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Comparison of Antibacterial Activity of Ozonated Olive Oil and Chlorhexidine Gluconate

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Comparison of Antibacterial Activity of Ozonated Olive Oil and Chlorhexidine Gluconate

by

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A Thesis submitted in partial satisfaction of
the requirements for the degree
Master of Science in Periodontics

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Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

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ABSTRACT

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Master of Science, Graduate Program in Periodontics
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Aim: The aim of this in vitro disk diffusion susceptibility test is to compare the antibacterial activity of ozonated olive oil (OzOO) with chlorhexidine gluconate (CHX) against periodontal pathogens, *Porphyromonas gingivalis* and *Aggregatibacter Actinomycetemcomitans*.

Materials and Methods: The antibacterial activity of two antiseptics, OzOO and CHX, and, two controls, olive oil and normal saline was assessed by disk diffusion susceptibility test. For disk diffusion susceptibility test, two periodontal pathogens, 20 petri dishes for *P. gingivalis* and 18 petri dishes for *A. actinomycetemcomitans*, were used. To assess the antibacterial activity, the diameters of the growth inhibition zone were measured with a caliper.

Results: The diameters of the growth inhibition zone against *P. gingivalis* were 31.4 ± 5.05 mm for OzOO and 20.9 ± 1.19 mm for CHX ($p<0.001$). The diameters of the growth inhibition zone against *A. actinomycetemcomitans* were 25.9 ± 1.69 mm for OzOO and 18.4 ± 1.55 mm for CHX ($p<0.001$). All the measured diameters for olive oil and normal saline against both periodontal pathogens were 6 mm, with no sign of the growth inhibition.

Conclusion: The antibacterial activity of OzOO against *P. gingivalis* and *A. actinomycetemcomitans* is superior to that of CHX.

CHAPTER ONE

INTRODUCTION AND REVIEW OF THE LITERATURE

In the mouth, teeth provide hard and non-shedding surfaces for the development of bacterial deposits. This bacterial colonization, dental plaque, is considered as the primary cause of periodontal disease and caries. Hundreds of different bacterial species are found in dental plaque. Among these bacteria, three red complex bacteria, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannnerella forsythia* and *Aggregatibacter actinomycetemcomitnas* are believed to be the major etiologic elements for the development of periodontal disease (1). These species have been recognized as the important periodontal pathogens in many scientific articles and also in the consensus report from the World Workshop of Periodontology (2).

The primary goal in the treatment of periodontal disease is the anti-infective therapy to eliminate oral pathogens, stop disease progression and maintain the long-term periodontal stability. Therapeutic regimes to achieve these aims include various combinations of oral hygiene techniques, scaling and root planing (SRP) as an initial non-surgical treatment, surgical elimination of periodontal pockets and anatomical defects, and, correction of inadequate dental restorations which aid bacterial retention and interfere with plaque removal.

The bacterial etiology of periodontal disease (3-5) and the hypothesis that specific bacteria are involved (6) have intensified the interest of using anti-bacterial agents as a complement to non-surgical and surgical periodontal therapy (7, 8). Among anti-bacterial agents, chlorhexidine gluconate (CHX) is often used as the

local vehicle. It has shown to inhibit the growth of various bacterial species isolated from subgingival plaque (9). Clinically, it has been widely used, as the antiseptic agent in the mouthrinse. Clinical trials also have been performed with daily use of CHX mouthrinse for up to 2 years (10, 11). In addition, a CHX chip product is also available as an adjunct to SRP (12, 13).

However, the prolonged use of CHX may cause epithelial desquamation, tooth staining, altered taste sensation, impairment of wound healing and reduced attachment of fibroblasts to root surfaces (14, 15).

Ozone is currently discussed as a possible alternative oral antiseptic agent with fewer side effects. Ozone is bactericidal, anti-viral and anti-fungal. When ozone is infused into unsaturated oil such as olive oil, it becomes ozonated olive oil (OzOO) and releases hydrogen peroxide. OzOO has been reported to be used topically for the treatment of war wounds, gingivitis, abscess, anaerobic and herpetic infections, fungal disease, trophic ulcers and burns, anal fissures, furunculosis and vulvo-vaginitis (16, 17). It has been hypothesized that the action of ozonated oil on wound healing may be connected in part to its antimicrobial effect but also to its ability to promote some growth factors, activate local anti-oxidant mechanisms and promote tissue repair (18).

Clinical effects of topical application of OzOO have also been reported. In one study, the efficacy of OzOO on free gingival graft surgical wounds was evaluated. A significant improvement in epithelial healing and gingival health after topical application to the surgical sites was reported (19). Also, the efficacy of OzOO as a

mono-therapy and an adjunct to SRP in the treatment of chronic periodontitis improved the periodontal conditions (20).

