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Original Article

The Anti-inflammatory Effect of flaxseed oil on Ligature Induced Periodontitis in Mandibular Molars of Wistar Rats (An Animal Study)

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Abstract

Aim: To evaluate therapeutic anti-inflammatory effect of intraperitoneally injected flaxseed oil in Wister rats exposed to ligature induced periodontitis in mandibular right first molar. **Methodolgy:** Eighteen male Wister rats were randomized into three groups: group I (control) was supplied with intraperitoneal injection of (3 ml/Kg) of saline each other day. Periodontitis was induced for group II, III by ligature placement under general anesthesia. After four weeks, group II was injected with saline intraperitoneally (3ml/kg) each other day, for 2 weeks, while group III was injected with flaxseed oil intraperitoneally (3ml/kg) each other day, for 2 weeks. **Results:** Histolopathological examination of group I showed normal architecture of both the periodontal ligament and alveolar bone tissues. While group II (untreated) revealed disruption of periodontal ligament fibers, inflammatory cell infiltration and marked alveolar bone resorption. Group III (treated group) showed re-organization of the periodontal ligaments, less inflammatory cells infiltration, regular bone surface lined by osteoblasts and obvious reversal lines were recognized denoting new bone formation. The quantitative real-time polymerase chain reaction results of Interlekin-10, transforming growth factor-β gene expression displayed a significant decrease in their expression in group II, while increase their expression in group III. Alternatively, osteopontin gene expression displayed a significant increase in its expression in group II and decrease its expression in group III. **Conclusion:** In a rat model, intraperitoneal injection of flaxseed oil aids in resolution of mechanically induced periodontal tissue inflammation and accelerates healing process.

Keywords: Periodontitis, Flaxseed oil, rats, Interlekin-10, transforming growth factor-β, Osteopontin.

I. INTRODUCTION

Periodontal disease is a global burden affecting about 743 million people throughout the world, it is considered the primary reason for teeth loss among adults. In 2014, the World Health Organization (WHO) reported a periodontitis prevalence of more than 80% of all the studied subjects in Egypt. Although this health problem affects a large portion of the Egyptian population, no official preventive steps have been done to address it. [1,2]

Periodontal disease is characterized by an infection that destructs the supporting tissue and induces a local

production of immune-inflammatory mediators in response to periodontal pathogens and their products. Periodontal diseases affect mastication as well as other oral functions like phonetics, and esthetics considerably. It also has a severe impact on the individual social life .[3,4]

Even though antibiotics play a major positive role in the management of periodontal disease. Microbial resistance to antibiotics is a major issue implicated with inadvertent drug usage. That's why the use of herbs in the treatment of periodontal disease is now given great attention in ongoing research. [5]

Flaxseed is rich in omega-3- polyunsaturated fatty acids (ω-3 PUFA), fat, fibers, proteins, carbohydrates, vitamins and minerals. It has been proven that ω-3 PUFA has a significant role in modulating cell signaling, gene expression and inflammatory processes. Hence, flaxseed is known for its nutritional importance which shows anti- bacterial, anti-inflammatory, anti-thrombotic, anti- cancer and anti-arrhythmic properties. [6,7,8]

The rich nutritional components and therapeutic benefits of flaxseeds have brought attention to its use in the human food system. According to its physicochemical composition, oil, protein, dietary fiber, soluble polysaccharides, lignans, phenolic compounds, vitamins (A, C, F and E) and minerals (P, Mg, K, Na, Fe, Cu, Mn, and Zn) are all components of flaxseed. Flaxseed oil is considered a rich plant source in PUFA with 59.61% Omega.3 a-linolenic acid (ALA), 14% Omega-6 linolenic acid, 15% monosaturated fatty acid, oleic acid, and Omega-9 fatty acid, while it is low in saturated fatty acids (8-10%). Flaxseed has been related to the therapy of several disorders, including cardiovascular disease, high blood pressure, atherosclerosis, diabetes, cancer, arthritis, osteoporosis, autoimmune diseases, and neurological issues.[6]

In vitro studies of the effect of flaxseed oil on periodontal ligament cells showed reduction of periodontal inflammation through periodontal ligament cell proliferation by inducing growth of immature PDL cells and that in turn, accelerates periodontal regeneration [9,10].

Based on the previous findings, The current study was conducted to investigate the potential therapeutic effect of flaxseed oil in treatment of induced periodontitis in a Wister rat model.

Flaxseed oil is likely safe for most adults. Nevertheless, adding flaxseed to the diet might increase the number of bowel movements each day. It might also cause side effects such as bloating, gas, stomach-ache, and nausea. Higher doses are likely to cause more side effects.[7]

II. MATERIALS AND METHODS

a. Flaxseed oil

Flaxseed oil (100% Natural – Cold Pressed) purchased from IMTENAN company, Egypt.

b. Animals

The animal care and experimental procedures were held in the animal house, Faculty of Medicine, Cairo University according to approval and recommendation of the Institutional Animal Care and Use Committee (IACUC) (approval no. CU-III -F -26- 21), Cairo University.

Eighteen adult healthy male Wister rats of age range from 3 to 4 months and weighing about 170-200 grams were used in the present study. The animals were housed under the same housing and feeding conditions in a sterile, controlled environment (temperature 21 ± 2°, 50-60% relative humidity) and fed with standard pellets diet and tap water. The animals were randomly distributed by the Random Sequence Generating Program (random.org) into three groups (one control and 2 experimental) each including 6 animals. Implementation of the allocation was done as follow: numbers from 1 to 18 were written on folded papers that were placed in opaque sealed envelopes, matching of the rats with the numbers were done blindly through the technician in charge at the animal house, each rat was attached to its number till the end, then the numbers were opened, and the rats were allocated in their groups according to the program recommendations.

c. Sample size

Sample size calculated depending on a previous study (Zhou et al., 2020) as a reference. According to this study, IL-10 levels was estimated in both flaxseed oil treated (413.52 ± 24.47) and induced ulcerative coilitis (358.00 ± 32.92) to elucidate the anti-inflammatory potential of flaxseed oil since the anti-inflammatory effect of flaxseed oil will be the primary outcome in the current research. If the difference in the flaxseed treated and positive control means is 55.52, accordingly minimally the study needed 6 rats in each group to be able to reject the null hypothesis with 80 % power at $\alpha = 0.05$. Sample size calculation was done using http://www.biomath.info/power/prt.htm

d. Induction of periodontitis

Animals were subjected to ligature placement under general anesthesia by an intraperitoneal injection of a mixture of ketamine 300-360 mg/kg and xylazine 30-40 mg/kg as described by the American Veterinary Medical Association (AVMA) AVMA Guidelines (2020). Intrasulcular incision using blade #15, then full thickness mucoperiosteal flap reflection was done using mucoperiosteal elevator. Bone removal was done using round bur and low-speed contra from the buccal aspect. After bone removal the edges of the flap were sutured by interrupted sutures using a 4/0 black silk suture material. A piece of 4/0 black silk suture placed in submarginal position around the right mandibular 1st molar teeth and kept there for four weeks to provoke the development of plaque accumulation, inflammation and finally, induce periodontitis.[11,12]

e. Study design and grouping

The animals were divided into 3 groups, each containing 6 rats as following: Group I: Negative control group (no periodontitis will be induced), it was injected by intraperitoneal saline (3ml/kg) starting from week 4 each other day. Group II: Ligature induced periodontitis; it was injected by intraperitoneal saline (3ml/kg) starting from week 4 each other day. Group III: Ligature induced periodontitis; it was injected by intraperitoneal Flaxseed oil (3ml/kg) starting from week 4 each other day.

Starting from the same day after ligature removal (after 4 weeks), group II, the rats received normal saline intraperitoneally (3ml/kg) each other day, for 2 weeks. For group III was injected with flaxseed oil intraperitoneally (3ml/kg) [13] each other day, for 2 weeks.

