as a means to assess potential differences in cytotoxicity, wound healing, as well as determine the anti-inflammatory effects of Zinc-C by evaluating the change in cytokine expression of gingival fibroblasts.

Materials and Methods:

Cells isolation and Culture:

Gingival fibroblasts isolated and established previously from a patient with healthy gingiva who underwent oral surgery at the Louisiana State University School of

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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 15h passage.

Mouth Rinses:

The mouth rinses used for this study are commercially available mouth rinses EO*, CHX‖, Zinc-based Mouth rinses, Zinc-A†, Zinc-B‡, and Zinc-C§. Cells were exposed to concentrations of 0-25% for 3 mins

Cell Survival to Rinses:

Fibroblasts were plated onto 48-well plates at 4,000 cells/well. Dilutions of Mouth rinses were added to wells for a three-minute exposure. Dilutions of all smart mouth rinses were 5%, 10%, 15%, 20%, 25%. Rinses were removed by aspiration and replaced with MEMα. Cells were incubated for up to 24 hours before assessment for cell survival. Cell survival was assayed using calcein-acetoxymethyl ester (Molecular Probes, Eugene, Or) for 1 hour; cells were rinsed in PBS, and fluorescence was recorded using a Bio Tek Synergy2 fluorescent multi-well plate read appropriately for 480nm excitation and 520nm emission.

LPS and Rinse Exposure and RNA Isolation:

Fibroblasts were plated onto 48-well plates at 4,000 cells/well. Dilutions of LPS were added to wells for 24-hour exposure. Dilutions of all E-coli LPS were 1g/ml, 2g/ml, 5g/ml, 10g/ml. Rinses were removed by aspiration and replaced with MEMα. Dilutions of 5% Mouth rinses were added to wells for a three-minute exposure. Rinses were removed by aspiration and replaced with MEMα. Cells were incubated for up to 24

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hours. 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 the 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 the 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 a 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 30 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. Thirty (30) cycles of PCR will be chosen to be within the linear range of detection for all the cytokines examined. *A statistical* test will be used to determine the amount of change seen before and after cells were exposed.

Measuring Cell Motility

Cells were plated into 12-well tissue culture plates and allowed to adhere for 24 hours. Using a P-1000 pipette tip, a 1.5mm wound was made in each well. Cells were immediately treated with between 5%, and the width of the wound was measured at 24-hour intervals. Untreated cells served as control. For each time point, 8 measurements were made for each condition. Cells were treated with Calcein AM for 1 hour to aid in visualization. Cells were examined using a Nikon inverted TE2000 microscope

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equipped with a CoolSNAP cfi camera and Metamorph software. For quantitative analysis, 8 measurements were averaged. The mean and standard deviation were compared to the control values for untreated cells.

Statistics: Two Tail Students t-tests were used to statistically compare differences between samples. Significance was defined as $P<0.05$.

Results:

Effects of Mouth Rinse Exposure on Cell Survival:

A three-minute exposure of gingival fibroblasts to mouth rinses at different dilutions resulted in different levels of cell survival. Initial experiments examining mouth rinses at concentrations of 5% and 10% revealed complete cell death after a 24-hour exposure for all mouth rinses except EO (Figure 1). Gingival fibroblast exposed to 0-25% dilutions for 3 min exposures, found to have significant cell death in Zinc-C and CHX for concentrations 10% or greater (Figure 2.) Gingival fibroblasts exposed to 5% dilution of mouth rinses displayed greater cell survival for all mouth rinses (Figure 2). After a 3-minute exposure at 5%; gingival fibroblasts displayed 100% survival for EO, 100% for Chlorhexidine, 100% for Zinc-A, 100% for Zinc-B, and 91% for Zinc-C. Thus, the majority of available mouth rinses were not toxic to cells at a 5% concentration for 3-minute exposure.