In another study, OzOO showed significantly greater growth inhibition against *P. gingivalis* and *Staphylococcus aureus* than 0.2% CHX, using a disk diffusion susceptibility test (21). However, the evidence of OzOO as an alternative to CHX is still lacking. To evaluate the antibacterial activity for both chronic and aggressive periodontitis, the effect of OzOO and CHX on red complex bacteria and *A. actinomycetemcomitans* should be assessed. In addition, the effect of another commercially available OzOO and CHX should be assessed.

The aim of this in vitro disk diffusion susceptibility test is to compare the antibacterial activity of a commercially available OzOO with CHX against two periodontal pathogens, *P. gingivalis* and *A. actinomycetemcomitans*. The null hypothesis is that there is no significant difference of the antibacterial activity between two antiseptics, OzOO and CHX, against two periodontal pathogens, *P. gingivalis* and *A. actinomycetemcomitans*.

CHAPTER TWO

MATERIAL AND METHODS

Microbial Species

Freeze-dried bacteria were obtained from Division of Microbiology and Molecular Genetics, School of Medicine, Loma Linda University. The bacteria strains were: *P. gingivalis* ATCC® 33277™ and *A. actinomycetemcomitans* ATCC® 33384™.

Culture Conditions

Frozen and isolated *P. gingivalis* was inoculated into the culture tube containing Brain-Heart Infusion (BHI) medium consisted of 18.5 g dehydrated BHI, 5 ug/ml hemin, 100 ul vitamin K, 2.5 g yeast extract and 0.5 g DL-cystein. Then the tube was incubated at 37˚c under anaerobic conditions (5% CO_2, 10% H_2, 85% N_2) for 24 hours.

Frozen and isolated *A. actinomycetemcomitans* was also inoculated in the culture tube having same medium and incubated at 37˚c under the same anaerobic conditions for 24 hours.

Antiseptics

The following antiseptics were tested: OzOO (PurO3®, Promolife Inc., Fayetteville, AR, USA) with a peroxide value of 224 meq/Kg (Figure 1), and, 0.12% CHX (Peridex®, 3M ESPE, St. Paul, MN, USA).

Figure 1. Ozonated olive oil (PurO3®, Promolife Inc., Fayetteville, AR, USA)

In addition to these two antiseptics, two control groups were tested: Olive oil (Promolife Inc., Fayetteville, AR, USA), and, normal saline (0.9% Sodium Chloride Irrigation, USP®, Baxter, Irvine, CA, USA).

Disk Diffusion Susceptibility Test

6 mm-diameter sterile disks (Antibiotic Sensitivity Disks, Blank, Sterile®, Carolina Biological Supply Company, Burlington, NC, USA) (Figure 2) were prepared and impregnated with two test groups, OzOO and CHX, and two control groups, olive oil and normal saline. 50 ul of each group was impregnated into each disk using a micropipette. Semi solid-liquid OzOO was warmed up in the heating bath circulator to be converted into liquid immediately prior to impregnation for ease of application. Then, all the sterile disks were kept for 24 hours for fully impregnation in room temperature.

Figure 2. 6 mm-diameter sterile disk (Antibiotic Sensitivity Disks, Blank, Sterile®, Carolina Biolgical Supply Company, Burlington, NC, USA)

Microbial suspensions, *P. gingivalis* and *A. actinomycetemcomitans*, were aseptically spread on the 20 petri dishes for *P. gingivalis* and 18 petri dishes for *A. actinomycetemcomitans*, respectively. Each dish contained BHI agar consisted of 18.5 g dehydrated BHI, 5 ug/ml hemin, 100 ul vitamin K, 2.5 g yeast extract, 0.5 g DL-cystein and 10 g bacto agar.

Previously impregnated sterile disks were placed onto BHI agar surface. In one petri dish, four disks impregnated with each group, OzOO, CHX, olive oil and normal saline respectively, were placed onto each quadrant. Overall, 144 total sterile disks were used for this in vitro study.

All the petri dishes spread with *P. gingivalis* and *A. actinomycetemcomitans* respectively with four disks were then incubated at 37˚c under anaerobic conditions (5% CO_2, 10% H_2, 85% N_2) for 48 hours.

The antibacterial activity was assessed by measuring the diameters of the growth inhibition zone surrounding each disk with a caliper in millimeters, after 48 hours of incubation period. When the shape of the growth inhibition zone surrounding disks was not a circle, the shortest and longest diameters were measured and the mean measurement was calculated as a representative diameter.

Statistical Analysis

The diameters of the growth inhibition zone of four groups against for each microbial suspension, *P. gingivalis* and *A. actinomycetemcomitans* were compared using Kruskal-Wallis test at alpha level of 0.05. Since there was no effect of olive oil and normal saline, a paired t-test was used to compare the effect of OzOO and CHX on each microbial suspension, *P. gingivalis* and *A. actinomycetemcomitans* at alpha level of 0.05.

CHAPTER THREE

RESULTS

The diameters of the growth inhibition zone of all groups against *P. gingivalis* and *A. actinomycetemcomitans* were measured. The mean and standard deviation (SD) of all petri dishes are presented in Tables 1, 2 and 3.