At the end of experimental period (6 weeks from start of ligation placement), all animals were sacrificed by 5% Isoflurane inhalation[14]. The mandibles then dissected, separated from muscle and soft tissues. The right half of the mandible was used for histopathological examination, quantitative RT-PCR reaction for localization of Interlekin-10 (IL-10), transforming growth factor-β (TGF- β), and osteopontin (OPN) proteins, as well as histomorphometric analysis for bone dynamic parameters.

f. Histopathological examination

The fixed tissue samples were dehydrated in ascending grades of alcohol, cleared in xylene, and paraffin embedded. Sections of 4-5µm thickness were obtained then mounted on clear glass slides. Staining of the specimen was done using hematoxylin and eosin stain. Histopathological changes in the stained sections were then examined using Leica DM300 light microscopic (Leica Microsystems, Inc., Switzerland).

g. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA extraction and reverse transcription

Rat bones were stored at -80C for one day. Bone samples were transferred from -80°C storage to 2 mL Eppendorf tubes containing 1.5 mL of pre-chilled RNAlater ice (InvitrogenTM, Carlsbad, CA, USA) and then stored at -20°C for at least 16 hours before RNA isolation. Once the initial overnight soak at -20°C was complete, bone samples were removed from RNAlater ice, sectioned into small pieces, weighted, and 100 mg was placed into homogenization tubes containing 1.5 mL of pre-chilled TRIzol Reagent (InvitrogenTM). RNA samples were subjected to RNA quantitation and purity assessment using the NanoDrop® (ND)-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). Reverse transcription (RT) was carried out on total RNA in a final volume of 20 uL RT reactions using the RT kit (Qiagen, Valencia, CA, USA).

Quantitative Real-time PCR (qPCR) for Detection of IL-10, TGF-β, Osteopontin

This step was carried out using SYBR® Green PCR kit and protocol for RNAs quantitative detection (Qiagen, Valencia, CA, USA). Reaction mix was prepared for a 20 µl per well reaction volume. The real-time cycler (Rotor-gene thermocycler, Qiagen, USA) was programmed.

Primer sequences used:

IL-10:
F:5- CAGGACTTTAAGGGTTACTTGG-3
R:5-GGGGAGAAATCGATGACAGC-3

TGF β1:
F:5-CAAAGACATCACACACAGTA-3
R:5-GGTGTTGAGCCCTTTCCAGG-3

Osteopontin:
F:5_CTTCAGCTCCACAGAGAAGAACTGC3
R:5_CACGATCATGTTGGACAACTGCTCC-3
β-actin:
F:5-GATATCGCTGCGCTCGTC-3
R:5-TGGGGTACTTCAGGGTCAGG-3

h. Calculation of results

After completion of the PCR cycles, melting curve analyses were performed to validate the specific generation of the expected PCR products. All samples were normalized against β-actin expression using the ΔCt method. The cycle threshold (Ct) value is the number of qPCR cycles required for the fluorescent signal to cross a specified threshold. ΔCt for IL-10, TGF-b and Osteopontin were calculated by subtracting the Ct values of B-actin from those of target RNAs. ΔΔCt was calculated by subtracting the ΔCt of the control samples from the ΔCt of the disease samples.

i. Statistical analysis

Data have been collected, tabulated and statistically analyzed using Microsoft Excel ® 2016, Statistical Package for Social Science (SPSS)® Ver. 24. and Minitab ® statistical software Ver. 16. Data obtained from histomorphometric analysis have been statistically described in terms of mean and standard deviation. Numerical data explored for normality by checking the data distribution using Kolmogorov-Smirnov and Shapiro-Wilk tests. One Way ANOVA test was used to make comparison between the studied groups. Unpaired t-test performed to make multiple 2 group comparisons. P values less than 0.05 were considered as statistically significant.

III. RESULTS

a. Histopathological results

Histopathological sections of the first molar area of the decalcified jaw of group I (control) showed normal architecture of the interdental papilla, periodontal ligament, cementum, and alveolar bone tissues with regular surfaces and normal size and shape of the marrow cavities (Fig.1a, b). The periodontal ligament showed intact collagen fibers parallelly arranged in wavy course with numerous fibroblasts running in the same direction of the fibrous bundles with normal interstitial tissue spaces (Fig.1c). Alveolar bone exhibited normal architecture with regular surfaces, normal size and shape of the marrow cavities and abundant number of osteocytes. Blood vessels appeared more localized adjacent to the bone surface (Fig.1d).

Group II (untreated ligature induced periodontitis group) revealed disruption of periodontal ligament fibers with loss of connective tissue organization and fibrous attachment between cementum and bone surface (Fig.2a). There is massive bone resorption leaving an isolated osteo-necrotic part of inter-radicular septum comprising empty lacuna of osteocytes (Fig.2b). Marked inflammatory cell infiltration, newly formed capillaries, resorption in cementum surface, and dilated congested blood vessels were also observed (Fig.2c). Moreover, marked alveolar bone resorption was evident with prominent enlargement in the bone cavities (Fig.2d).

On other hand, group III (flaxseed treated ligature induced periodontitis group) showed re-organization and re-attachment of the periodontal ligament's fibers to both bone and cementum surfaces, with less inflammatory cells infiltration in comparison to group II and normal blood vessels appearance (Fig.3a, b). Regular bone and cementum surfaces lined by osteoblasts and cementoblast were obvious with formation of new bone bridges (Fig.3c). Also, reversal lines were recognized denoting new bone formation and normal number of osteocytes in their lacunae. Increased thickness of bone trabeculae in comparison to group II was also observed with normal size of bone marrow cavities (Fig.3d).