Effects of Mouth Rinse Exposure on Transcript Expression:

Mouth rinses were tested for changes in the expression of specific interleukins, cytokines, and Growth Factors. Primer expression for IL-1B, IL-4, IL-10 and TNF-α, MIP-1b, G-CSF, and BMP-7 was not expressed following exposure to any of the mouth rinses or the control. Statistical significance changes in expression of MCP-1 and GM-CSF were both found to be decreased in all mouth rinses, however, there was no statistically significant change in GM-CSF in Zinc-B and MCP-1 in Zinc-C (Figure 3). For

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interleukins, IL-6 showed a statistically significant decrease in all mouth rinses (Figure 4). Growth factors were evaluated and found that BMP-2 showed a statistically significant increase in expression when exposed to Zinc-C (Figure 5). Growth factors GDF5, FGF-2, and CTGF showed a statistically significant decrease in expression in EO, CHX, and Zinc-A (Figure 5). GDF5 and CTGF showed a statistically significant decrease in expression in Zinc-B and Zinc-C mouth rinses (Figure 5).

Cell Motility

Cell motility was observed for 3 days in 24-hour intervals. It was found that at Day 0 there was no statistical difference in wound between any of the mouth rinse groups and the control. On Day 1, the percent wound closures were 73% control, 77 EO, 55% CHX, 59%, Zinc-A, 48% Zinc-B, and 35% Zinc-C. There was a statistically significant decrease in wound closure of all mouth rinses except EO (Figure 6). EO was found to be similar to control at all time points. On day 2, the percent wound closures were 92% for control, 94% for EO, 85% for CHX, 93% for zinc-A, 82% for Zinc-B, and 48% for Zinc-C. On day 3, the percent wound closures were 100% for control, 99% for EO, 93% for CHX, 94% for zinc-A, 96% for Zinc-B, and 62% for Zinc-C. On Day 3, no statistical difference in percent wound closure in EO, CHX, Zinc-A, or Zinc-B (Figure 6).

Discussion

Summary of Data

Our study found that a 3-minute exposure of 10% or greater dilution of Zinc-C to gingival fibroblasts had a significant decrease in cell survival and was significantly more cytotoxic than the other mouth rinses examined. For transcript expression, cell exposure to mouth rinses had a similar effect on cytokine expression with anti-inflammatory properties. Cell motility and percent wound closure was decreased for all mouth rinses

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on day 1, however, by day 3, Zinc-C was the only mouth rinse showing decreased wound closure compared to control.

Cell Survival

Zinc-C had the highest cytotoxic effect on cells of mouth rinses in our study, with EO being the least toxic after 24-hour exposure. When mouth rinses were diluted and exposure time decreased to 3min exposures, it was found that Zinc-C had the lowest cell survival at all dilutions. Zinc-C, an over-the-counter mouth rinse, was found to be more cytotoxic at lower concentrations than CHX which is a prescriptive antimicrobial mouth rinse.

PCR Transcripts

Evaluation of cytokine expression found a decrease in MCP- 1 and GM-CSF expression in all mouth rinses. A study found that an increase in severity of periodontal disease was related to an increasing concentration of MCP-1 (9). GM-CSF has been shown to be a marker for the "potential to discriminate between early and advanced stages of periodontitis" (10). A decrease in both MCP-1 and GM-CSF may indicate the anti-inflammatory properties of mouth rinses. This study found that all mouth rinses decreased the expression of IL-6. IL-6 is a pro-inflammatory and anti-inflammatory 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. (11). IL-6 "has been shown to be "related to tissue destruction at the periodontal site" (12). A decrease in IL-6 expression may be related to a decrease in periodontal tissue destruction and indicate anti-inflammatory properties of mouth rinses. Zinc-C was found to increase the expression of BMP-2, a bone morphogenic protein that can be found localized in alveolar bone and can aid in periodontal regeneration (13). An increase in BMP-2, which is seen only in the Zinc-C group, could imply better periodontal regeneration properties. The growth factor, FGF-2,

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was found to be elevated, however, this was found not to be statistically significant. FGF-2 is related to “accelerate periodontal regeneration” and “induces the growth of immature hPDLCs, which is a competitive inhibitor of epithelial down growth, and suppresses their differentiation into mineralized tissue” (14).

Cell Motility

Zinc-C dilutions of 10% were found to be toxic at 3 min exposures, however, at 5% dilution, it was not initially toxic. It was found that at 5% dilution after 3 min exposure of Zinc-C mouth rinse, cell motility was inhibited. It was found that on day one, all mouth rinses had delayed healing compared to control however by day three all mouth rinses had similar to control except for Zinc-C rinse. Zinc-C rinse had significantly less wound closure and cell motility by day three. Chlorhexidine has been found to interrupt wound healing and impair gingival fibroblast migration and long-term survival (15). Zinc-C had an even greater decrease in cell motility than CHX. Zinc-C may not be recommended mouth rinse after periodontal surgery due to inhibition of cell motility.