The mean ± SD diameters of the growth inhibition zone against *P. gingivalis* were 31.4 ± 5.05 mm for OzOO, and, 20.9 ± 1.19 mm for CHX (Figure 3 and 4). The mean ± SD diameters of the growth inhibition zone against A. actinomycetemcomitans for OzOO and CHX were 25.9 ± 1.69 mm and 18.4 ± 1.55 mm respectively (Figure 3-8). All the measured diameters for olive oil and normal saline against both microbial suspensions were 6 mm, same as the diameter of a sterile disk, with no sign of the growth inhibition (Figure 5-8).

While the growth inhibition zone for CHX was a full circle shape in all petri dishes, it was not always for OzOO. In addition, the growth inhibition zone for OzOO and CHX against *A. actinomycetemcomitans* was fully and/or partially blurred (Figure 7, 8). The growth inhibition zone for OzOO was not fully clear but blurred, and the degree of blurring varied between petri dishes. The growth inhibition zone for CHX was a clear circle with a blurred edge.

Kruskal-Wallis test showed a significant difference of the growth inhibition zone between the groups. A paired t-test was conducted to compare the growth inhibition zone for OzOO and CHX against both *P. gingivalis* and *A. actinomycetemcomitans*. For both microbial suspensions, there was a significant difference between two groups with $p<0.001$ (Table 4).

Table 1. Diameters in millimeters of the growth inhibition zone for OzOO, CHX, olive oil and normal saline against *P. gingivalis*

No. of petri dish	Diameter					
	CHX	OzOO			Olive oil	Normal saline
		Short	Long	Mean		
1	21.2	30.3	38.6	34.5	6	6
2	21.8	31.0	41.5	36.3	6	6
3	23.5	33.8	47.1	40.5	6	6
4	21.7	29.7	39.2	34.5	6	6
5	21.7	31.9	47.6	39.8	6	6
6	21.3	31.3	41.2	26.3	6	6
7	20.0	25.6	29.1	27.4	6	6
8	20.3	28.6	29.3	29.0	6	6
9	22.2	32.6	25.9	34.3	6	6
10	19.4	28.2	31.3	29.8	6	6
11	19.5	26.5	28.1	27.3	6	6
12	19.4	24.5	25.9	25.2	6	6
13	19.7	29.8	32.4	31.1	6	6
14	19.4	28.8	29.6	29.2	6	6
15	22.0	31.2	31.7	31.5	6	6
16	19.6	18.8	18.8	18.8	6	6
17	21.7	32.0	33.6	32.8	6	6
18	21.8	29.7	31.6	30.7	6	6
19	20.0	23.5	32.3	27.9	6	6
20	20.8	32.1	32.1	32.1	6	6

Table 2. Diameters in millimeters of the growth inhibition zone for OzOO, CHX, olive oil and normal saline against *A. actinomycetemcomitans*

No. of petri dish	Diameter					
	CHX	OzOO			Olive oil	Normal saline
		Short	Long	Mean		
1	19.1	26.7	26.7	26.7	6	6
2	20.0	24.1	24.1	24.1	6	6
3	18.9	26.1	26.1	26.1	6	6
4	19.9	27.8	27.8	27.8	6	6
5	18.4	26.9	26.9	26.9	6	6
6	20.0	24.5	24.5	24.5	6	6
7	20.9	27.2	27.2	27.2	6	6
8	18.9	27.9	27.9	27.9	6	6
9	17.2	20.9	21.4	21.2	6	6
10	18.2	22.1	29.0	25.6	6	6
11	17.8	25.0	27.3	26.2	6	6
12	18.5	22.1	25.8	24.0	6	6
13	18.2	24.5	26.6	25.6	6	6
14	20.1	26.2	28.0	27.1	6	6
15	15.8	27.0	27.0	27.0	6	6
16	15.6	24.0	25.6	24.8	6	6
17	18.1	24.6	28	26.3	6	6
18	15.8	22.1	32.5	27.3	6	6

Table 3. Mean diameter and SD of the growth inhibition zone for OzOO, CHX, olive oil and normal saline against *P. gingivalis* and *A. actinomycetemcomitans*

Antiseptic	*P. gingivalis*		*A. actinomycetemcomitans*	
	Mean	SD	Mean	SD
CHX	20.9	1.19	18.4	1.55
OzOO	31.4	5.05	25.9	1.69
Olive oil	6	0	6	0
Normal saline	6	0	6	0

