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THE ANTI-INFLAMMATORY POTENCY OF VITAMIN E AND COCONUT OILS ON LIGATURE-INDUCED PERIODONTITIS IN ALBINO RATS

(HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Plaque induced periodontitis is the second most common oral disease worldwide after dental caries. Adjunctive methods such as mouthwashes have been developed as an aid to routine scaling for treating periodontal inflammation.

Aim: The aim of this study was to evaluate the anti-inflammatory potency of vitamin E and coconut oils when used for oil gum massage therapy in periodontitis and compare them to the conventionally used chlorhexidine.

Methods: 105 adult male albino rats were divided into 5 groups of 21 rats each: The negative control group (group 1) that received no intervention, the positive control group (group 2) that received ligature induced periodontitis without any treatment, the chlorhexidine experimental group (group 3) that received ligature induced periodontitis and chlorhexidine for 10 days, the vitamin E experimental group (group 4) that received ligature induced periodontitis and vitamin E for 10 days. And the coconut oil group (group 5) that received ligature induced periodontitis and coconut oil for 10 days. Groups 2-5 were further divided into subgroups A,B, and C according to day of termination (day 3, 7, &10). After rats were terminated, maxillary molar regions were excised and stained with H&E and Masson's trichrome stain. Specimens were also examined immunohistochemically for the expression of MMP-1 antigen.

Results: Histological and immunohistochemical examination showed an inflammatory reaction with ligature induced periodontitis that decreased gradually from day 3 until day 10. Statistical analysis regarding the immunohistochemical expression of MMP-1 inflammatory marker showed that it gradually decreased over the course of the treatment in all experimental groups with chlorhexidine showing the greatest decrease in inflammation while there was no significant difference between vitamin E and coconut oil.

Conclusion: Oil gum therapy using either chlorhexidine, vitamin E, or coconut oil was associated with variable grades of statistically significant reduction of inflammation.

KEYWORDS: ligature induced periodontitis, inflammation, MMP-1, chlorhexidine, coconut oil, vitamin E, immunohistochemistry

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INTRODUCTION

Plaque induced periodontitis is the second most common oral disease worldwide after dental caries **(Petersen 2003)**. Mechanical removal of plaque via routine scaling is critical to maintain the health of the periodontal tissues **(Sharma et al., 2004)**. However, since most people do not consistently control plaque accumulation adequately, adjunctive methods such as antimicrobial mouthwashes and dentifrices have been developed for treating periodontal inflammation **(Nadkerny et al., 2015)**. Among these adjunctive methods, chlorhexidine has long been considered the "gold standard" for treating gingival inflammation, owing to its broad spectrum action on plaque-causing bacteria **(Indurkar et al., 2016)**.

Since it has been found that most tissue destruction in periodontal disease is dependent more on the host's response to the plaque-causing microorganism rather than the microorganism itself **(Lamster et al.,1992; Dahiya et al., 2013)**, the use of antioxidant rich oils for oil pulling and oil gum massage presents itself can be seen as a potential alternative to chlorhexidine. Vitamin E oil and coconut oil are two natural products that are currently gaining popularity for their powerful antioxidant properties, enabling them to oxidize free radicals and reactive oxygen species generated during periodontal inflammation (**Atalay et al., 2000; Singla et al., 2014**).

By exploring the effectiveness of vitamin E and coconut oil when used as anti-inflammatory regimens for periodontitis, we hope to provide patients with a substitute to chlorhexidine that is natural, more acceptable, and evades the disadvantages of chlorhexidine. Over and above, as antibiotic drug resistance continues to become an emerging global threat, this study may open doors for treating oral bacterial infections using antioxidants rather than antimicrobials.

MATERIALS AND METHODS

One hundred and five adult, male Wistar albino rats (*rattus albus*) weighing 150-200 grams were used in this study.

The animals were divided into the following groups:

Group 1: Negative control group: This group consisted of 21 rats that were kept on a normal diet and did not receive any intervention.

Group 2: Positive control group: This group consisted of 21 rats which received a plain, dry cotton pellet without any intervention rubbed on the gums for 10 days.

Group 3 (Chlorhexidine experimental group): This group consisted of 21 rats which received 500 mg/kg (about 100 mg) of 1% chlorhexidine gel twice a day for 10 days.

Group 4 (Vitamin E experimental group): This group consisted of 21 rats which received 500 mg/kg (about 100 mg) of vitamin E oil twice a day for 10 days.

Group 5 (Coconut oil experimental group): This group consisted of 21 rats which received 500 mg/kg (about 100 mg) of 100% extra virgin coconut oil twice a day for 10 days.

Periodontitis was induced in the rats of groups 2-5 via the ligature induced periodontitis technique, which involves the use of silk ligatures around the teeth to encourage local accumulation of plaque, thereby enhancing bacterial infection and alveolar bone loss **(Graves et al., 2008).** The procedure for ligature induced periodontitis was carried out as was mentioned in **(Abe & Hajishengallis, 2013).** Over the course of the 10 days, 7 rats from each group were terminated on day 3, day 7 and day 10. Hence, the 5 groups were further evenly divided into the following subgroups according to day of terminated:

Subgroup A: terminated on day 3

Subgroup B: terminated on day 7

Subgroup C: terminated on day 10

Rats were terminated by I/V administration of anesthetic overdose of sodium thiopental 80 mg/kg. The maxillae of the rats were excised to allow for laboratory processing and examination. Examination was performed via H&E stain, Masson's trichrome stain to examine newly formed collagen fibers as an indicator of healthy periodontal tissue according to the technique mentioned in **(Kiernan 2008)**, and immunohistochemical expression of matrix metalloproteinase-1 (MMP-1) in the gingiva using a monoclonal antibody to MMP-1 (clone SB12e; Santa Cruz Biotechnology, USA) according to the technique mentioned in **(Lv et al., 2014)**.

Statistical Analysis

Slides were examined under a light microscope at a magnification of 400x. Fields showing the highest immunopositivity were selected and photomicrographs were captured. This was performed using a digital camera (C5060 Olympus, Japan) which was mounted on a light microscope (BX60, Olympus, Japan). Images were then transferred to the computer system for analysis. Statistical analysis was then performed using a commercially available software program (SPSS 19; SPSS, Chicago, IL, USA).

RESULTS

Hematoxylin & Eosin Staining

Negative Control Group

Examination of the (H&E) stained sections of the periodontium surrounding the upper first molar of rats of the negative control group revealed collagen fiber bundles of the periodontal ligament (PDL) which were dense and rich in fibers with little ground substance in between them. The bundles showed a regular arrangement, taking their characteristic wavy course as they traveled from cementum to bone.

Observation of the principal collagen fiber bundles of the PDL showed that they were arranged in different groups which were: gingival group connecting the cervical cementum to the lamina propria of the gingiva, transseptal group connecting adjacent teeth together, alveolar crest group radiating from the crest of the alveolar process to the cervical cementum, horizontal group running horizontally between the cementum and alveolar bone in the neck and midroot section, oblique group running obliquely and apically from the alveolar bone to the cementum, apical group radiating from the apical region of the root to the surrounding bone, and interradicular group extending from the crest of the interradicular septum to the furcation of the tooth. All of the collagen fiber groups showed their regular course and orientation.

Parts of the collagen fiber bundles were found inserted into the cementum and bone and are known as Sharpey's fibers. Between the collagen bundles, interstitial tissue is found containing blood vessels, nerves and lymphatics. There does not appear to be any dilation in the blood vessels of the interstitial tissue. Fibroblasts were found arranged along the axes of the collagen fiber bundles. They were elongated and tapering with thin cytoplasmic extensions. The nuclei of the fibroblasts were oval in shape and ran parallel to each other. Cementoblasts which appeared round and plump in shape lined the cementum surface and were arranged in a single continuous layer.

The alveolar bone showed a smooth surface lined by osteoblasts which were cuboidal in shape with an eccentric nucleus. Osteocytes are seen entrapped within the bone matrix, and bone marrow spaces are found containing blood vessels. Zuckerkandl and Hirschfeld canal is seen perforating the bone carrying blood vessels and nerves to it.

Positive Control Group

Examination of the (H&E) stained sections of periodontium surrounding the upper first molar

of the rats belonging to the positive control group revealed collagen fiber bundles of PDL whose collagen component was not abundant. The bundles showed thin, deficient fibers with wide spaces in between them. Arrangement of the bundles was distorted, leading to loss of their normal wavy course from bone to cementum.

Observation of the principal collagen fiber groups of the PDL showed irregular arrangement of the different collagen fiber groups. Collagen fiber bundles of the gingival group, alveolar crest group, the horizontal group, the oblique group and the interradicular group showed disorientation in the directions of the fibers as well as severing of collagen fiber bundles with large spaces of periodontal dissociation. Meanwhile the transseptal and apical groups did not show changes in the direction and density of the fibers and assumed their regular wavy course. Sharpey's fibers inserting into the cementum and bone were severed in many areas where they should be attached. Between the collagen fiber bundles, interstitial tissue showed many dilated blood vessels with extravasation of red blood cells from the blood vessels into the tissue.

Inflammatory cells are scattered throughout the periodontal ligament appearing as darkly stained nuclei scattered throughout the tissue. Within the bundles, the nuclei of the fibroblasts were not parallel to each other and assumed many different directions.

The alveolar bone of the interdental septum showed an irregular surface scalloped by several Howship's lacunae. Multinucleated osteoclasts were found in their Howship's lacunae on the bone surface. Multiple reversal lines were seen in bone.

Chlorhexidine Group

Histological examination of the specimens that received chlorhexidine revealed that after 3 days of treatment (subgroup A), collagen fiber bundles were distorted with spaces between the fibers indicative of periodontal disease, there was extravasation of inflammatory cells, and scalloping of the alveolar bone with presence of osteoclasts, indicating bone resorption. Subgroup B, in which specimens received chlorhexidine for 7 days revealed more parallel collagen fibers, fewer inflammatory cells and less scalloped alveolar bone with few osteoclasts. Subgroup C, in which specimens received chlorhexidine for 10 days showed further organization of the collagen fibers along with reversal lines in bone, indicating the residing of inflammation and the initiation of bone repair. (Fig. 1)

Vitamin E Group

Examination of the histological structure of the rats which received vitamin E showed that subgroup A had irregularly arranged collagen fibers dissociated from the alveolar bone, red blood cell extravasation and scalloped alveolar bone with osteoclasts indicative of bone resorption. Subgroup B then showed more organized collagen fibers, but continued to show inflammatory cells and resorption lacunae harboring osteoclasts. Finally subgroup C showed a histological picture that featured thicker, more organized, collagen fiber bundles. However, inflammatory cells and RBC extravasation were still present. Osteoclasts were absent and reversal lines were seen, indicating bone repair. (Fig 2)

Coconut Oil Group

The histological structure of the rats which received coconut oil also featured unorganized collagen fibers with spaces in between, inflammatory cells and bone resorption in subgroup A. After 7 days of treatment, subgroup B showed more organized collagen fibers but continued to show an inflammatory cell infiltrate and resorption lacunae harboring osteoclasts. Finally, at 10 days of treatment, subgroup C showed further organized collagen bundles along with a mild inflammatory cell infiltrate. (Fig 3)

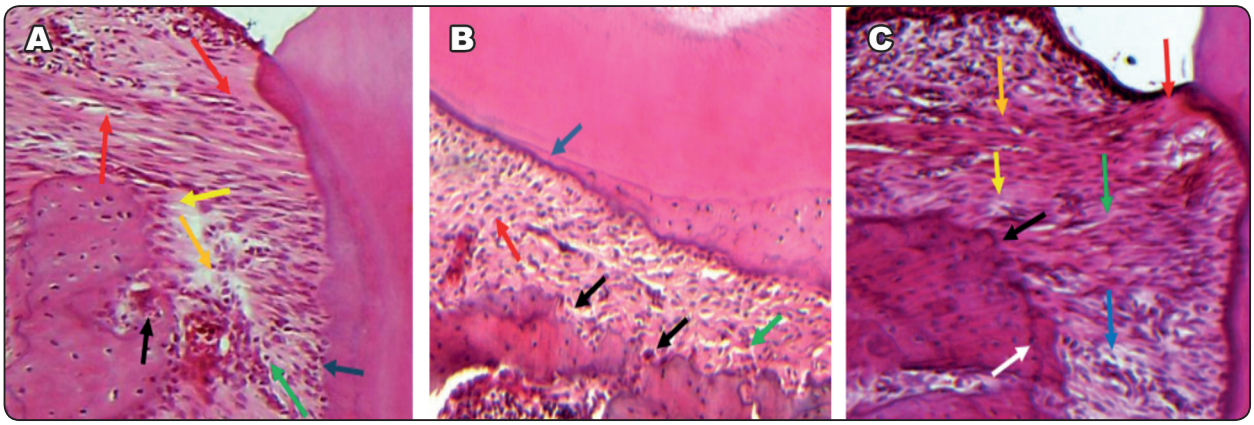

Fig. 1A: A photomicrograph of the periodontium of chlorhexidine group subgroup A showing dense collagen bundles of gingival and transseptal groups (red arrows) with disoriented collagen fibers of the alveolar crest, horizontal, and oblique groups showing nonparallel direction of fibroblast nuclei (orange arrow), detachment of Sharpey's fibers from bone (yellow arrow), inflammatory cells (green arrow), disrupted cementoblast layer (blue arrow), and irregular bone surface scalloped by Howship's lacunae harboring osteoclasts (black arrow). **1B:** subgroup B showing more dense collagen bundles of oblique group in wavy course and more parallel fibroblast nuclei (red arrow), inflammatory cells (green arrow), more organized cementoblast layer (blue arrow) and irregular bone surface (black arrow). **1C:** subgroup C showing gingival (red arrow), transseptal (orange arrow), alveolar crest (yellow arrow), horizontal (green arrow) and oblique group (blue arrow) of PDL fibers featuring dense fibers with minimal interstitial tissue with less scalloped alveolar bone (black arrow) with reversal lines (white arrow). **(H&E x100)**