Conclusion:

This study found Zinc-C to have the most cytotoxic effects on gingival fibroblasts after only three minutes of exposure. It was found that Zinc-C increased BMP-2 expression often found in periodontal regeneration as well as FGF-2, which regulates osteoclast. cell migration. When human gingival fibroblasts are exposed to Zinc-C, there was a statistically significant decrease in percent wound closure compared to control on day 3.

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

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Figure 1. Effects of mouth rinses on cytotoxicity after 24-hour exposure

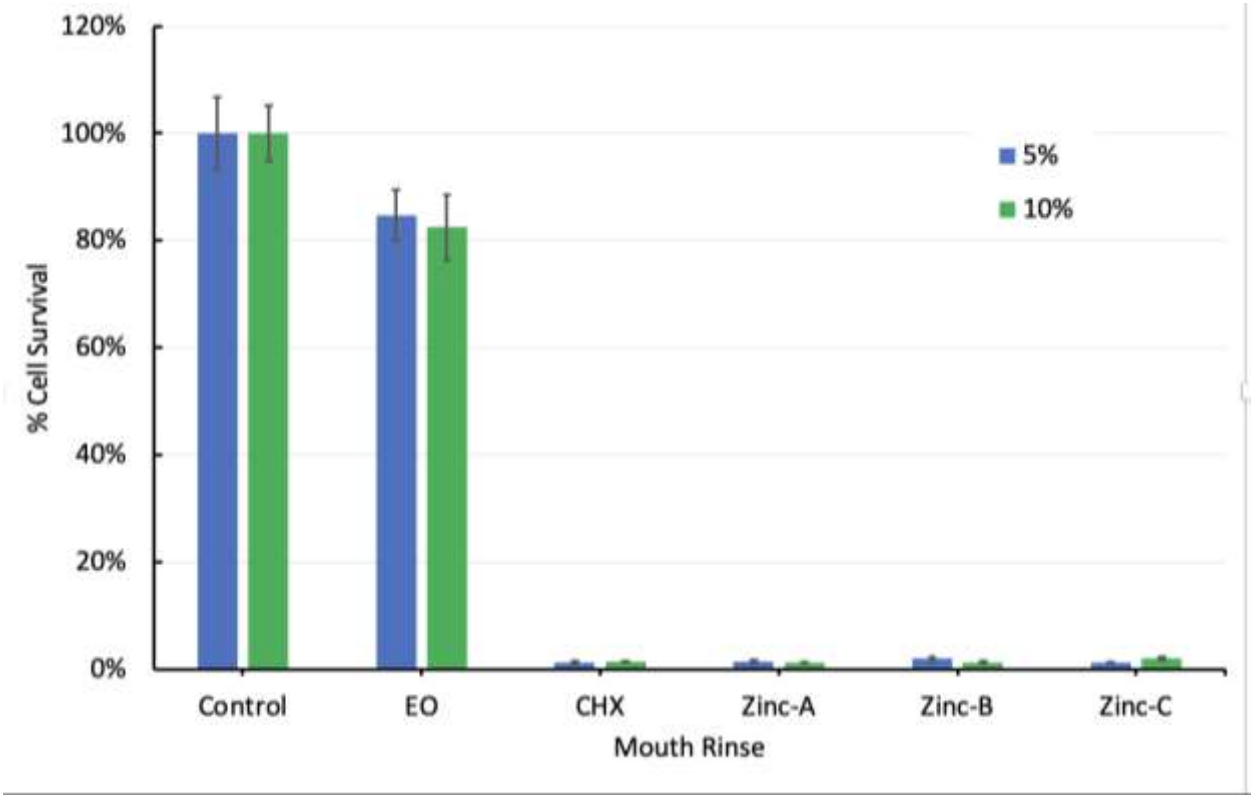

Figure 1: Survival of gingival fibroblasts exposed to a variety of mouth rinse dilutions for 24 hours at 5% and 10%. Mouth rinses examined were EO, CHX, Zinc-A, Zinc-B, Zinc-C. Cell were exposed at dilutions of 0%, 5%, 10%, 15%, 20%, 25%.