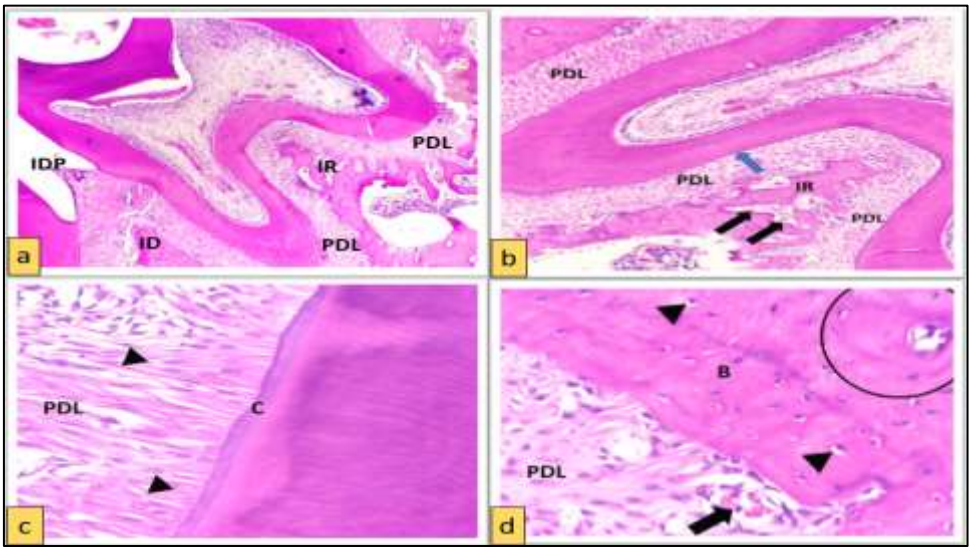

Figure 1: A Photomicrograph of the rat's lower first molar of the control group showing: (a) Normal structure of the interdental papillae (IDP), normal orientation of periodontal ligament fibers (PDL), and normal bone architecture with normal interdental (ID) & inter-radicular septa (IR) (**H & E, orig. Mag.X40**). (b) Higher magnification showing normal cementum (arrow), periodontal ligamnet (PDL) surrounding the roots of the teeth, and normal interradicular septa (IR) architecture with regular surfaces, normal size and shape of the marrow cavities (**H & E, orig. Mag.X100**). (c) Higher magnification showing well organized periodontal ligament fibers (PDL) arranged in a wavy course, fibroblasts (arrow heads), and a thin layer of cementoid tissue (C) covering the root surface (**H & E, orig. Mag.X400**). (d) Higher magnification showing well organized periodontal ligament fibers (PDL) attached to bone surface, normal bone architecture with smooth and regular surface (B), abundant number of osteocytes in their lacunae (black arrow heads), Haversian system (circle), and interstitial tissue with a small sized blood vessel (arrow) (**H & E, orig. Mag.X400**).

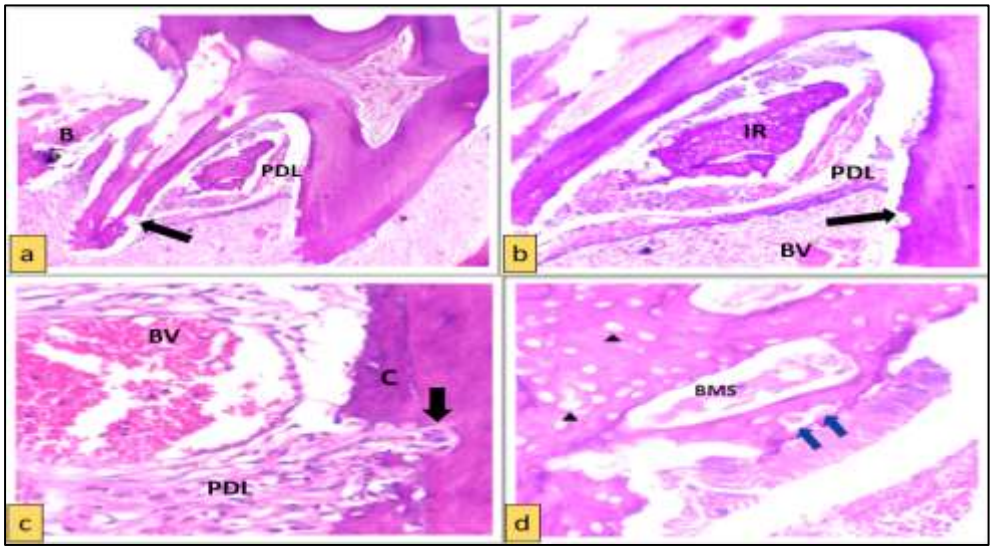

Figure 1: A Photomicrograph of the rat's lower first molar of group II showing: (a) Prominent disruption and separation of the periodontal ligaments (PDL), massive bone resorption and loss of architecture (B), and cementum resorption (arrow) **(H & E, orig. Mag.X40).** (b) Higher magnification showing isolated osteo-necrotic part of inter-radicular bone with empty lacuna of osteocytes (IR), loss of periodontal ligaments attachment (PDL), dilated blood vessels (BV) and cementum resorption (arrow) **(H & E, orig. Mag.X100).** (c) Higher magnification showing disorientation of periodontal ligaments fibers with prominent inflammatory cells infiltration within the periodontal tissue (PDL), dilated blood vessels with congested blood (BV), resorption in cementum surface (C), and cementoclasts inside their lacunae (arrow) **(H & E, orig. Mag.X400).** (d) Higher magnification showing osteoclasts in their Howship's lacunae (arrows), empty lacunae of osteocytes (arrow heads) and bone marrow spaces (BMS) filled with degenerated tissue **(H & E, orig. Mag.X400).**

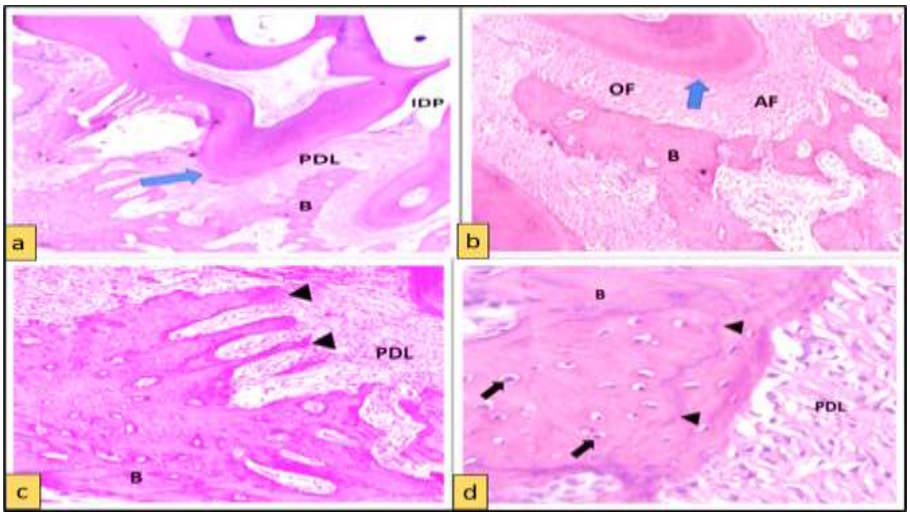

Figure 2: Photomicrograph of the rat's lower first molar of group III showing*:* (a) Re-organization of periodontal ligaments fibers (PDL), normal bone architecture (B), regeneration of the interdental papillae (IDP) and regular surface of cementum (arrow) **(H & E, orig. Mag.X40).** (b) Higher magnification showing re-attachment of both oblique fibers (OF) and apical fibers (AF) of periodontal ligaments to surfaces of cementum and alveolar bone, regeneration of bone architecture (B), smooth and regular surface of cellular cementum at the apex of the root (arrow) **(H & E, orig. Mag.X100).** (c) Higher magnification showing formation of new bone bridges (arrow heads) to reform normal architecture of bone (B) with reorganization of periodontal ligaments fibers (PDL) **(H & E, orig. Mag.X400).** (d) Higher magnification showing re-attachment of periodontal ligaments (PDL) to bone surface (B) with collagen fibers arranged in a wavy course, bone with obvious reversal lines (arrow heads) were seen denoting new bone formation with abundant number of osteocytes in their lacunae (arrows) **(H & E, orig. Mag.X400).**

b. PCR results

One-way ANOVA was used to compare levels of IL-10, TGF-β and OPN between the various groups as $P < 0.05$ was used to indicate that there was a significant difference between them. A multiple comparison Tukey's Post Hoc test was conducted afterward, and the results showed a significant difference in means with different superscript letters as $P < 0.05$ and an insignificant difference in means with the same superscript letters as $P > 0.05$.as presented in (Fig.4).

In regards of IL-10, group II was significantly the lowest, while there was insignificant difference between group I & III (fig.4a). In regards TGF- β, group II was significantly the lowest, while there was insignificant difference between group I & III (fig.4b). In regards of OPN, group II was significantly the highest, while there was insignificant difference between group I & III (fig.4c).

IV. DISCUSSION

Ligature induced periodontitis was selected to be used in this investigation as it has been used extensively as a rat model by many investigators for experimental induction of periodontitis. Meanwhile, ligature placement allows bacteria to accumulate around the ligature, leading to rapid induction periodontitis.[11,15,16,17]

In the ongoing study, periodontitis was induced by a piece of 4/0 black silk suture placed in submarginal position around the right mandibular first molar and kept there for four weeks to provoke the development of plaque accumulation, inflammation and, subsequently, induce periodontitis. This leads to soft tissue haemorrhage and erosion, and development of a periodontal pocket and bone loss surrounding the teeth. These findings showed that a model of periodontitis with a clinical course resembling that of humans has been successfully established. [18]

Cytokines play a vital role in initiation and regulation of the inflammatory process of periodontitis. There is high expression of proinflammatory cytokines such as interleukin IL-1, IL-6, IL-12, osteopontin (OPN) and TNF-α, and anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β.[19,20,21,22]

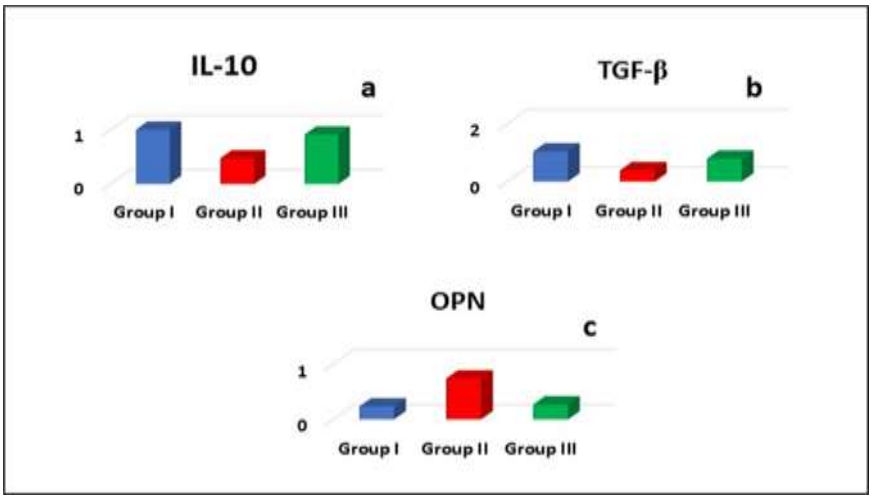

Figure 4: Comparison of the expression levels of IL-10, TGF-β and OPN between the various groups (a) bar chart showing mean of IL-10 in all groups and comparison between them. (b) bar chart showing mean of TGF-β in all groups and comparison between them. (c) bar chart showing mean of OPN in all groups and comparison between them.

Flaxseed oil was the treatment of choice to be used in the current study. This was based on what has been proved in literature that flaxseed oil is one of the richest sources of ω-3 PUFAs, which contributes to its wound healing properties. It is also known for its nutritional profile which shows anti- bacterial and anti-inflammatory properties [7].

The histopathological results of group II came in agreement with Galvão et al.; Di Paola et al.; Cekici et al.; and Garcia et al., 2016 [23,24,25,26]. Their results showed; increase in number of inflammatory cells, looseness and disorganization of PDL fiber bundles, in addition to abundant congested blood vessels. They suggested that the host- mediated response in periodontal disease results in the activation of the innate immunity, specifically by upregulation of proinflammatory cytokines like IL-1, 1L-6 and TGF-α from monocytes/polymorphonuclear leukocytes, and down regulation of growth factors from macrophages.

The histopathological results of group III (flaxseed treated group) showed re-organization and re-attachment of the periodontal ligament's fibers to both bone and cementum surfaces, with less inflammatory cells infiltration in comparison to group II and normal blood vessels appearance. It was observed that treatment with flaxseed oil can stimulate the healing process by inducing proliferation of fibroblast cells in the granulation tissue, elevation of collagen levels and lowering cell damage, this might be attributed to the presence of high flavonoids content of flaxseed which has a potent antioxidant activity via the reduction of oxidative stress [27].

Group III results were in coincidence with that of Draganescu et al. [28] who mentioned that flaxseed oil inhibits cell necrosis, elevates angiogenesis and increase blood flow which allows more nutrients transportation efficiently that crucial to tissue regeneration. This could be owing to the high content of lignans and phenolic compounds that possess antimicrobial and antioxidant properties enabling the flaxseed in scavenging of the free radicals and reactive oxygen species.

The results of the here in study were also in line with Zhu et al. [29] who investigated the anti-inflammatory properties of dietary flaxseed oil on streptozotocin induced diabetic rats. It has been shown that supplementation of flaxseed oil for 8 weeks caused a significant decrease in the levels of proinflammatory cytokines including IL-1β and TNF-α. This was attributed to high content of ω-3 PUFAs (rich in ALA) which has been shown to have

anti-inflammatory properties. According to statistical findings from qRT-PCR, group II (untreated group) had significantly less IL-10 than group III (treated group). This was in line with findings by Luo et al., and Zhang et al.[30,31] indicating that low levels of IL-10 may provoke the inflammatory response, accelerate alveolar bone resorption and reduce bone formation, as well as promote gingival enlargement and the development of periodontal disease.

The level of IL-10 increased significantly in qRT-PCR results for group III in comparison to group II. This was in agreement with Al-Za'abi et al.[32] who stated that the administration of flaxseed oil increased the concentration of IL-10 level in streptozotocin induced diabetic rats. Similar findings were reported by Bashir et al.[33] who evaluated the effect of flaxseed oil supplementation on adipose tissue inflammation in mice model. the severity of periodontitis in humans, found the opposite of the findings of the present investigation. According to their study, TGF-β may play a significant role in the development and progression of periodontitis and bone resorption. As the authors showed, the degree of periodontitis increased when TGF-β level increased. This could be attributed to the difference in the study model selection.

There was a marked elevation in the level of TGF-β in qRT-PCR results for group III. These findings supported results obtained by Morshedzadeh et al. [36] who stated that the patients who received flaxseed oil have showed marked increase in TGF-β and reduced levels of the inflammatory markers.

Concerning OPN level; there was a marked increase in group II in comparison to group III. This was in line with Sharma & Pradeep[37] who showed that the concentrations of OPN level in gingival crevicular fluid increased gradually from healthy to periodontitis groups. The results of the herein study, was also in accordance with that of Kido et al.[38] who reported increasing OPN levels in in gingival crevicular fluid with the progression of periodontal disease.

They found that flaxseed oil treatment results in inhibition of pro-inflammatory cytokines (IL-2, TNF-α and IFN-γ) production and upregulation of anti-inflammatory cytokines (IL-4, IL-10).

Concerning TGF-β; Its level was significantly decreased in group II in comparison to group III. This was in agreement with Al-Rubaie [34] who stated that TGF-β contributes to regulation and remodeling of the inflammatory events during periodontal disease. This might be owing to the fact that TGF-β over expression could lead to excessive deposition of collagen by exceeding its physiological effect on healing by increasing the number of fibroblasts and increasing their capacity for synthesis of collagen.

In contrast to herein research Li et al.[35] who observed the association between TGF-β and

In the present study, results of qRT-PCR revealed significant decrease in OPN expression in group III as compared to the group II which indicates that flaxseed oil has an anti-inflammatory potential. These results in line with Karcher et al.[39] who proved that supplementation of flaxseed oil on cytokine gene expression in cows lead to reduced OPN level in acute inflammation.

V. CONCLUSION

According to the results of the present study, it could be concluded that intraperitoneal injection of flaxseed oil results in obvious anti-inflammatory effect on resolution of mechanically induced periodontitis by ligature placement in Wister rats. Also, elevation in the amount of the anti-inflammatory markers (IL-10, TGF-β) is a strong indicator for healing process of periodontitis and reduction in OPN cytokine level which indicates reduction in rate of bone resorption and decrease number of osteoclasts.

Recommendation

Flaxseed oil is recommended to be produced in different formulation products to

enable its usage in treatment of periodontitis. The dietary control using fatty acids in the form of flaxseeds and its oil can help in the reduction of periodontal inflammation. Consequently, further long-term prospective studies involving a different sample size and different dosages are recommended to be carried out before its use in clinical trials and consequently, its clinical applications could be further expanded.

Conflict of Interest

The authors declare no conflict of interest.

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Ethics

This study protocol was approved by the ethical committee of the faculty of dentistry-Cairo university on: July 2021, approval number: CU-III -F -26- 21.

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