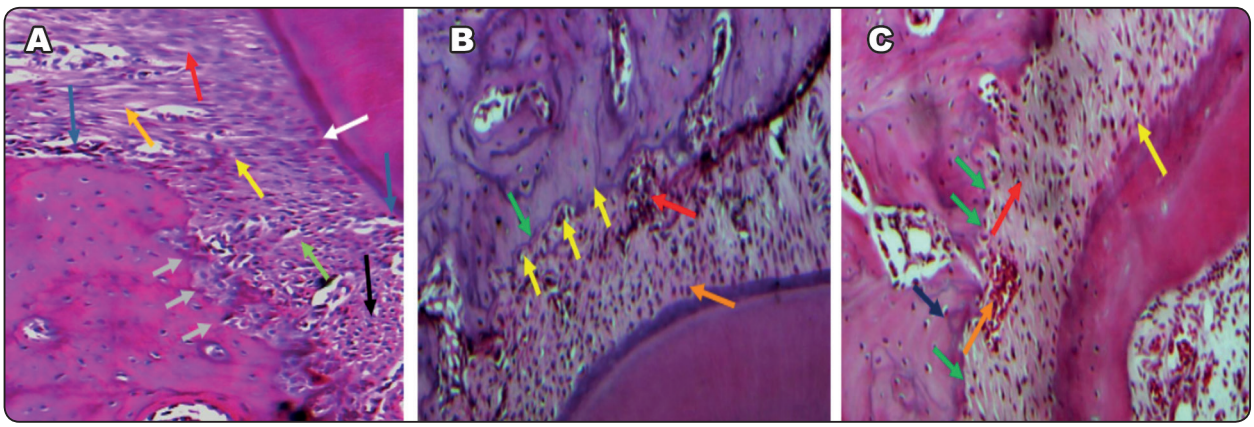

Fig. 2A: a photomicrograph of Vitamin E subgroup A showing transseptal (red arrow), alveolar crest (orange arrow), horizontal (yellow arrow) and oblique group (green arrow) of PDL fibers, detachment of Sharpey's fibers from bone and cementum (blue arrows), nonparallel fibroblast nuclei (black arrow), distorted cementoblast layer (white arrow) and irregular bone surface and osteoclast (grey arrows). **2B:** subgroup B showing oblique fibers featuring thicker fiber bundles with minimal interstitial tissue, mild inflammatory reaction (red arrow), more linear arrangement of cementoblasts (orange arrow), scalloped alveolar bone with osteoclasts (yellow arrows), and reversal lines in bone (green arrow). **2C:** subgroup C showing thick collagen bundles of oblique fibers with minimal interstitial tissue (red arrow), blood vessel dilatation with extravasation of RBCs (orange arrow), linear arrangement of cementoblasts (yellow), smooth alveolar bone surface (green arrow), and reversal lines in bone (blue arrow). **(H&E x 100)**

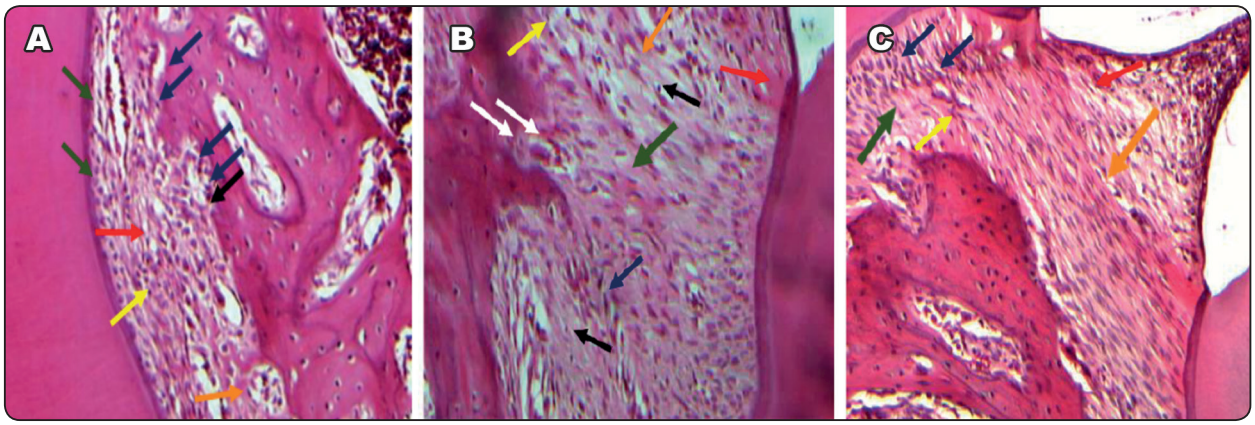

Fig. 3A: a photomicrograph of the periodontium of coconut oil subgroup A showing thin disoriented collagen fibers of oblique group with nonparallel direction of fibroblast nuclei (red arrow), blood vessel dilation (orange arrow), inflammatory cells (yellow), disrupted cementoblast layer (green arrows), irregular bone surface (blue arrows) and osteoclast (black arrow). **3B:** subgroup B showing gingival group (red arrow), transseptal group (orange arrow), alveolar crest (yellow arrow), horizontal (green arrow) and oblique group of PDL fibers (blue arrow) with inflammatory cells (black arrows) and resorption lacuna harboring osteoclast (white arrows). (H&E x100) . **3C:** subgroup c showing gingival group (red arrow), transseptal group (orange arrow), alveolar crest (yellow arrow), horizontal (green arrow) and oblique group of PDL fibers (blue arrows).

Collagen fiber assessment by Masson's Trichrome Staining

Negative Control Group

Examination of the MT stained sections of the periodontium surrounding the upper first molar of the rats of the positive control group revealed thin, deficient collagen fiber bundles of PDL with minimal density and wide spaces in between them. These collagen fibers were weakly stained in bluish green color and showed a disoriented arrangement. Sharpey's fibers inserting into the cementum and bone which were stained red were cut in many areas where they should be attached.

Observation of the principal collagen fiber groups of the PDL showed disorientation of the different collagen fiber groups. Collagen fiber bundles of the gingival group, alveolar crest group, the horizontal group, the oblique group and the interradicular group showed disorientation in the directions of the fibers as well as severing of collagen fiber bundles with large spaces of periodontal dissociation. Meanwhile the transseptal and apical groups did not show changes in the direction and density of the fibers and assumed their regular wavy course.

Between the collagen fiber bundles, interstitial tissue showed many dilated blood vessels with extravasation of red blood cells from the blood vessels into the tissue. Inflammatory cells with darkly stained nuclei are scattered throughout the periodontal ligament tissue.

Positive Control Group

Examination of the MT stained sections of the periodontium surrounding the upper first molar of the rats of the positive control group revealed thin, deficient collagen fiber bundles of PDL with minimal density and wide spaces in between them. These collagen fibers were weakly stained in bluish green color and showed a disoriented arrangement. Sharpey's fibers inserting into the cementum and bone which were stained red were cut in many areas where they should be attached.

Observation of the principal collagen fiber groups of the PDL showed disorientation of the different collagen fiber groups. Collagen fiber bundles of the gingival group, alveolar crest group, the horizontal group, the oblique group and the interradicular group showed disorientation in the directions of the fibers as well as severing of collagen fiber bundles

with large spaces of periodontal dissociation. Meanwhile the transseptal and apical groups did not show changes in the direction and density of the fibers and assumed their regular wavy course.

Between the collagen fiber bundles, interstitial tissue showed many dilated blood vessels with extravasation of red blood cells from the blood vessels into the tissue. Inflammatory cells with darkly stained nuclei are scattered throughout the periodontal ligament tissue.

Chlorhexidine Treated Group

Examination of the MT stained sections of the periodontium surrounding the upper first molar of the rats of subgroup A (3 days post treatment) revealed weakly stained collagen fiber bundles of PDL with thin fibers and wide spaces in between them. The fiber bundles were disoriented in arrangement rather than assuming their characteristic wavy course from cementum to bone. At the site of inflammation, Sharpey's fibers are detached from the cementum and bone which were stained red. Observation of the principal collagen fiber groups of the PDL showed weakly stained, largely disoriented arrangement of the different collagen fiber groups. Along with disorientation of the fiber direction, collagen fiber bundles of the gingival group, alveolar crest group, the horizontal group, the oblique group and the interradicular group showed large spaces of periodontal dissociation. Meanwhile the transseptal and apical groups did not show changes in the direction and density of the fibers and assumed their regular wavy course. Between the collagen fiber bundles, interstitial tissue featured blood vessel dilation with extravasation of red blood cells from the blood vessels into the tissue. Inflammatory cells with darkly stained nuclei are seen throughout the periodontal ligament tissue.

By the end of the treatment, MT stained sections of the periodontium surrounding the upper first molar of the rats showed dense collagen fiber bundles of the periodontal ligament (PDL) rich in fibers intensely stained in bluish green color. Furthermore, mature, denser collagen fiber bundles were found stained in red. The fibers ran into the organic matrix of the cementum stained in red and also passed through the bone crest of the interdental septum as Sharpey's fibers. Regular arrangement of the fibers was seen as they followed their characteristic wavy course. The principal collagen fiber bundles of the PDL showed fibers of the gingival group, transseptal group, alveolar crest group, horizontal group, oblique group, apical group, and interradicular group All of the collagen fiber groups showed their regular course and orientation. Between the collagen bundles, interstitial tissue is found containing blood vessels, nerves and lymphatics. A well vascularized periodontal ligament was seen enriched with blood vessels in the interstitial tissue. No dilation in the blood vessels of the interstitial tissue was seen nor were any inflammatory cells present.

Vitamin E Treated Group

Examination of the MT stained sections of the periodontium surrounding the upper first molar of the rats of subgroup A revealed disoriented, weakly stained collagen fiber bundles of PDL with deficient fibers with wide spaces in between them. Interruption in the insertion of Sharpey's fibers inserting into alveolar bone was noted. Observation of the principal collagen fiber groups of the PDL showed weakly stained, largely disoriented arrangement of the different collagen fiber groups. Along with disorientation of the fiber direction, collagen fiber bundles of the gingival group, alveolar crest group, the horizontal group, the oblique group and the interradicular group showed large spaces of periodontal dissociation. Meanwhile the transseptal and apical groups did not show changes in the direction and density of the fibers and assumed their regular wavy course. Between the collagen fiber bundles, interstitial tissue showed dilated blood vessels and extravasation of red blood cells into the tissue. Throughout the periodontal ligament tissue, an inflammatory cell infiltrate of inflammatory cells with darkly stained nuclei was seen.

By the end of the treatment, MT stained sections of the periodontium surrounding the upper first molar of the rats showed collagen-rich fiber bundles positively stained in an intense bluish green color. Furthermore, mature, denser collagen fiber bundles were found stained in red. Fibers were regularly arranged as they followed their characteristic wavy course. All of the collagen fiber groups showed their regular course and orientation. Between the collagen bundles, interstitial tissue is found rich in blood vessels. No blood vessel dilation. Inflammatory cells were not seen throughout the tissue.

Coconut Oil Treated Group

Examination of the MT stained sections of the periodontium surrounding the upper first molar of the rats of subgroup A revealed collagen fiber bundles of PDL lightly stained in a pale bluish green color. Fiber bundles were deficient in fibers with wide spaces in between them. The insertion of Sharpey's fibers into alveolar bone was severed. Observation of the principal collagen fiber groups of the PDL showed weakly stained fibers that were not oriented in their characteristic wavy course. Along with disorientation of the fiber direction, collagen fiber bundles of the gingival group, alveolar crest group, the horizontal group, the oblique group and the interradicular group showed large spaces of periodontal dissociation. Meanwhile the transseptal and apical groups did not show changes in the direction and density of the fibers and assumed their regular wavy course. Interstitial tissue in between the collagen fiber bundles showed blood vessel dilation and extravasation of red blood cells into the tissue. Inflammatory cells were seen scattered throughout the tissue.

By the end of the treatment, MT stained sections of the periodontium in this group showed thick, dense collagen fiber bundles deeply stained in an intense bluish green color. Additionally, some strands of collagen fibers were found stained in red indicating more mature, denser fibers. Regular arrangement of the fibers was seen as they followed their characteristic wavy course. All of the principle collagen fiber groups showed bundles that were dense and positively stained with minimal interstitial tissue in between. Between the collagen bundles, a well vascularized interstitial area rich in blood vessels filled with blood cells stained red was seen. Inflammatory cells were not seen throughout the tissue. (Fig. 6C)

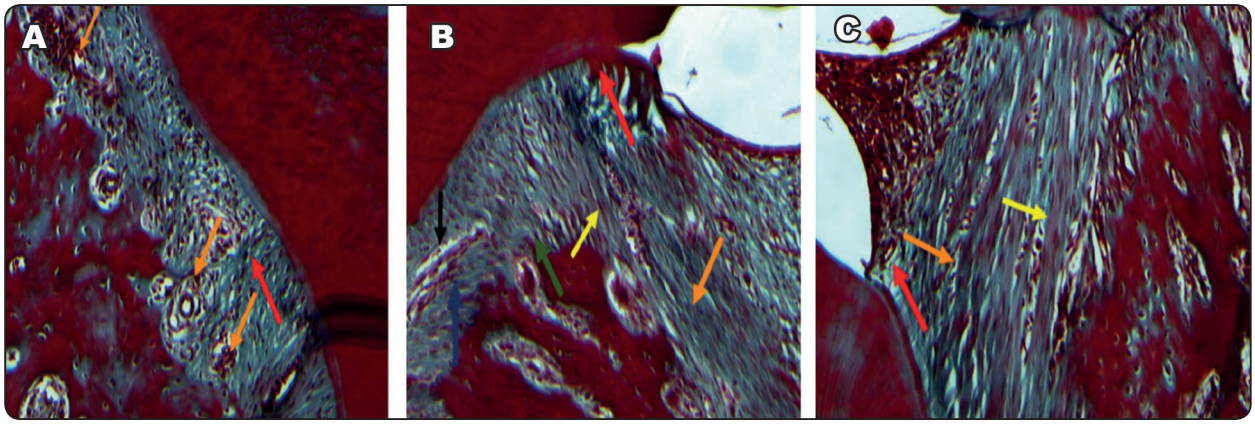

Fig 4A: A photomicrograph of the chlorhexidine subgroup A showing the oblique group featuring thicker, less spaced, moderately stained fibers (red arrow) with blood vessel dilation and RBC extravasation (orange arrows). **4B:** subgroup B showing gingival (red arrow), transseptal (orange arrow), alveolar crest (yellow arrow), horizontal (green arrow), and oblique group (blue arrow) of PDL featuring thicker, less spaced, moderately stained fibers with minimal interstitial tissue in between (black arrow). **4C:** subgroup C showing gingival group (red arrow) and transseptal group (orange arrow) featuring dense intensely stained blue fiber bundles (orange arrow) and more mature fibers stained red (yellow arrow) with regular arrangement. **(MTC x100)**

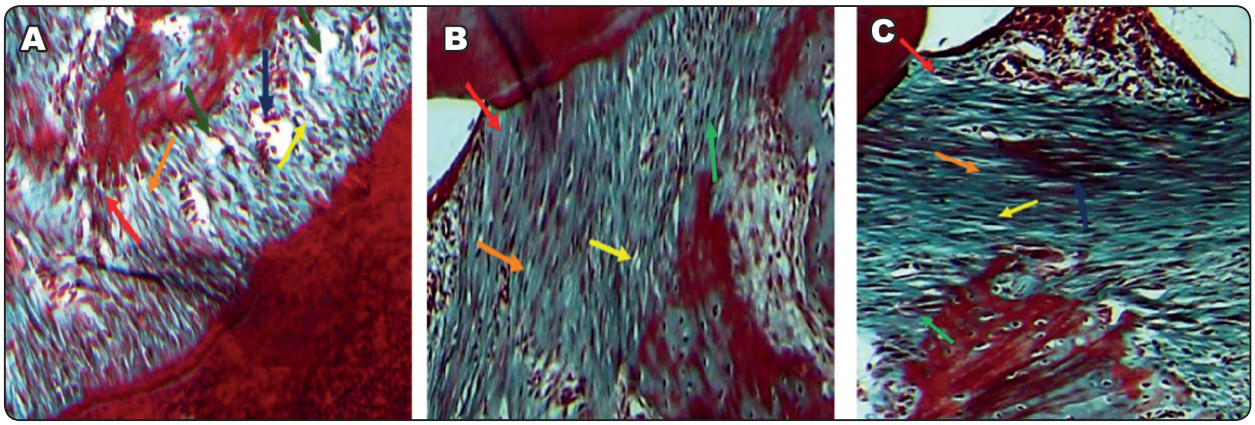

Fig 5A:A photomicrograph of the vitamin E subgroup A showing alveolar crest (red arrow), horizontal (orange arrow) and oblique (yellow arrow) groups featuring newly formed thin, light blue collagen fibers with irregular arrangement, spacing between bundles (green arrows) and RBC extravasation (blue arrow). **5B:** subgroup B showing gingival (red arrow), transseptal (orange arrow), alveolar crest (yellow arrow), and horizontal fibers (green arrow) showing moderately stained, more arranged fibers with less spacing. **5C:** subgroup C showing gingival (red arrow), transseptal (orange arrow), alveolar crest (yellow arrow), and horizontal fibers (green arrow) showing moderately stained, more arranged fibers with more mature red collagen fibers (blue arrow). **(MTC x100)**

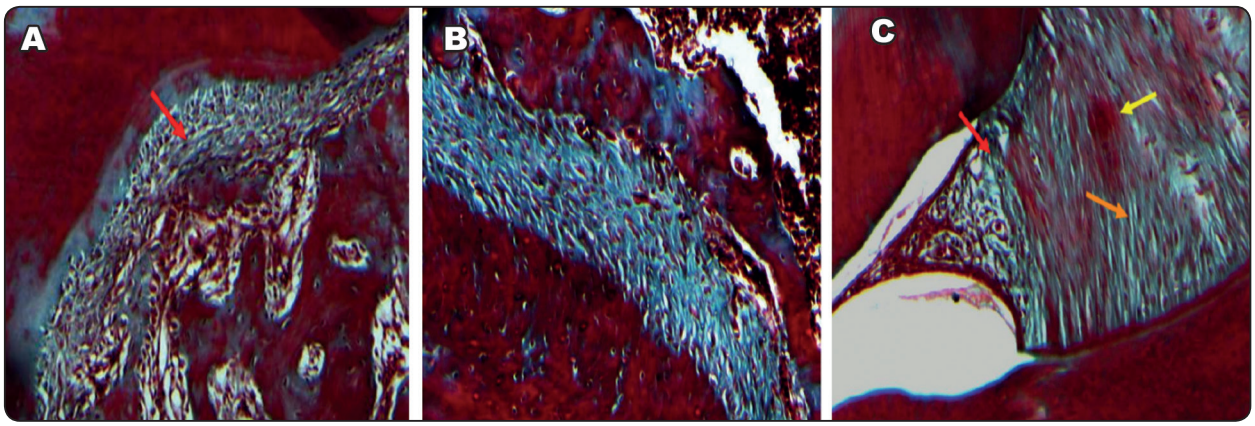

Fig. 6A: A photomicrograph of coconut oil subgroup A showing interradicular fibers of PDL new lightly stained collagen fibers (red arrow). **6B:** subgroup B showing oblique fibers of PDL with newly formed collagen fibers stained in bluish green stain. **6C:** subgroup C showing gingival (red arrow) and transseptal groups of PDL (orange arrow) showing deeper stained, more arranged fibers with more mature red collagen fibers (yellow arrow). **(MTC x100)**

Immunohistochemical Results

In the present study, MMP-1 expression was evaluated in the periodontal ligament in accordance with **(Kubota et al., 2008)**, who observed enhanced expression of MMP-1 in the fibroblasts of periodontally diseased tissue. Active MMP-1 expression highlighted as brown cytoplasmic staining in the cytoplasm of fibroblast and fibroblast like cells.

Negative Control

Immunohistochemical analysis of the periodontal ligament tissue of the rats belonging to the negative control group regarding the expression of MMP-1 antigen showed a negative cytoplasmic MMP-1 reaction in the fibroblasts of the periodontal ligament of all examined cases.

Positive Control:

Immunohistochemical examination of the periodontal ligament of the positive control group revealed active MMP-1 expression highlighted as brown cytoplasmic staining in the periodontal ligament fibroblasts where they were densely located in the cytoplasm of fibroblast and fibroblast like stromal cells.

Chlorhexidine treated group:

The periodontal ligament of subgroup A (day 3) of the chlorhexidine treated group showed a less immunopositive reaction to MMP-1 than the positive control. It was seen as cytoplasmic staining of the fibroblast cells of the periodontal ligaments less than that seen in the positive control group. Immunohistochemical examination of the periodontal ligament of subgroup B showed an obvious reduction in MMP-1 positive cytoplasmic staining of fibroblast and fibroblast like cells of the periodontal ligament. By day 10, the periodontal ligament of subgroup C also revealed a further reduction in the fibroblast and fibroblast-like cells immunopositive for MMP-1 antigen. (Fig. 7)

Vitamin E treated group:

The periodontal ligament of subgroup A of the vitamin E treated group showed the periodontal ligament fibroblasts positive for MMP-1 as cytoplasmic staining.Meanwhile, the periodontal ligament of subgroup B continued to show MMP-1 positive staining in the fibroblasts of the periodontal ligament but to a slightly lesser extent than in subgroup A. Finally, The periodontal ligament fibers of subgroup C showed more reduction in the staining of the tissues by MMP-1 in the cytoplasm of fibroblast and fibroblast-like cells of the periodontal ligament. (Fig. 8)

Coconut Oil treated group

The periodontal ligament of subgroup A of the coconut oil treated group showed cytoplasmic staining of the fibroblasts of the periodontal ligament. Meanwhile, subgroup B revealed slightly less MMP-1 positive staining in the fibroblasts of the periodontal ligament. Finally, subgroup C showed a lesser cytoplasmic immunopositive reaction to MMP-1 in the fibroblasts. (Fig. 9)

Statistical Analysis:

The mean area fraction in **negative control** remained unchanged by time**.** In **positive control**, the mean area fraction gradually increased by time. However, ANOVA test revealed that this difference was not statistically significant (p=0.423). In **Chlorhexidine**, the mean area fraction gradually

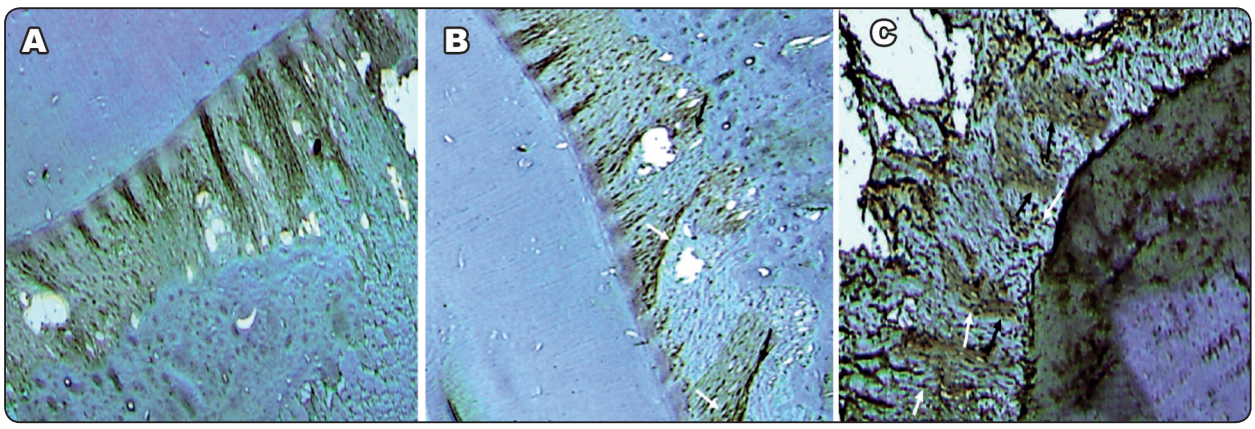

Fig. 7A: A photomicrograph of a mesiodistal section of the periodontal ligament fibers of chlorhexidine subgroup A showing a positive cytoplasmic reaction in the fibroblasts. **7B:** subgroup B showing a reduction in immunohistochemical reacrtion. **7C:** subgroup C showing a further reduction in immmunohistochemical reaction. **(anti-MMP-1 x400) counterstain: Hematoxylin**

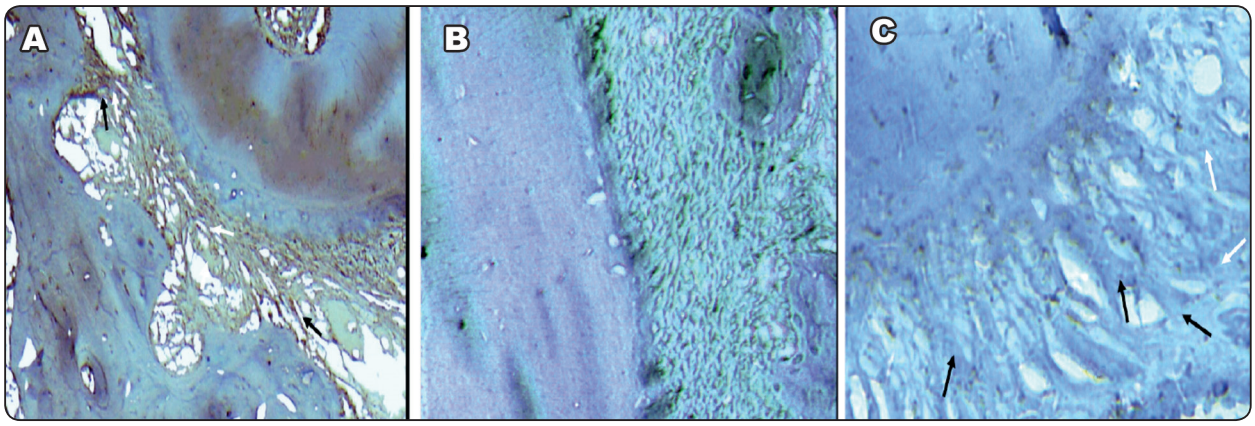

Fig. 8A: A photomicrograph of a mesiodistal section of the PDL of vitamin E subgroup A showing a positive cytoplasmic reaction to MMP-1 in the fibroblasts. **8B:** subgroup B showing a less positive staining to MMP-1. **8C:** subgroup C showing more reduction in staining of periodontal tissues. **(anti-MMP-1 x400) counterstain: Hematoxylin**

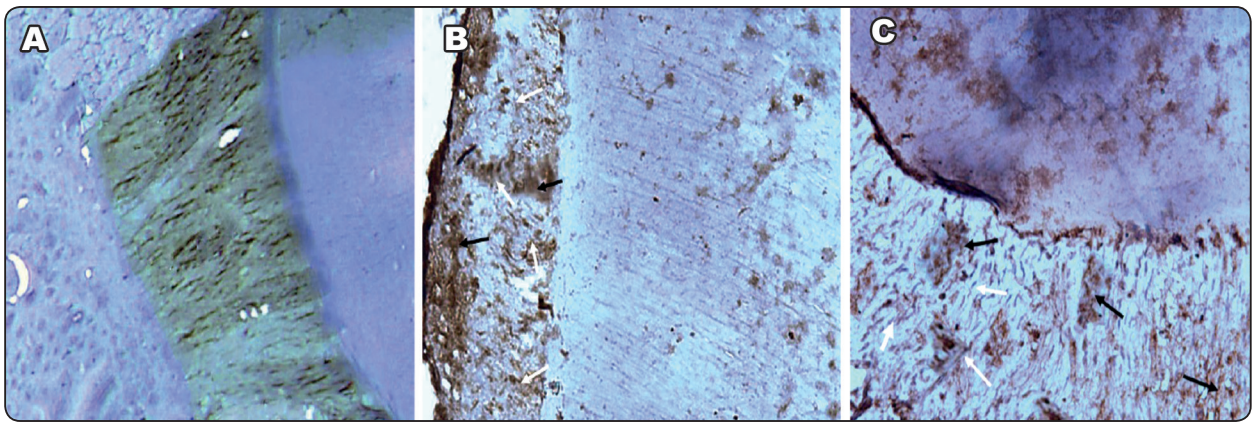

Fig. 9A: A photomicrograph of a mesiodistal section of the PDL of coconut oil subgroup A showing a positive cytoplasmic reaction of the fibroblasts of the lamina propria of the periodontium. **9B:** subgroup B showing a reduction in cytoplasmic staining. **9C:** subgroup C showing lesser cytoplasmic immunopositive reaction. **(anti-MMP-1 x400) counterstain: Hematoxylin**

decreased by time. ANOVA test revealed that this difference was statistically significant ($p<0.0001$). Tukey's post hoc test revealed no significant difference between B and C. In **Vit. E,** the mean area fraction gradually decreased by time. ANOVA test revealed that this difference was statistically significant ($p=0.0019$). Tukey's post hoc test revealed that B was not significantly different from A and C. In **coconut oil,** the mean area fraction gradually decreased by time. ANOVA test revealed that this difference was statistically significant ($p=0.0003$). Tukey's post hoc test revealed no significant difference between A and B.