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Figure 2. Effects of mouth rinse on Gingival Fibroblast Cell Survival after 3min exposure

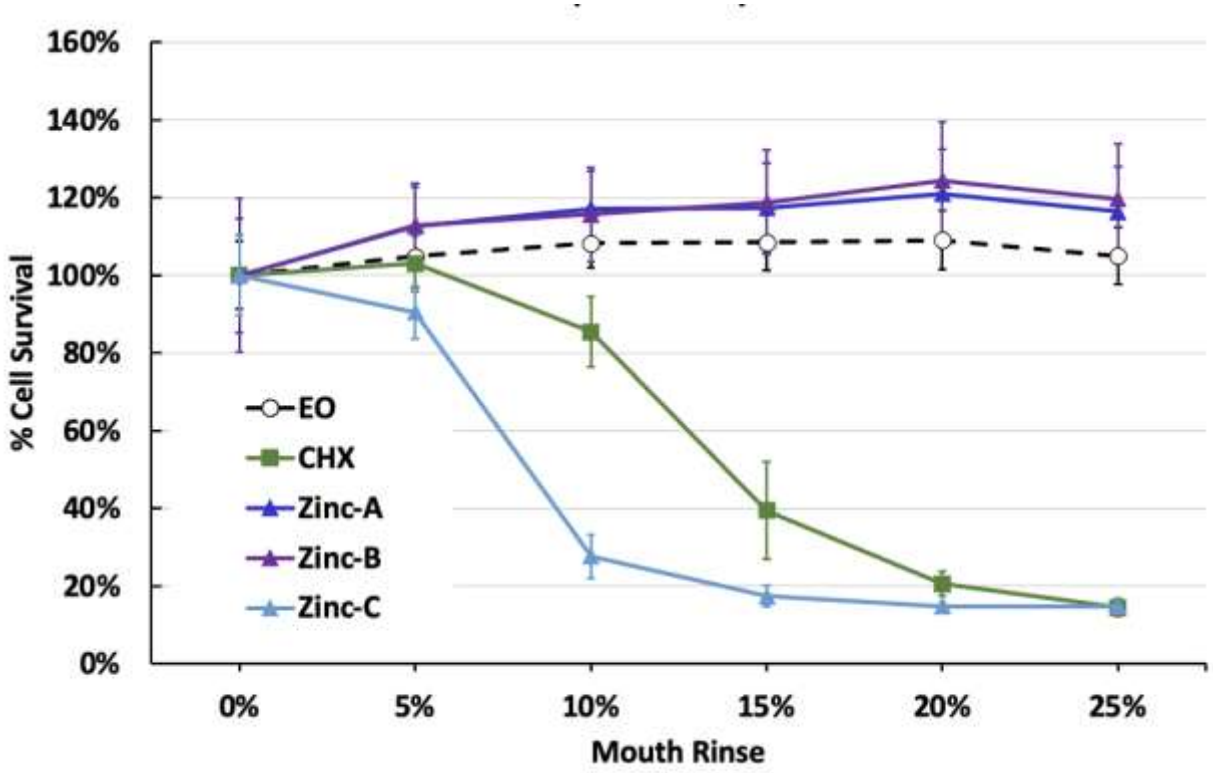

Figure 2: Survival of gingival fibroblasts exposed to a variety of mouth rinse dilutions (0-25%) for 3 minutes. Mouth rinses examined were EO, CHX, Zinc-A, Zinc-B, Zinc-C. Cell were exposed at dilutions of 0%, 5%, 10%, 15%, 20%, 25%.

