Impact of Ozonated Olive Oil on Neutrophil Cells in Periodontitis: Experimental Insights from Male Wistar Rats

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ABSTRACT

Background: *Periodontal tissue, comprising gingiva, periodontal ligament, cementum, and alveolar bone, supports and surrounds teeth. Periodontitis, an inflammation affecting these tissues, manifests through gingival inflammation, pocket formation, and loss of attachment and bone.This research aims to investigate whether ozonated olive oil influences neutrophil cell levels in the gingiva of male Wistar rats with periodontitis.*

Method: *Employing a posttest-only control group design, this laboratory study included 24 male Wistar rats with concentrated periodontitis divided into six groups based on the day of observation. Histological analysis was conducted on days 1, 3, and 5, with three control groups and treatment groups.*

Result: *The hypothesis test revealed a significant difference in neutrophil cell count between the ozonated olive oil treatment group and the control group ($p < 0.05$). The treatment group exhibited superior outcomes compared to control and other treatment groups, as confirmed by the LSD Post Hoc Test ($p < 0.05$).*

Conclusion: *Administration of ozonated olive oil can influence neutrophil cell involvement in periodontitis healing among male Wistar rats, suggesting its potential therapeutic efficacy.*

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INTRODUCTION

Periodontal tissue was the intricate tissue serves as a crucial support system for our teeth, encompassing various components such as the gingiva, periodontal ligament, cementum, and alveolar bone[1]. However, this support structure can be compromised by periodontitis, one of the most prevalent periodontal diseases[2]. Periodontitis wreaks havoc on the connective tissue and alveolar bone, leading to significant destruction and undermining the stability of our teeth. Characterized by inflammation in the tissues surrounding the teeth, periodontitis presents itself through distinct clinical signs, including gingival inflammation, the formation of pockets, loss of periodontal attachment, and deterioration of alveolar bone. Today, we delve into the complexities of this condition and explore potential avenues for its management and treatment[3].

The occurrence of inflammation activates leukocytes. Leukocytes are cells that function as a defense system against infectious disease agents. Leukocytes are divided into several cells, one of which is neutrophil cells[4]. Neutrophil cells are the first cells to migrate to blood vessels during inflammation so that these cells function as the body's defense against microorganisms[3]. Ozonated olive oil was effective in reducing inflammation. The ozonides formed during the ozonation process help to modulate the body's inflammatory response. By decreasing the production of pro-inflammatory cytokines, it can reduce swelling, redness, and pain associated with various inflammatory conditions

Olive oil was a versatile and health-promoting ingredient with a rich composition of healthy fats, antioxidants, and essential nutrients. Its origins in the Mediterranean ensure high quality and traditional production methods, while its indications for use range from culinary applications to health and beauty benefits. Ozonated Olive Oil contains carbon double bonds in unsaturated fatty acids which results in the formation of many oxygenated compounds. This compound helps in the wound healing process, related to antimicrobial activity and activation of the NFkB transcription factor which controls growth factor genes such as *Vascular Endothelial* (VEGF) and *CyclinD1* which play an essential role in wound healing[5].

Oxygen compounds that enter the blood vessels increase oxygenation in areas of tissue that are inflamed, so the number of blood vessels increases [6], Applying ozonated olive oil will improve oxygenation and counteract the damaging anoxic phase of tissue trauma, allowing normal tissue repair to occur[7,8]. The purpose of this study is to ascertain how much ozonated olive oil affects the quantity of neutrophil cells in the gingiva of male Wistar rats who are suffering from periodontitis. It is hoped that ozonated olive oil will become an alternative ingredient for curing periodontitis for patients who are hypersensitive to chemical therapy and patients with systemic diseases.

RESEARCH METHOD

This study encompasses two control groups: a treatment group and a standard control group, employing an actual experimental laboratory research design with a posttest-only control group approach. Conducted at the Laboratory of Pharmacology and Anatomical Pathology, Faculty of Medicine, Muhammadiyah University, Surakarta, the research received approval from the Health Research Ethics Committee of RSUD Dr. Moewardi under No. 739/V/HREC/2023.

The ozonated olive oil used in the research was PurO3 LLC, Fayetteville, AR USA, which consists of 100% organic Olea europaea (olive) and activated oxygen (ozone). This ozonated olive oil was applied using a micropipette in an amount of 0.1 ml around the subgingival area of the lower jaw incisors of male Wistar rats

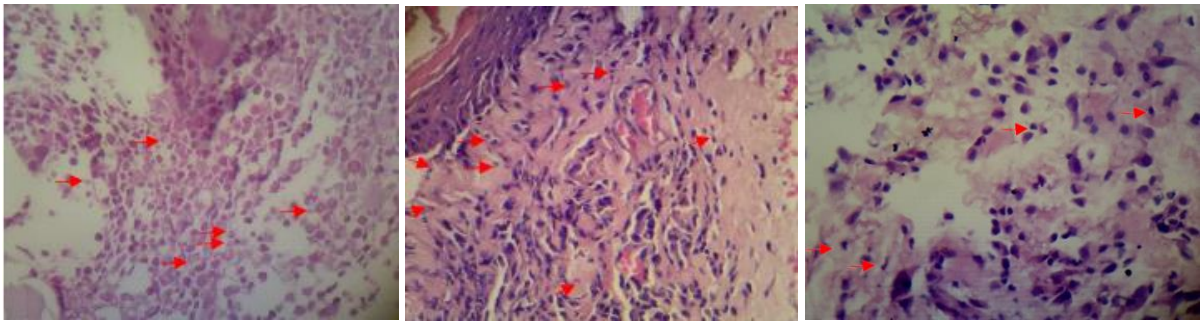
suffering from periodontitis. The experimental animals used were male Wistar rats aged 2 months, weighing 150-200 grams, obtained from the Pharmacology Laboratory, Faculty of Medicine, Muhammadiyah University, Surakarta. Induction of periodontitis in Wistar rats was carried out by anesthesia first using an intramuscular injection of ketamine hydrochloride (0.2 ml/200g body weight) to cause a sedative effect.[7]. After that, periodontitis was induced using a 3.0 mm silk ligature tied to the subgingiva of the mandibular incisors with ties forming a figure of eight. Then, the Porphyromonas Gingival bacteria was applied using an insulin syringe and irrigated on the silk ligature that had been installed in the subgingiva of the rat's mandibular teeth for every 2 days. After the occurrence of periodontitis on the 7th day the silk ligature is removed. The treatment group of rats was treated with periodontitis for 5 days with 0.1 ml of Ozonated Olive Oil irrigation in the gingival sulcus.

On days 1, 3, and 5 after treatment the animals were sacrificed by decapitation. The gingival tissue of the jaw carrying the anterior teeth that has been treated is taken. The periodontitis-affected tissue is fixed using 10% formalin and left for 24 hours. The tissue is cut using a surgical scalpel with a thickness of 4 mm. The water contained in the tissue is removed using an automatic tissue processor. The next stage, namely the dehydration stage using 70% to 100% alcohol, is carried out in phases to remove remaining tissue in the fixation process. They are cleaning remaining alcohol by clearing xylol. After infiltration, the tissue is placed in a base mold filled with liquid paraffin blocks at a temperature of 57°C. The hardened paraffin blocks were cut using a microtome with a thickness of ±3-4 µm. The resulting pieces are put into water and then placed on the slide. Next, incubate using a hot plate for 15 minutes to evaporate the water in the tissue. Then, the incubated slides were stained using Hematoxylin Eosin (HE), then the mounting process was carried out using DPX liquid. The slide was covered with a desk glass and then labeled for each sample preparation[8].

Observations of neutrophil cells were carried out using a light microscope with 400x magnification on tissue preparations that had been stained using H&E staining and counted using image raster software. Neutrophil cell observations were carried out at the Anatomical Pathology Laboratory, Faculty of Medicine, Muhammadiyah University, Surakarta. Each preparation was observed in five different fields of view in the gingival sulcus and connective tissue under the junctional epithelium and sulcular epithelium. The average of the observation results was calculated and analyzed using One Way Anova and LSD.

RESULTS

The results of the research were observed under a microscope and images of gingival tissue and neutrophil cells attached to the connective tissue were visible.



a) **b)** **c)**

Figure 1. Neutrophil cells magnification 400 times a) day 1 b) day 3 c) day 5

Table 1 presents the average neutrophil cell counts in male rats afflicted with periodontitis, depicting the mean counts for each group over the course of five days. Statistical analysis confirms the normal distribution of data for all groups (p>0.05) and homogeneity in data variance (p<0.05).