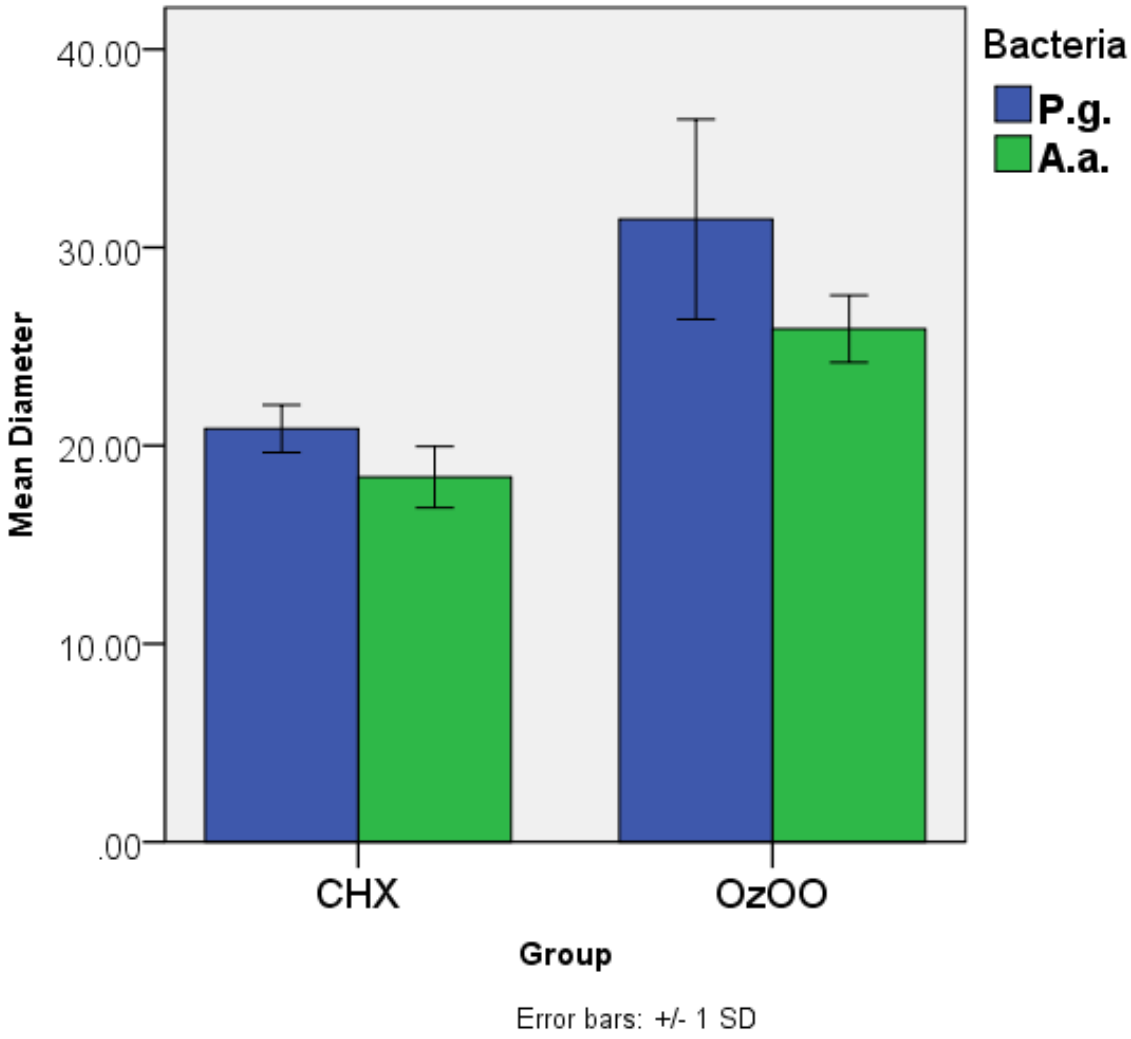

Figure 3. Bar chart of the mean diameter of the growth inhibition zone with error bars representing SD for OzOO and CHX against *P. gingivalis* and *A. actinomycetemcomitans*

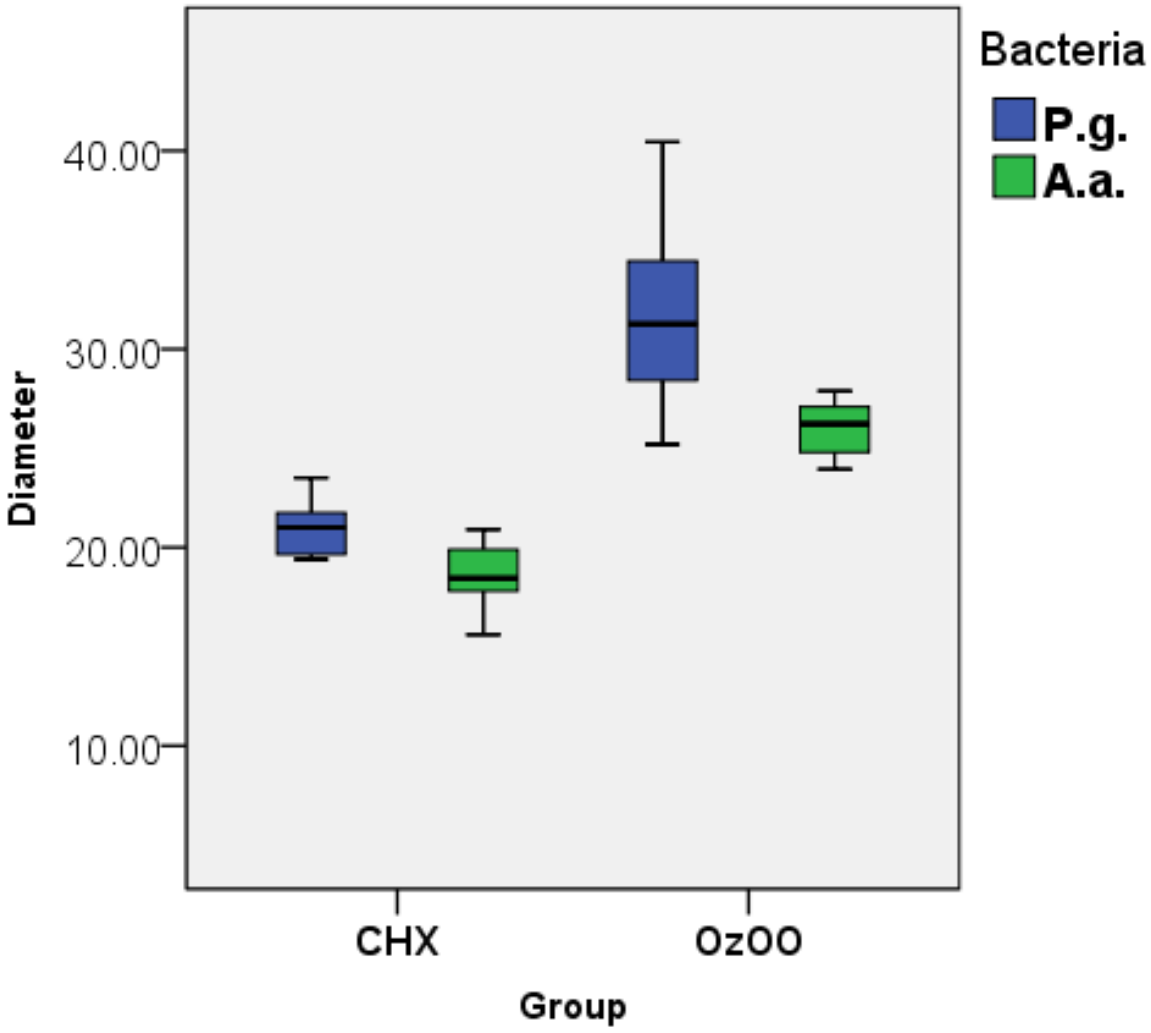

Figure 4. Box-and-whisker plot of the mean diameter of the growth inhibition zone with median, 25% and 75% quartiles, and, minimum and maximum for OzOO, CHX, olive oil and normal saline against *P. gingivalis* and *A. actinomycetemcomitans*