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Figure 3: RT-PCR gene expression of Cytokines

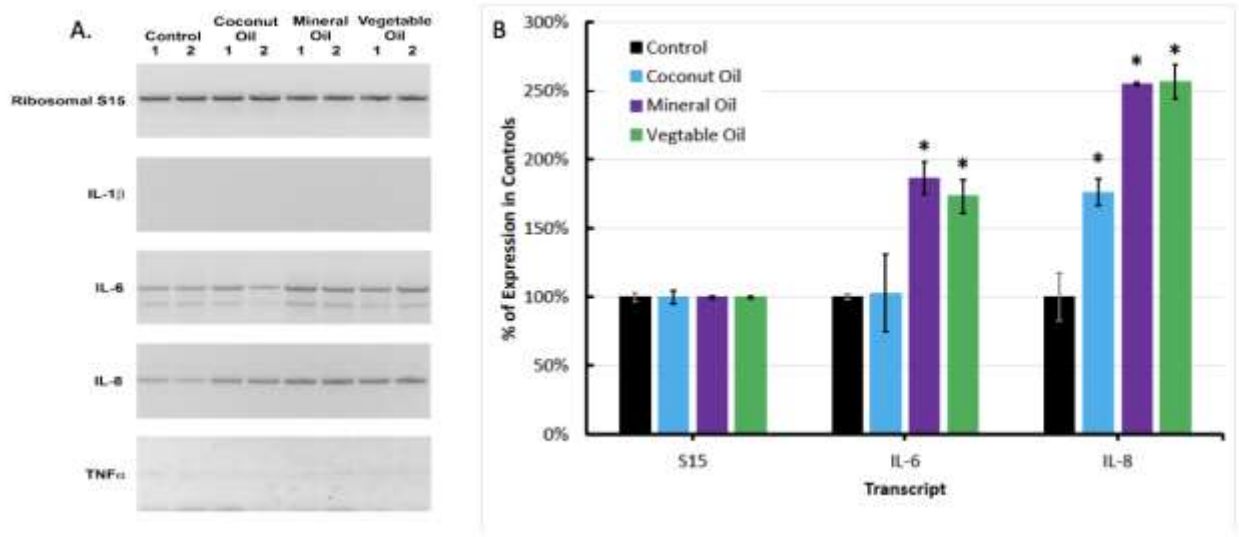

Figure 3. Transcript expression analysis for cytokines using RT-PCR (A). Gingival fibroblasts were exposed to EO, CHX, Zinc-A, Zinc-B, and Zinc-C for 3 minutes. (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks (*) represent statistically significant differences from controls ($P<0.05$).

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Figure 4: RT-PCR gene expression of Interleukins

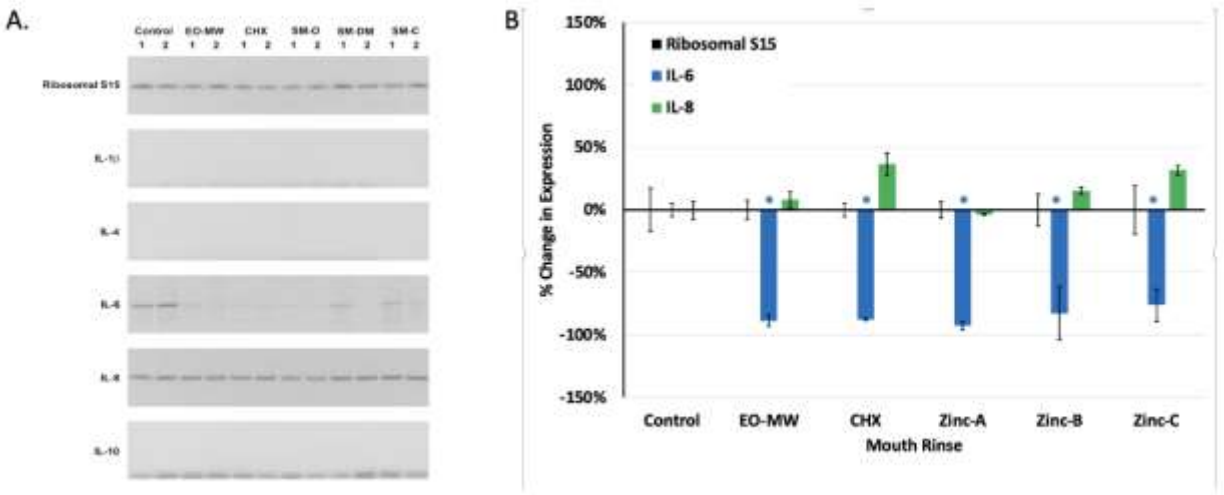

Figure 4. Transcript expression analysis for interleukins using RT-PCR (A). Gingival fibroblasts were exposed to EO, CHX, Zinc-A, Zinc-B, and Zinc-C for 3 minutes. (B) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks (*) represent statistically significant differences from controls ($P<0.05$).

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Figure 5: RT-PCR gene expression of growth factors

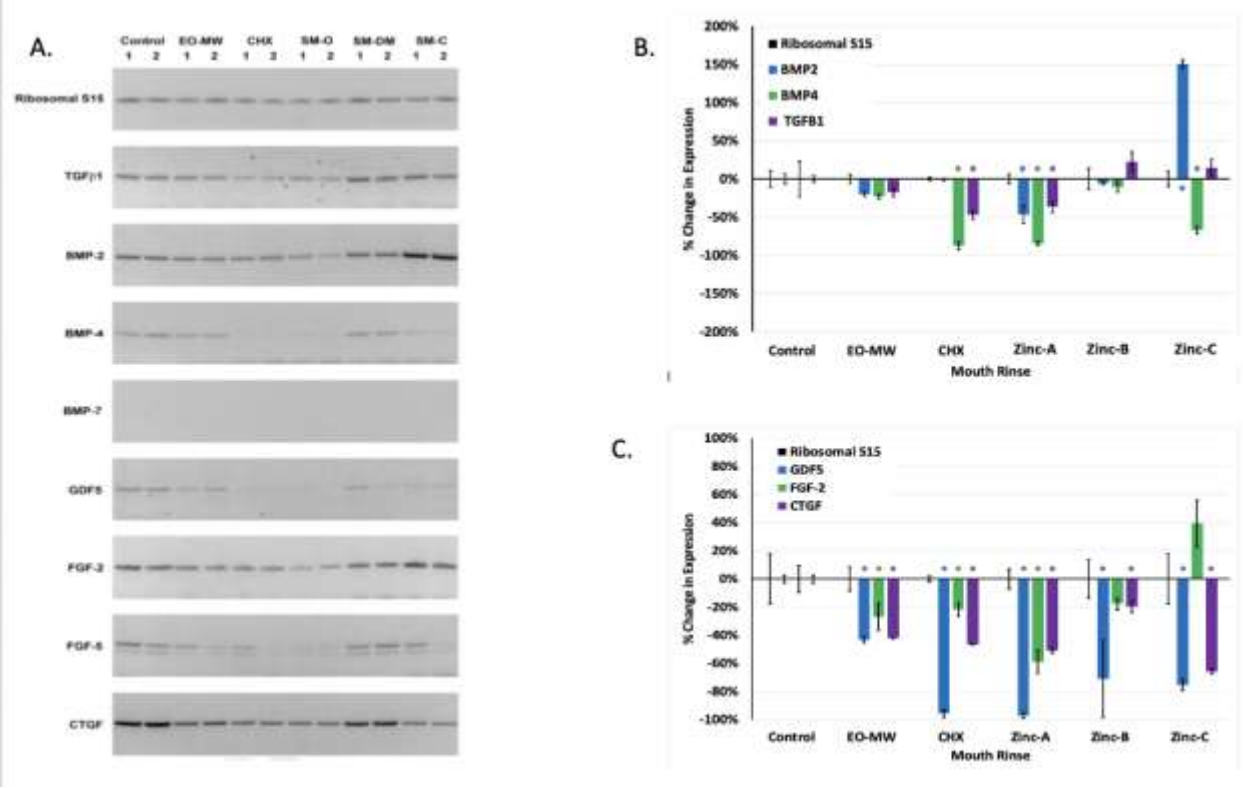

Figure 5. Transcript expression analysis for growth factors using RT-PCR (A). Gingival fibroblasts were exposed to EO, CHX, Zinc-A, Zinc-B, and Zinc-C for 3 minutes. (B and C) Quantification of transcript expression where bars represent the mean and standard deviation of two samples. Asterisks (*) represent statistically significant differences from controls ($P<0.05$).