DISCUSSION

Chlorhexidine antibacterial mouthwashes have long been used as adjunctive methods alongside routine scaling to minimize periodontal inflammation. However, with the development of antibiotic resistance which became alarmingly high, research is now being directed towards natural alternatives with similar anti-inflammatory potency to this long called "gold standard" mouthwash **(Indurkar et al., 2016; Singla et al., 2014)** . Our study experimented with two potential natural alternatives proven to have strong anti-inflammatory properties, coconut oil and vitamin E, tested against

the conventionally used chlorhexidine. Regimens were massaged onto the gingivae of rats where ligature induced periodontitis was established within a period of 7 days. This is a technique known as oil gum massage therapy.

The "ligature" model of periodontitis, which involves placing a ligature in the gingival sulcus around the molar to act as an irritant, inducing plaque accumulation and subsequently periodontitis, was chosen for this experiment for several reasons. In the ligature model, alveolar bone loss in the rats is dependent upon bacteria just like in human periodontitis since the ligature induces food impaction and plaque accumulation, which is in agreement with **(Rovin et al., 1966)**, who observed that the use of ligatures for inducing periodontitis in germ-free rats was not capable of developing periodontal disease. Additionally, this model is reproducible and induces periodontitis in a short time, with initial signs of gingivitis occurring as early as 3 days after ligature placement and pronounced clinical signs of periodontitis with bone resorption seen as early as 7 days. This is in accordance with **(Ionel et al., 2015)**, **(Bezerra et al., 2002)**. The maxillary molars rather than the anterior teeth were chosen for executing the ligature-induced periodontitis model because they were identified as the most appropriate teeth for evaluating inflammation and inflammatory bone loss resulting from periodontitis, which is in accordance with **(Abe & Hajishengallis, 2013)**. The first molar was chosen rather than the second molar for ease of accessibility, given that the small size of the rat's oral cavity presents technical challenges in executing the ligature placement surgery.

Ligatures were removed after 7 days in accordance with **(Yang et al., 2013)**, who found that most alveolar bone loss occurs in the first 7 days after induction and then stabilizes after that. After removing the ligatures, several histological changes were noted in the periodontium of the rats in our study, mainly periodontal degradation, inflammatory response, and alveolar bone loss. This came in agreement with **(Araújo et al, 2017)** who also found inflammatory response, oxidative stress, periodontal degradation and bone loss in the periodontal tissue induced for periodontitis.

In the periodontal ligament tissue, spaces along with dissociation of the fiber bundles from the alveolar bone were observed. Our study found that fibers alveolar crest, horizontal, and oblique PDL fibers were most affected by ligature induced periodontitis. The effect of ligature induced periodontitis was less severe on the interradicular fibers, and the apical fibers of PDL showed mainly an inflammatory cell infiltrate without much damage to the orientation and density of the PDL fibers. This came in agreement with **(Lee et al., 2013)** who found that the more coronal attachment sites of the PDL showed more PDL degeneration than their apical counterparts given that they are in closer proximity to the site of the ligature and plaque accumulation than the apical fibers. **(Lee et al., 2013)** also explained the less severe effect of ligature induced periodontitis on the interradicular fibers by pointing out that the PDL space in this site is narrower with more compact fibers. Our study also found that transseptal fibers were less affected than alveolar crest, oblique and horizontal fibers. This is in agreement with the findings of **(Newman et al., 2014)** who claimed that transseptal fibers are always present, even in extreme cases of periodontal disease due to their continuous tendency to recreate farther along the root as disease progresses.

Our findings also included inflammatory cell infiltration along with blood vessel dilation and red blood cell extravasation, which indicated inflammation. This is in accordance with **(Wu et al, 2018),** who found that the large amount of plaque and sulcular epithelium ulceration that is induced by ligature placement stimulates the production of an inflammatory cell infiltrate which releases a variety of inflammatory factors, mainly TNF-α and IL-1, which in turn stimulate the release of MMPs responsible of collagen fiber degradation of PDL.

The alveolar bone also showed a scalloped surface harboring multinucleated osteoclasts indicating bone resorption. These results coincide with **(Emerit & Michealson, 1982)**, who found that ligature induced periodontal disease triggers reactive oxygen species (ROS) in which the hydroxyl radical of the ROS initiates a chain reaction known as lipid peroxidation which is what leads to bone resorption. Additionally, **(Emerit & Michealson, 1982)** also found that superoxide generated at the osteoclast-bone interface causes bone matrix degradation. Our study found that bone resorption was noted mainly in the coronal region of the alveolar bone. This is in accordance with **(Lee et al., 2013)**, who found that the expression of TRAP and RANKL(+) bone resorption markers was higher in the coronal part of the alveolar bone due to its proximity to the ligature.

The oil gum massage technique was used for this study rather than the conventional mouth washing as a modification of the ancient Indian tradition, oil pulling. Oil gum massage therapy, which involves rubbing the oil onto the gingiva was used for this study due to its added advantages of mechanically removing plaque and stimulating blood circulation in the gingiva. This technique is in accordance with **(Singla et al. 2014).** The suggested mechanism of action of this technique is that the viscosity of the oils inhibits plaque aggregation bacteria adhering to the tooth surface. An additional mechanism is the hydrolysis of the fat in the oils which occurs when salivary alkalis act upon the oil. This causes the fats to disintegrate into small droplets in water. This process is known as emulsification and greatly enhances the cleansing property of the oil, allowing less bacteria and plaque to remain on the tooth surface, thereby minimizing inflammation. The rubbing of the oil against the gingiva has also been claimed to activate enzymes and draw out toxins from the blood **(Asokan et al, 2011).**

The experimental period during which the treatment regimens were applied to the periodontium of the rats was 10 days in accordance with **(Asokan et al. 2009)**. This treatment duration could not be extended for a longer time as was done with other experiments on human models because the ligature induced periodontitis rat model is not optimal for studying the evolution of disease histologically over long periods due to the continuous growth and migration of teeth **(Struillou et al, 2010)**. Regimens were massaged onto the gingiva of the rats twice a day in accordance with **(El-Housseiny et al., 2007)**.

Histological examination of the specimens revealed that after 3 days of treatment (subgroup A), collagen fiber bundles were distorted with spaces between the fibers indicative of periodontal disease, there was extravasation of inflammatory cells, and scalloping of the alveolar bone with presence of osteoclasts, indicating bone resorption. Subgroup B, in which specimens received therapy for 7 days revealed more parallel collagen fibers, fewer inflammatory cells and less scalloped alveolar bone with few osteoclasts. Subgroup C, in which specimens received the treatment regimen for 10 days showed further organization of the collagen fibers along with reversal lines in bone, indicating the residing of inflammation and the initiation of bone repair.

Our results coincided with **(Peedikayel et al, 2015)**, which showed that oil pulling with chlorhexidine and coconut oil effectively reduced inflammation of the periodontal tissues, and **(Carvalho et al, 2013)** which showed that vitamin E decreased the inflammatory reaction induced by ligature induced periodontitis.

Masson's trichrome stain was used in our study to evaluate the progress of healing in terms of collagen formation, as it stains collagen blue-green, while cytoplasm and RBCs are stained red and is typically used to assess the advancement of collagen deposition during tissue healing and matrix remodelling **(Brainman-Wiksman et al., 2007)**.

Based on these histological parameters, it was observed in the present study that collagen fiber deposition was present in all groups in the earliest stages of healing (day 3) and gradually increased

with time until day 10 in accordance. Examination of the MTC stained sections of the 3 treatment groups revealed new, lightly stained, short, wavy, poorly oriented collagen fiber bundles at the beginning of treatment, and gradually more organized collagen fiber bundles at the end of the treatment period. This is indicative of the formation of new collagen fiber bundles in the periodontal ligaments as treatment progressed. Our results came in agreement with **(Saguier et al, 2000)** and **(Almeida et al., 2015)** who stated that activation of inflammatory cells in periodontitis leads to loss of collagen fibers while healthy periodontal tissue showed a higher expression of collagen fibers.

Matrix metalloproteinases (MMPs) are important mediators of connective tissue destruction in periodontal disease, specifically MMP-1,-3.-8, and -9 **(Birkedal-Hansen et al., 1993)**. Pro-inflammatory cytokines as TNF alpha and interleukins produced during periodontal inflammation lead fibroblasts to respond by overproduction of MMPs. In our study, MMP-1 (also known as fibroblast collagenase) was selected as the immunohistochemical marker to evaluate the progression of inflammation because it was found to initiate extracellular matrix destruction and more specifically collagen degradation in cooperation with other MMPs, accounting for most of the collagenase activity performed by gingival fibroblasts **(Beklen et al., 2007)**. The high expression of MMP-1 provokes periodontal and alveolar damage in accordance with another study conducted by **(Claesson et al. 2002)**.

In the present study, MMP-1 expression was evaluated in the epithelium and lamina propria of the gingiva in accordance with **(Kubota et al., 2008)**, who observed enhanced expression of MMP-1 in gingival keratinocytes which extended to the fibroblasts, PMNLs, and macrophages of the lamina propria in periodontally diseased tissue.

In our study, immunohistochemical evaluation revealed a positive expression of MMP-1 in all 3 treatment groups. Over the course of the treatment, there was a statistically significant decrease in the expression of MMP-1 in all of the treatment groups, indicating that all regimens were capable of reducing inflammation. By the end of the treatment period, chlorhexidine showed the lowest value of immunopositivity among the treatment groups with an insignificant difference between the values of vitamin E and coconut oil.

This is in agreement with **(Singla et al, 2014)**, who found that there was a significant decrease in inflammation using both oil gum massage therapy with coconut oil and chlorhexidine gel. This is also in agreement with **(Carvalho et al, 2013)**, who found that vitamin E decreased the inflammatory response of ligature induced periodontitis. To our knowledge, however, no study has compared these 3 regimens together.

The possible mechanism for explanation of our results may be the scavenging of reactive oxygen species (ROS) by the antioxidants in the different treatment regimens tested. Given that it is an infectious disease with inflammatory cell infiltration, periodontal disease generates various kinds of ROS, which destroys the periodontal ligaments and adjacent tissues **(Nakamura et al, 1998, Yasunari et al, 2006). (Brock et al, 2004)** found that total antioxidant capacity is decreased in the gingival crevicular fluid of periodontitis patients, suggesting enhanced ROS production in the diseased tissue. Since ROS is important in the pathogenesis of periodontal diseases, the development of ROS scavengers (i.e. antioxidants) is vital to the control and prevention of periodontal disease.

Yeung et al, 2007 found that chlorhexidine effectively scavenges the superoxide radical and exhibits some antioxidant property to hydroxyl radicals of the ROS generated from periodontitis. These results suggest that chlorhexidine exhibits antioxidant properties. It is by this mechanism that chlorhexidine gains its preventive effect on inflammatory periodontal destruction. In addition to its anti-inflammatory potency derived from its

antioxidant property, chlorhexidine has long been known for its strong antibacterial property and superior substantivity in the oral cavity **(Asokan et al, 2011)**.

The anti-inflammatory potency of vitamin E can also be explained by several mechanisms. **(Schneider & Pose, 1969)** found that vitamin E was capable of reducing the inflammatory cell infiltrate in ligature induced periodontitis. Vitamin E also has the ability to inhibit neutrophil function and reduce pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. This is in accordance with **(Tahan et al, 2011)**, who found that vitamin E decreased the production of IL-1β, IL-6 and TNF-α in the colonic tissue of rats induced for ulcerative colitis. In addition to that, **(Carvalho et al, 2013)** found that vitamin E caused a decrease in superoxide dismutase (SOD) activity, implying that there was a reduction in ROS and consequently reduced oxidative stress. SOD is a powerful antioxidant enzyme in the body that catalyzes superoxide radicals and protects cells against oxidative stress that exhibit high activity when ROS levels are augmented in the tissue. Vitamin E also reduces inflammation via the inhibition of lipid peroxidation, the oxidative degradation of lipids from the cell membrane by free radicals **(Kay et al, 1986)**.

Coconut oil has also been found to exhibit its anti-inflammatory potency through several processes. Lauric acid, a medium chain fatty acid found in large quantities in coconut oil, has been found to react with sodium hydroxide in saliva during the oil gum massage procedure forming sodium laureate. This compound is responsible for the cleansing action and decreased accumulation of plaque, thereby minimizing inflammation **(Asokan et al, 2011)**. This is in agreement with **(Kaliamoorthy et al, 2018)**, who found that oil pulling with coconut oil reduced the severity of gingival inflammation as early as 7 days from the start of treatment. Most importantly, coconut oil acts by scavenging free radicals generated by oxidative burst, a process involving the production of superoxide, hydrogen peroxide and other oxidizing radicals in inflamed tissue. This is according to **(Padumadasa et al, 2016)**, who found that the the ethyl acetate soluble proanthocyanidins (EASPA) fraction of coconut oil was able to scavenge superoxide radicals, and hence exhibited antioxidant activity as well as anti-inflammatory activity similar to that of ibuprofen, which is one of the most widely used drugs for inflammatory diseases. Alongside its anti-inflammatory potency, coconut oil has also been found to have a strong antibacterial effect via monolaurin, which disrupts the lipid membranes of microorganisms **(Thaweboon et al, 2014)**.

CONCLUSIONS

1. Ligature induced periodontitis was associated with remarkable histological inflammatory reaction in the periodontal ligament.
2. Oil gum massage therapy using either chlorhexidine gel, vitamin E oil or coconut oil was associated with variable grades of statistically significant reduction of inflammation.
3. Chlorhexidine presented the greatest reduction in inflammation. However, no statistically significant difference between coconut oil and vitamin E was noted.
4. The potency of coconut and vitamin E oils as alternative therapies to chlorhexidine should be further considered and investigated.
5. Further studies with longer duration of treatment are recommended.

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