Table 1. Average Results (Mean ± SD) Number of Neutrophil Cells

Group	Mean ± SD Number of Neutrophil Cells		
	Day 1	Day 3	Day 5
K (-)	48.00±2.8	40.00±2.8	32.00 ±3.6
K(+)	32.00±7.1	28.00±4.3	24.00 ±1.6
OZO	35.00 ±1.9	30.00±2.5	24.00±1.6

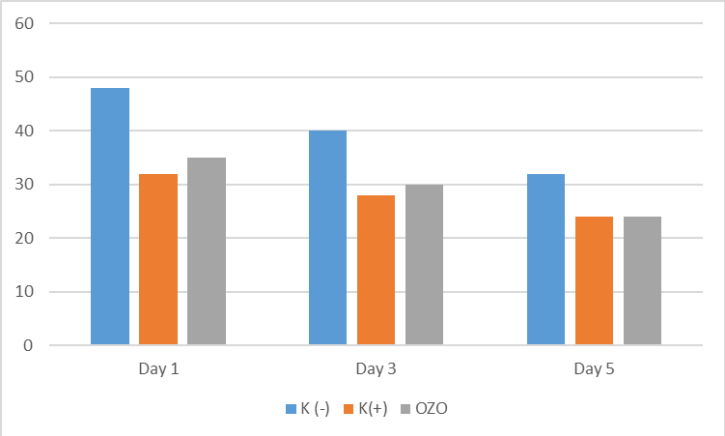


Figure 2. Number of Neutrophil Cells

The calculations presented in Table 1 indicate a reduction in neutrophil cell numbers as the observation period progresses. Specifically, the negative control group exhibited the highest neutrophil count on day 1, averaging 48 cells. Contrasting this, the treatment groups showcased declines in neutrophil counts, with differences of 16 and 13 on day 1, 12 and 10 on day 3, and 8 and 8 on day 5.

Table 2. Results of LSD Test

Group	K (-) 1	K (-) 3	K (-) 5	K(+) 1	K(+) 3	K(+) 5	OZO 1	OZO 3	OZO 5
K (-) 1	-	0.124	0.004*	0.004*	0.000*	0.000*	0.016*	0.001*	0.000*
K (-) 3	0.124	-	0.124	0.124	0.025*	0.004*	0.330	0.058	0.004*
K (-) 5	0.004*	0.124	-	1.000	0.434	0.124	0.557	0.695	0.124
K(+) 1	0.004*	0.124	1.000	-	0.434	0.124	0.557	0.695	0.124
K(+) 3	0.000*	0.025*	0.434	0.434	-	0.434	0.176	0.695	0.434
K(+) 5	0.000*	0.004*	0.124	0.124	0.434	-	0.038*	0.244	1.000
OZO 1	0.016*	0.330	0.557	0.557	0.176	0.038*	-	0.330	0.038*
OZO 3	0.001*	0.058	0.695	0.695	0.695	0.244	0.330	-	0.244
OZO 5	0.000*	0.004*	0.124	0.124	0.434	1.000	0.38*	0.244	-

The Parametric One Way Anova test yielded a significant p-value of p=0.00 (p<0.05), indicating notable differences among the positive, negative, and inert control groups. After the data is collected, it is examined using the Post Hoc LSD test as a follow-up measure to determine whether days and treatment groups exhibit statistically significant variations. In the Post Hoc Test, the control group (-), day one and day 1, 3, 5 treatment

groups, day three and day 3, and 5 treatment groups, and day 5 with day 5 of the treatment group were shown to vary significantly (p-value <0.05). Day 1 and Day 1 of the therapy group showed a significant difference ($p < 0.05$). Significant difference with a p value <0.05 between the treatment group on day 1 and day 1 of the control group (-) and day 5 of the control (+); day 3 with day 1, 3 control groups (-); and day 5 with days 1, 3, and 5 of the control group (-). These results show that the ozonated olive oil treatment group had fewer neutrophils than the control group.

DISCUSSION

The observed differences in neutrophil cell counts among the groups of male Wistar rats with periodontitis suggest that ozonated olive oil exerts an influence on neutrophil levels in gingival tissue. A lower score in neutrophil cell count correlates with reduced inflammation. This effect is supported by the One Way Anova test, which yielded a significant p-value of P=0.00 ($p<0.05$), indicating that ozonated olive oil had an effect on neutrophil cell count relative to the positive or negative control group that was significant between these mouse groups. In this study, the positive control was chosen Chlorhexidine gluconate 0.2%. This is because Chlorhexidine is a mouthwash that has been proven to inhibit the growth of oral bacteria[9]. Chlorhexidine is effective as an antiplaque so it can prevent caries[10]. The mechanism of Chlorhexidine as an antibacterial is by increasing the permeability of bacterial cell membranes and coagulating cytoplasmic macromolecules[11].