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Figure 6:Cell Motility

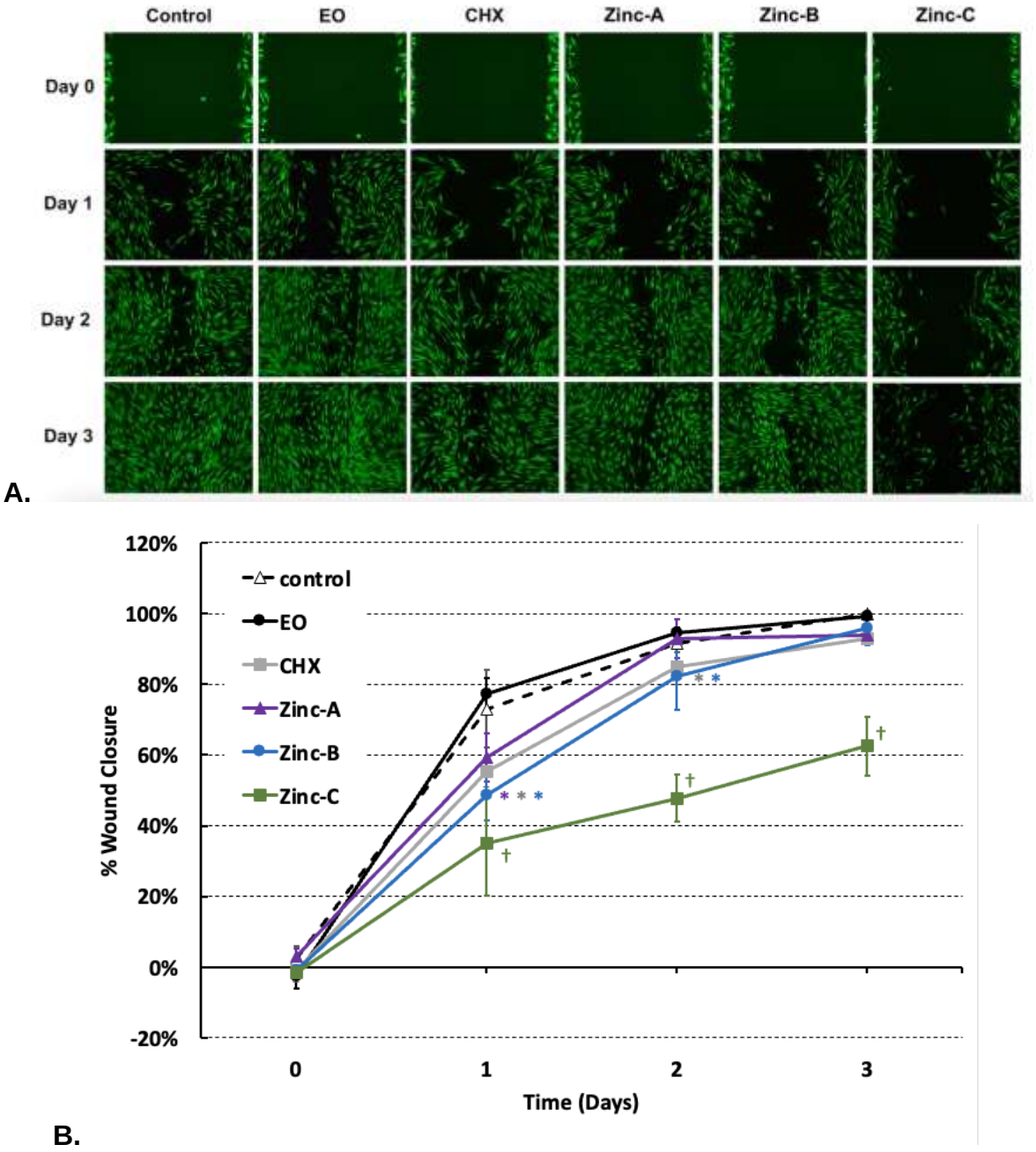

Figure 6. A . Confluent monolayers were scraped to create a 250-mm cell-free zone, and cells were observed immediately after the initial wounding. Photomicrographs of calcein-labeled, live GFs exposed to 5% dilution of EO, CHX, Zinc-A, Zinc-B, and Zinc-

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C mouth rinses for 3 mins. Untreated cells served as controls. Quantification of GF migrations was measured as a function of the reduction of the width of the cell-free zone over time. Points represent the mean and SD of 10 measurements. Stain: Calcien AM. Significant differences in cell movement from untreated control cells are indicated: P <0.01 and P <0.001.

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