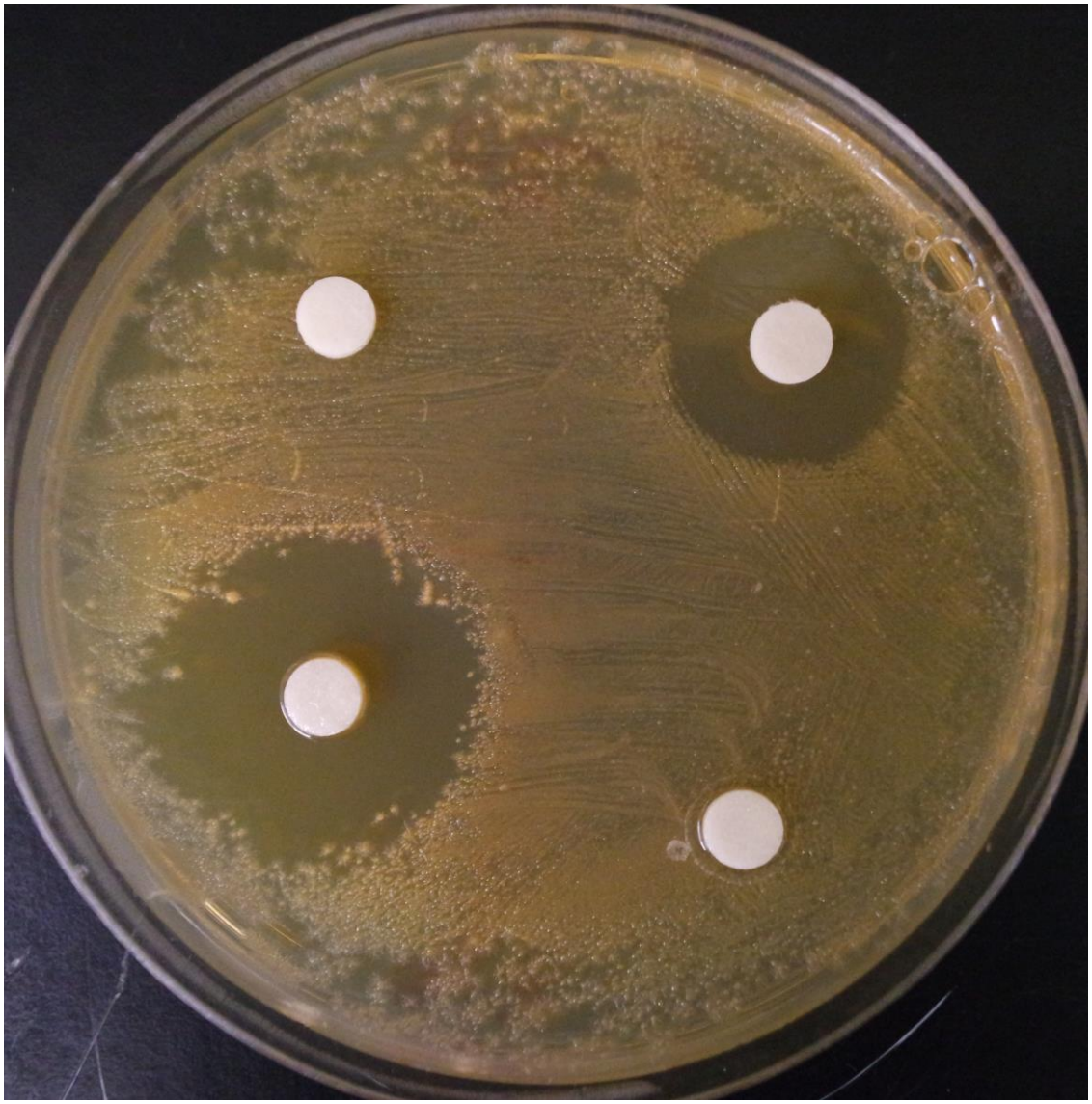

Figure 5. Petri dish (dish no. 12) for *P. gingivalis*, upper left quadrant – sterile disk impregnated by normal saline, upper right quadrant – sterile disk impregnated by CHX, lower right quadrant – sterile disk impregnated by olive oil, lower left quadrant – sterile disk impregnated by OzOO

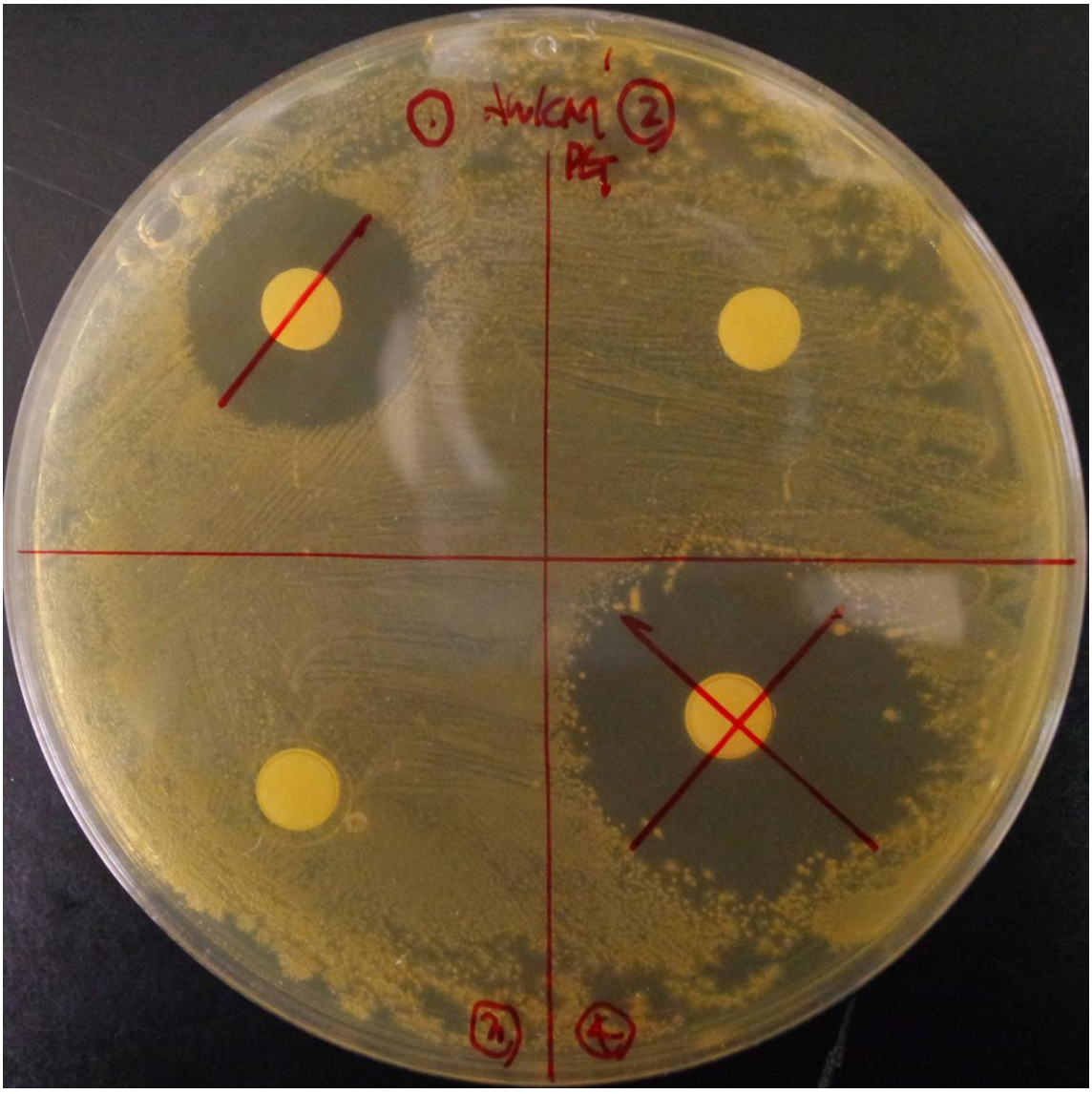

Figure 6. Petri dish (dish no. 12) for *P. gingivalis*, for measuring the diameter, upper left quadrant – sterile disk impregnated by CHX, upper right quadrant – sterile disk impregnated by normal saline, lower right quadrant – sterile disk impregnated by OzOO, lower left quadrant – sterile disk impregnated by olive oil

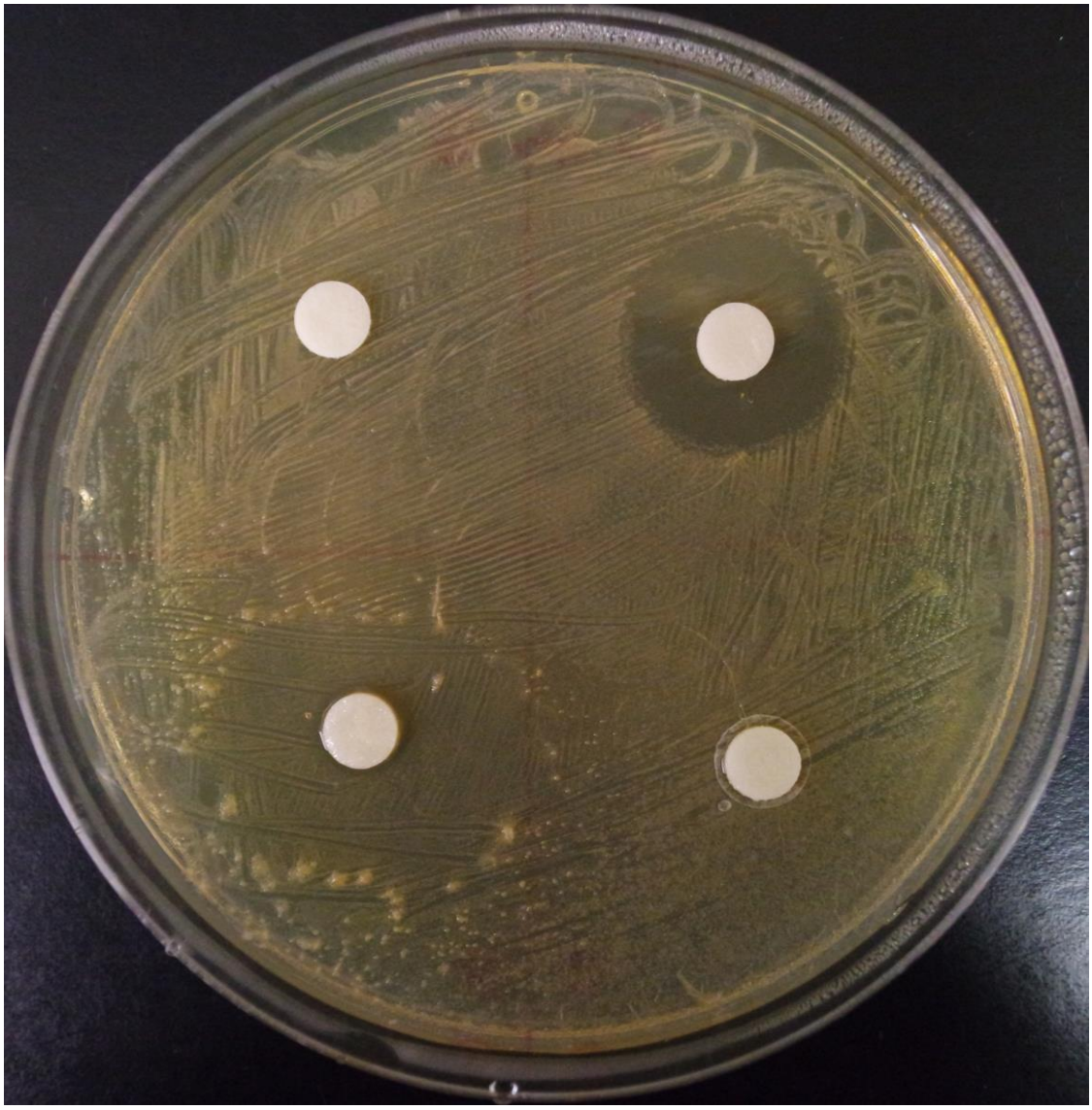

Figure 7. Petri dish (dish no. 12) for *A. Actinomycetemcomitans*, upper left quadrant – sterile disk impregnated by normal saline, upper right quadrant – sterile disk impregnated by CHX, lower right quadrant – sterile disk impregnated by olive oil, lower left quadrant – sterile disk impregnated by OzOO

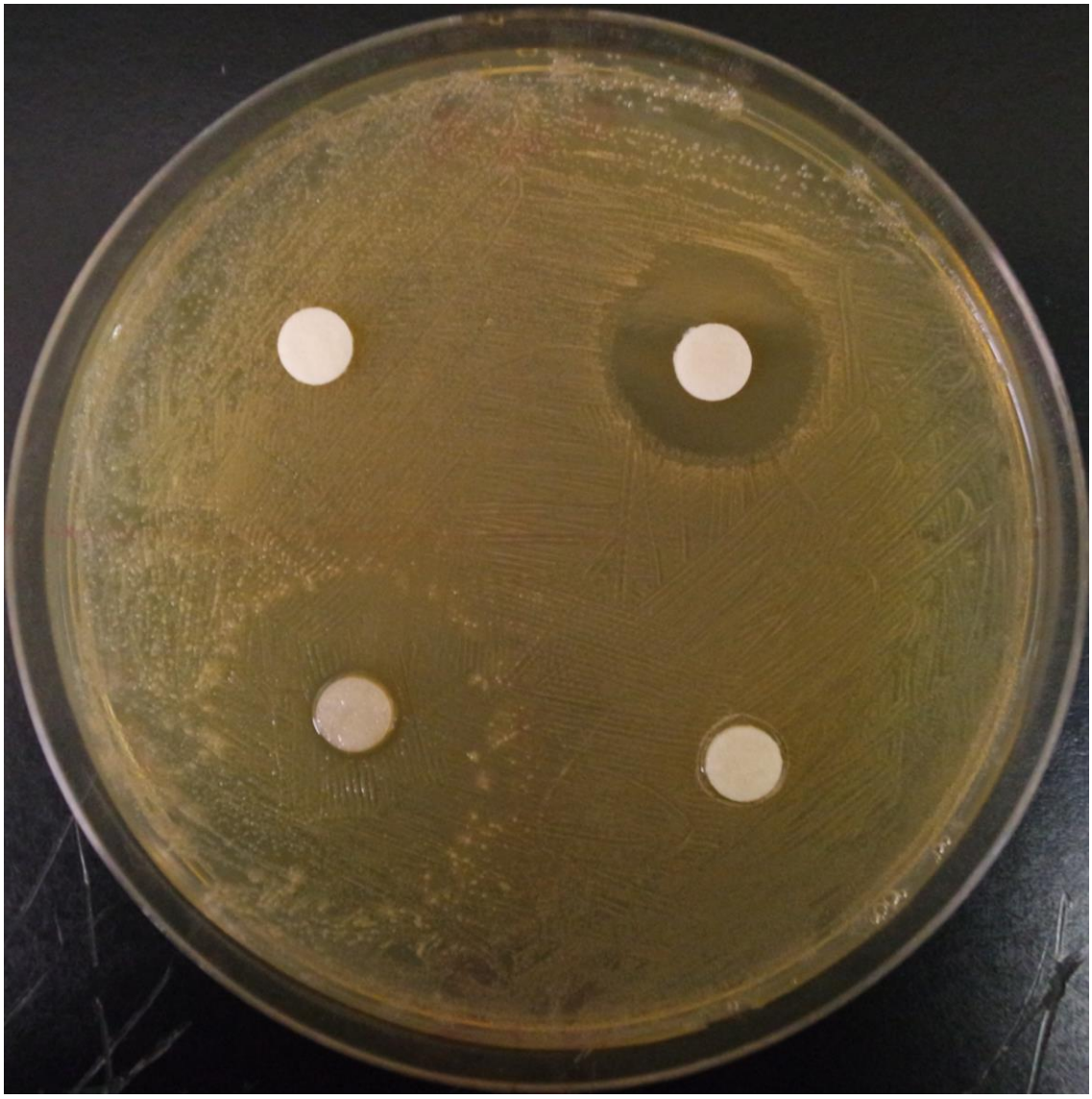

Figure 8. Petri dish (dish no. 15) for *A. Actinomycetemcomitans*, upper left quadrant – sterile disk impregnated by normal saline, upper right quadrant – sterile disk impregnated by CHX, lower right quadrant – sterile disk impregnated by olive oil, lower left quadrant – sterile disk impregnated by OzOO

Table 4. