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Application of Ozonated Olive Oil as Adjunctive Therapy After Periodontal Pocket Curettage Towards Collagen Density of Alveolar Bone in Periodontitis Healing Process (In VivoStudy with *Sprague dawley*)

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ABSTRACT

Periodontitis is an inflammatory disease of the supporting tissue of the teeth caused by specific microorganisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with periodontal pocket formation. The application of ozonated olive oil in dentistry is based on the actions such as antimicrobial and therapeutic agents, needed as adjunctive therapy after periodontal pocket curettage. Collagen is the main constituent of the alveolar bone extracellular matrix and is needed as a scaffold in the formation of a mineralized matrix. This study aimed to determine the density of collagen in alveolar bone on the periodontitis healing process after adjunctive topical application of ozonated olive oil in periodontal pocket curettage. In this study, 32 *Sprague dawley* rats were randomly divided into two groups: curetted-topical application of ozonated olive oil as treatment groups and curetted-topical application of 1% CMC-Na as the placebo group. Periodontitis induced by placing silk-ligature around submandibular incisors for 7 days. Subsequently, the rats were sacrificed on days 3, 5, 7, and 14 after curetted and topical application, and each group was represented by four rats. The staining was done using Mallory staining method. All the results were statistically analyzed using Kruskal-Wallis and Mann-Whitney tests. The results of the Kruskal-Wallis test showed that the length of time of application affects the density of collagen ($p<0,05$) and there were significant differences ($p<0,05$) in the density of collagen between two groups based on Mann-Whitney test. The study concluded that adjunctive topical application of ozonated olive oil after periodontal pocket curettage significantly increases the density of collagen in alveolar bone on the periodontitis healing process in *Spraque dawley.*
Key words: periodontitis; curetted; ozonated olive oil; collagen; *Sprague dawley*

INTRODUCTION

Periodontitis is defined as an inflammatory disease of supporting tissues of teeth caused by specific micro-organisms or groups of specific microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with periodontal pocket formation, gingival recession, or both (Newman *et al.,* 2012). The resorption of the alveolar crest bone initiated by periodontitis can gradually lead to tooth loss and edentulousness (Xu *et al.,* 2014).

One of the procedures to eliminate local factors in periodontal disease is curettage, which can reduce inflammation and periodontal pocket. Curettage is a scraping procedure on lateral walls of the periodontal pockets which aim to remove calculus, bacteria, and granulation tissues that are expected to form new attachments (Newman *et al.,* 2012). The limitations of curettage are mainly in accessing furcation areas, convexed teeth, and distal areas of molars. Local addition of antibiotics is more effective at accelerating healing than curettage alone. The use of antibiotics has a risk of developing bacteria that are resistant to antibiotics (Aoki *et al.,* 2004; Johnson and Perez, 2010).

Ozone (O_3) is a natural gaseous molecule made up of three oxygen atoms. It leads to lysis of the cell membrane of most of the bacteria that cause dental problems due to its oxidant and oxidizer properties (Kumar and Chandni, 2021). The word ozone originates from the Greek word ozein, which means odor and was first used by German chemist Christian Friedrich Schonbein, father of ozone therapy in 1840 (Gopalakrishnan and Parthiban, 2012). Ozone therapy opens a new vista in the treatment of dental problems due to its atraumatic, biologically based treatment (Kumar and Chandni, 2021). Potential applications of ozone in the clinical practice of dentistry ~~is~~ are based on the actions such as antimicrobial: bactericidal, viricidal, and fungicidal; anti-inflammatory; immunomodulating, biosynthetic:

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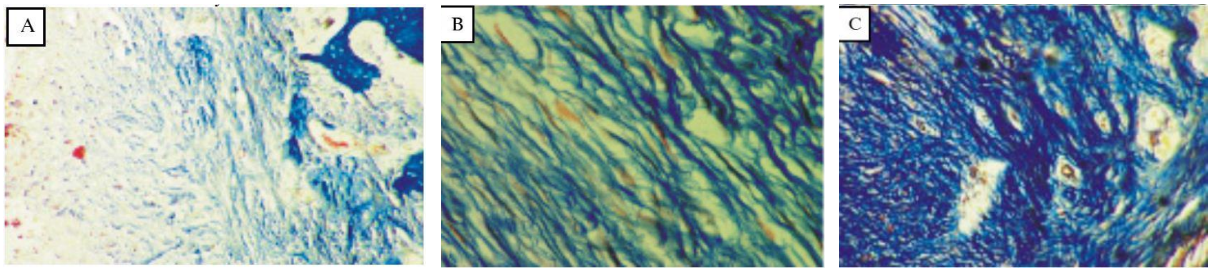

Figure 1. Criteria for valuation collagen density A=score 1, B=score 2, C=score 3 (Tandelilin *et al.,* 2006)

activation of the metabolism of carbohydrates, proteins, lipids; bioenergetic; antihypoxic; analgesic; and hemostatic effects (Ahmed *et al.,* 2013). In stomatology, three different forms of ozone are used: gaseous ozone, ozonated water, and ozonated oil (sunflower oil, olive oil, groundnut oil) (Lubojanski *et al.,* 2021). Ozone is an unstable gas that can only last a few minutes and cannot be stored, if it is dissolved in oil it can last for years. Ozonated olive oil (O3) is pure olive oil that has been ozonized using a mixture of ozone-oxygen flow in a ratio of 5: 95% until olive oil changes from a greenish liquid to a white gel (Shoukheba and Ali, 2014).

Ozonated olive oil by topical application increases the number of alveolar bone osteoblasts, blood vessels in the healing process of periodontitis on *Sprague dawley* (Herawati *et al.,* 2020). Collagen is the main constituent of the bone extracellular matrix (Sudatri, 2010). Collagen provides elasticity and structure for the component tissues (Viguet-Carrin *et al.,* 2006). Type I collagen is produced at high levels by differentiated osteoblasts and is required for the formation of a mineralized bone matrix. Collagen synthesis in bone is modulated by a variety of hormones, growth factors, and cytokines, some of which are produced locally by osteoblasts. Insulin, insulin-like growth factor, and transforming growth factor-β increase type I collagen synthesis (Kream and Lichtler, 2011). This study aimed to determine the density of collagen in alveolar bone on the periodontitis healing process in adjunctive topical application of ozonated olive oil after periodontal pocket curettage. A successful parameter in the wound healing process and bone formation process is the presence of collagen (Andriani *et al.,* 2020). Increased collagen deposition in the wound area is generally related to the faster wound healing process (Putra *et al.,* 2020).

METHODOLOGY

Materials

Ozonated olive oil was obtained from PurO3 LLC (Fayetteville, AR USA). Male *Sprague dawley* rats obtained from Laboratorium Penelitian dan Pengujian Terpadu (LPPT) Universitas Gadjah Mada, Yogyakarta, Indonesia.

Methods

The study was approved by the Committee for Research Ethics and Integrity of the Dentistry Faculty Universitas Gadjah Mada (Registration number: 00462/KKEP/FKG-UGM/EC/2015). In this study, 32 *Sprague dawley* rats were randomly divided into two groups: placebo and treatment groups. Rats were injected with ketamine hydrochloride (0.2 mL/ 200 g body weight) intra-muscular in the upper thigh to give a sedative effect before induced periodontitis by placing silk-ligature around sub-mandibular incisors. Tissues inflammation was curetted after 7 days inducing periodontitis, ozonated olive oil (PurO3 LLC, Fayetteville, AR USA) was applied to treatment groups and 1% Sodium Carboxymethylcellulose (CMC-Na) was applied to placebo groups, twice a day to mandible incisors periodontal pocket using explorer tip (Herawati *et al.,* 2020).

The rats were sacrificed on days 3, 5, 7, and 14 after topical application, and each group was represented by four rats. The staining was done using Mallory staining method. The observation on collagen fiber density was conducted on five view fields under light microscope with the 4(X) 100 objectives by two observers. Collagen appears to be light blue to dark blue with varied densities (Herawati *et al.,* 2020). Criteria for valuation are: score 1 showed collagen fiber density is less than 50% with less dense tissue structure, vascularization, mononuclear cells, and many cells can be found (Figure 1-A); score 2 showed collagen fiber density is more than 50% with more dense

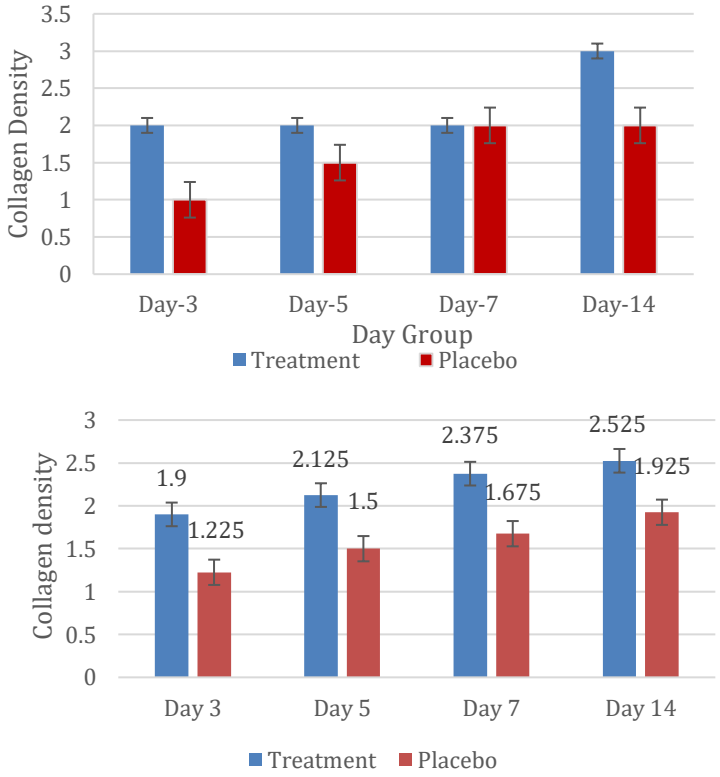

Figure 2. The increasing pattern of the collagen density based on observation days between the treatment group and placebo group

Table I. Results of the Kruskal–Wallis test on the mean and standard deviation of collagen density in treatment and placebo groups

Observation days	Treatment	Placebo
Day 3	1.900±0.496	1.225±0.423
Day 5	2.125±0.404	1.500±0.506
Day 7	2.375±0.586	1.675±0.474
Day 14	2.231±0.552	1.925±0.474
Asymp. Sig. (p)	0.000*	0.000*

*p < 0.05

tissue structure, less inflammatory reaction (Figure 1-B); and score 3 showed avascular and acellular collagen fibrous density (Figure 1-C) (Tandelilin *et al.,* 2006; Andriani *et al.,* 2020). All the results were statistically analyzed using Kruskal-Wallis and Mann-Whitney tests.

RESULT AND DISCUSSION

Means and standard deviations of the collagen density treatment and placebo in each group were based on observation days (Figure 2).

Kruskal-Wallis test was conducted to determine the difference of collagen density between days groups in ozonated olive oil treatment groups or 1% CMC-Na placebo groups. The results of Kruskal-Wallis test on of ozonated olive oil treatment groups and 1% CMC-Na placebo groups obtained p = 0.000 (p <0.05), there was a significant difference in collagen density between day 3, 5, 7, and 14 in ozonated olive oil treatment groups and 1% CMC-Na placebo groups. Based on the results of the Kruskal-Wallis test, it can be concluded that the length of time of application affects the density of collagen.

The next comparison test was Mann-Whitney test. It was used to determine the difference of collagen density between ozonated olive oil treatment group and the 1% CMC-Na placebo group in each day group. The result of Mann Whitney test shown p value <0.05, collagen density between ozonated olive oil treatment group and 1% CMC-Na placebo group was significantly different on days 3, 5, 7, and 14.

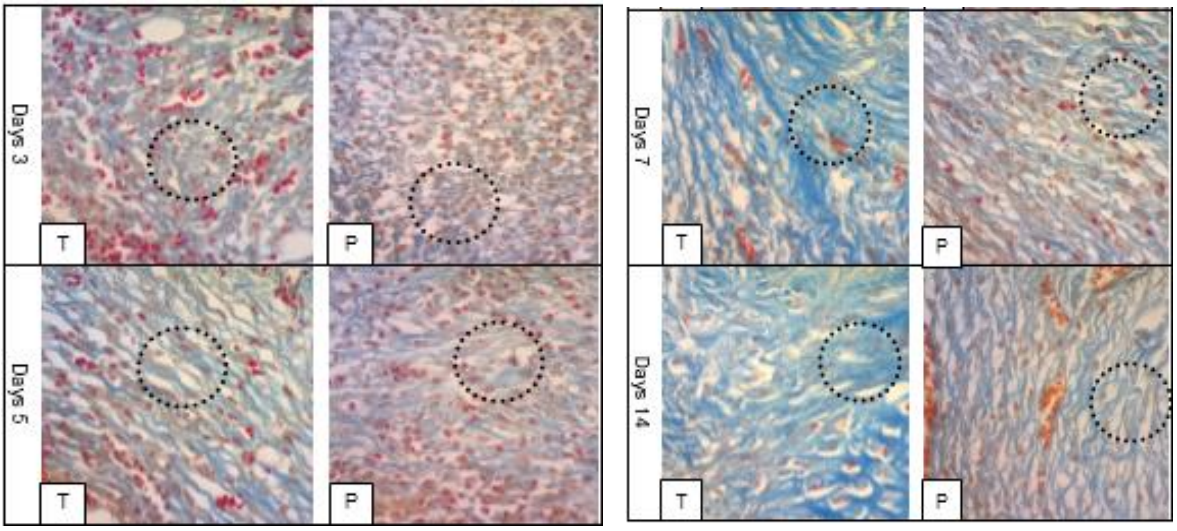

Figure 3. Histological microphotograph of collagen density in alveolar bone on treatment groups (T) and placebo groups (P), 400(X) magnifications.

Table II. Results of the Mann Whitney test on the mean and standard deviation of collagen density between treatment and placebo groups in each day groups

Day Groups	Asymp. Sig. (p)
Day 3	0.000*
Day 5	0.000*
Day 7	0.000*
Day 14	0.000*

*$p < 0.05$

The result showed collagen density increase from days 3, 5, 7, and continue up to days 14 after treatment. The increase of collagen density was clarified by Kruskal-Wallis test, so it can be concluded that there was a significant effect of topical application of ozonated olive oil on the density of collagen in alveolar bone on the periodontitis healing process.

Collagen density of 1% CMC-Na placebo groups increased during the normal healing process. The tissue healing process occurred overlapping in 4 phases: vascular response, inflammation response, proliferation, and maturation. Angiogenesis in vascular response stimulated by macrophages activity and hypoxia during injury. Macrophages produced many substances to stimulate angiogenesis, such as transforming growth factor (TGF) and tumor necrosis factor (TNF). Transforming growth factors increased tissue formation and TNF stimulated proliferation. Angiogenesis was a fundamental process in the formation of new bone. The bloodstream was responsible for nutritious substances delivery and bone progenitor cells recruitment (Flanangan, 2000; Mangano *et al.,* 2015).

The difference of collagen density between ozonated olive oil treatment groups and 1% CMC-Na placebo groups due to ozone contained in ozonated olive oil can stimulate the release of some growth factors, including PDGF, TGF-β1, IL-8, and TBX2. These growth factors regulated a series of bone formation processes and wound healing processes. Transforming Growth Factor (TGF-β1) also had an important role in the initial phase regulation and coordination of the wound healing process. Transforming growth factor-β1 (TGF-β1) was important in cell proliferation, chemotaxis, angiogenesis, extracellular matrix synthesis, and collagen synthesis (Filippi, 2001; Re *et al.,* 2010). Ozone oil also activated fibroblasts by increasing the critical genes (collagen-I, α-SMA, and TGF-β1) so fibroblasts' activities were gradually enhanced during the initial stage of new tissue formation and ozone oil promoted the wound healing via regulating the fibroblast functions. Fibroblast produces extracellular matrix (Collagen, Elastin fibers, and Reticular fibers). These fibers fill the

wound cavity and provide scallops for keratinocyte cell migration and osteoblasts (Xiao *et al.,* 2017; Ismardianita *et al.,* 2020)

Days 3 healing process was dominated by the end of the inflammatory phase. Macrophages migrated to the area of bone damage to phagocytes and secrete growth factors that will affect directly increased the synthesis of collagen, osteocalcin, and alkaline phosphate by osteoblasts. Collagen fibers synthesis on day 3 was primarily mediated by IL-4 from the macrophage. Furthermore, in the proliferation phase, osteoblasts start synthesized collagen by stimulation of TGF-β, especially type III collagen fibers. Collagen deposition seen in this phase was arranged randomly (Hernandez-Gill *et al.,* 2006; Polimeni *et al.,* 2000; Fratzl, 2008).

Based on Mann-Whitney test, there was a significant difference in collagen density between ozonated olive oil treatment groups and 1% CMC-Na placebo groups. The difference was due to the content of the application materials.

Ozonated olive oil contained three molecules of oxygen that stabilized in the form of ozonides and bind with unsaturated fatty acids. ozonated olive oil was used extensively because it had a therapeutic and antimicrobial effect. Olive oil was obtained from the olive tree and had a high content of oleic acid (65-85%). Ozone would react chemically with carbon double bond substances of unsaturated fatty acids and produced ozonide molecules. Ozonide is effective as an antimicrobial agent and stimulates tissue repair and regeneration at the cellular level. Ozonide molecules bind to cellular substances with double bonds in cells, body fluids, or tissues. These interactions produced derivates that acted as a second messenger in enzymes and chemical mediators activation (Kim *et al.,* 2009; Mosallam *et al.,* 2011; Schwartz and Sanchez, 2012).

Sodium Carboxymethylcellulose (CMC-Na) did not contain active substances that can affect healing. Sodium Carboxymethylcellulose (CMC-Na) was an additional material used in various industries as a coagulant agent. Carboxymethylcellulose (CMC) did not react in the body so it did not affect the periodontitis healing process (Tjay and Rahardja, 2007; Kamal, 2010; Reeves *et al.,* 2010).

Days 5 after application showed that there were significant differences in collagen density between ozonated olive oil treatment groups and 1% CMC-Na placebo groups. Ozone stimulated the release of growth factors that directly affected collagen synthesis by osteoblasts. The interaction of ozone with body fluid stimulated the formation of new blood vessels through a mechanism mediated by Reactive Oxygen Species (ROS) and increased inflammatory responses through the release of proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). Interleukin-1 (IL-1) stimulated proliferation and differentiation of pre-osteoblasts into osteoblasts to synthesize collagen (Hernandez-Gill *et al.,* 2006; Franscino *et al.,* 2013).

Days 7 after application showed the density of collagen in ozonated olive oil treatment groups and 1% CMC-Na placebo groups increased compared to days 3 and 5. The synthesis of collagen would continue to increase from days 3 up to 2 weeks after injury. Days 14 after application showed the highest density of collagen than days 3, 5, and 7 (Figure 2). This was due to the remodeling phase that occurred on day 14, the synthesis of collagen type III being replaced by collagen type I that had a ribbon-shaped and stronger tensile strength. Reorganization and cross-linking arrangement of collagen that occurred in remodeling phase provided strength and density of new tissue so the collagen density in days 14 denser than day 3, 5, and 7 (Kiani *et al.,* 2014; Sabirin *et al.,* 2013).

The results of the study demonstrated that osteoblasts number increased from day 3, 5, 7, and continued up to day 14 both in ozonated olive oil treatment and placebo group, which indicated by the observation time that affected the number of osteoblasts significantly. The number of blood vessels peaked on day 7 decreased up to day 14, both in the treatment and the placebo group (Herawati *et al.,* 2020). The collagen density continued up to day 14, the pattern like to osteoblast.

CONCLUSION

The study concluded that topical application of ozonated olive oil as adjunctive therapy in periodontal pocket curettage significantly increases the density of collagen in alveolar bone on the periodontitis healing process in *Spraque dawley.*

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