Periodontitis caused by Porphyromonas gingivalis cause a chronic inflammatory disease affecting the tissues surrounding the teeth, is associated with a dysbiotic oral microbiome. Individuals with diabetes often exhibit a different oral microbiome compared to non-diabetic individuals, with increased levels of periodontal pathogens. This altered microbiome is believed to contribute to both the onset and progression of periodontitis. The presence of periodontal pathogens triggers an inflammatory response in the gums, leading to tissue destruction and bone loss. This inflammation can spill over into the systemic circulation, contributing to the systemic inflammation observed in diabetes. Chronic inflammation from periodontitis can increase insulin resistance, making it more difficult for diabetic patients to control their blood glucose levels. The systemic inflammatory mediators released from periodontal tissues can interfere with insulin signaling pathways. Diabetic patients with periodontitis show increased production of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6, which contribute to both periodontal destruction and insulin resistance.

The bulk of leukocytes drawn to inflammatory tissue, such as the gingival crevice or periodontal pocket, which is home to numerous subgingival bacterial populations, are neutrophils (≥95%). The concentration of chemokines and adhesion molecules in normal human gingiva rises from the layer of neighboring epithelial basal cells towards the outer surface, forming the gingival floor. When bacteria cause gingival tissue to become inflamed, the tissue reacts with extracellular matrix binders like intercellular adhesion molecules and guides the recruitment of neutrophils from the junctional epithelial circulation to the gingival crevice. This process is known as chemotactic and hapotactic chemical properties. The neutrophil recruitment process is necessary for homeostasis of inflamed tissue[12].

Placing a silk ligature in the gingival sulcus surrounding the lower incisor teeth of rats functions to help the plaque buildup process so that the inflammatory process caused by bacteria (*P.gingivalis*), which has previously been induced becomes faster, so it can cause periodontitis. In other words, the use of silk ligature

can help speed up the inflammation process in periodontal tissue[13]. Clinical evidence shows that in inflammation neutrophils are responsible for inflamed tissue and their number neutrophil cell count shows a positive correlation with the severity of periodontitis[14]. The excessive activity or surplus of neutrophils can lead to connective tissue deterioration due to the release of inflammatory and toxic substances, such as reactive oxygen species (ROS). These compounds, including collagenase, can contribute to the onset of bone resorption by degrading connective tissue[15,16]. Antibody-mediated depletion of neutrophils reduces periodontitis in experimental mice, whereas uncontrolled neutrophil cells worsen periodontitis in experimental mice[12].

A decrease in neutrophil chemotaxis is linked to periodontitis. Dysfunctional neutrophil chemotaxis may worsen neutrophil-mediated collateral host tissue damage[16]. The lack of neutrophils in the tissue is brought on by poor recruitment and extravasation, which may result in long-term inflammation in the periodontal tissue and the breakdown of the alveolar bone. Regardless of whether an excess of neutrophils in the tissue is a cause of periodontitis, this illustrates the critical role that neutrophil balance plays in maintaining periodontal homeostasis[17]. Table one displays the average neutrophil cell counts obtained in this study, revealing that the highest count across all groups occurred on day 1. There is an increase in the number of neutrophil cells on the first day because neutrophils are the first defense cells whose numbers increase at the start of inflammation as a sign of the body's response. Neutrophils then invade the inflamed tissue area within the first few hours after inflammation begins. This inflammatory phase begins with hemostasis and platelet clotting[18]. These platelets release Platelet-Derived Growth Factor (PDGF) and Transforming Growth Factor Beta (TGF-β) which function to attract neutrophil cells and macrophages to areas experiencing inflammation. Transformation was causing the neutrophil cells to increase on day one or before 24 hours[19]

The lowest number of neutrophil cells is also shown in table 1 and figure 2, namely, in the group of mice treated with ozonated olive oil it was lower on the 5th day compared to the control group, as in previous research, the treatment group was more effective. Due to the use of ozonated olive oil as an antimicrobial agent and is able to stimulate blood circulation and immune responses in inflamed tissue. Olive oil's ozone concentration has the potential to influence collagen formation by inducing the release of growth factors[20]. Reactive oxygen species (ROS) ozone mediates the formation of new blood vessels, increasing the number of blood vessels and facilitating the transfer of oxygen to the injured tissue area via the blood vessels. Additionally, the active inflammatory response releases pro-inflammatory cytokines, prostaglandins, and interleukin-1 (IL-1), which in turn helps to reduce inflammation, speed up wound healing, and increase tissue metabolism, thereby increasing tissue oxygenation[21.22]. To ensure that tissue healing proceeds without hiccups, the damaging anoxic phase of tissue damage and alveolar bone can be neutralized by increasing the oxygen content of inflamed tissue and injecting ozonated olive oil[7].

Our results unveiled a significant disparity in neutrophil cell numbers, particularly within the gingival tissue of male Wistar rats suffering from periodontitis and subjected to ozonated olive oil treatment. This discrepancy was underscored by a significance value of $p=0.00$ ($p<0.05$). Remarkably, this suggests that ozonated olive oil holds the potential to augment blood flow to inflamed tissue, thereby facilitating the delivery of vital nutrients, oxygen, inflammatory cytokines, and white blood cells essential for expediting the healing process.Moreover, our exploration extended beyond mere observation, delving into the efficacy of ozonated olive oil irrigation following scaling and root planing (SRP). The amalgamation of these procedures yielded promising outcomes, notably altering the landscape of periodontal flora. This effect can be attributed to the

antibacterial properties inherent in ozonated olive oil, which disrupt bacterial cytoplasmic membranes via ozonolysis of double bonds, consequently inducing alterations in intracellular contents. Consequently, this mechanism serves to mitigate the virulence population of specific periodontal infections, heralding a potential breakthrough in periodontal treatment modalities[23].

The data that has been carried out by the One Way Anova analysis test is then analyzed using a follow-up test, namely the Post Hoc LSD test, which aims to find out whether the control and treatment groups show significant differences. Our analysis unraveled compelling insights into the efficacy of ozonated olive oil in modulating neutrophil counts, particularly when compared to the negative control group devoid of medication. On day 1, the administration of ozonated olive oil led to a substantial reduction in neutrophil numbers, with an average difference of 13 compared to the control group without medication. This trend persisted on day 3, with the treatment group exhibiting a notable decrease of 10 neutrophils compared to the untreated control group. By day 5, this disparity further accentuated, with the treatment group showcasing an average difference of 8 neutrophils fewer than the control group without medication. These findings underscore the potent anti-inflammatory properties of ozonated olive oil, indicative of its ability to mitigate neutrophil infiltration into inflamed tissues. By orchestrating a reduction in neutrophil counts, ozonated olive oil holds promise as a therapeutic intervention for periodontitis, potentially ameliorating the inflammatory response and fostering a conducive environment for tissue healing[24.25], The significance level of $p<0.05$ observed in our study signifies a robust statistical foundation, compelling us to reject the null hypothesis (H0) and accept the alternative hypothesis (H1). This pivotal decision is grounded in the tangible decrease in neutrophil numbers subsequent to the administration of ozonated olive oil. Such a decline not only validates the efficacy of ozonated olive oil as a therapeutic agent but also provides compelling evidence of its ability to influence cellular immunity and the humoral system. Neutrophils serve as primary responders to inflammatory stimuli, orchestrating an immune response vital for combating infections and promoting tissue repair. The observed reduction in neutrophil counts post-ozonated olive oil administration underscores the oil's capacity to modulate the intricate balance of cellular immunity. By diminishing the influx of neutrophils into inflamed tissues, ozonated olive oil exhibits a nuanced regulatory effect on the immune system, potentially mitigating excessive inflammation and fostering a more harmonious immune response. Furthermore, the implications extend beyond cellular immunity to encompass the humoral system, which orchestrates the production and circulation of antibodies essential for immune defense. The ability of ozonated olive oil to elicit such a profound impact on neutrophil numbers suggests its potential to modulate broader aspects of immune function, including antibody production and immune signaling pathways.[26], the immunomodulatory properties of ozonated olive oil extend beyond the direct regulation of neutrophil activity, encompassing a broader enhancement of immune function. By stimulating the proliferation of immunocompetent cells and augmenting the synthesis of immunoglobulins, ozonated olive oil holds promise as a multifaceted therapeutic agent for addressing periodontal inflammation and promoting optimal oral health [27], activates macrophages[28,29]and increases the sensitivity of microorganisms to phagocytosis[30]. By boosting oxygenation and lowering local inflammatory processes, this enhances the metabolism of inflammatory tissues. [31].

CONCLUSION

The study's findings suggest that, in comparison to the control group, the number of neutrophil cells in the gingiva of male Wistar rats with periodontitis may be impacted by the application of ozonated olive oil.

Suggestions that can be given are that further research needs to be done regarding the concentration of ozone contained in olive oil that is effective and safe for healing periodontitis.

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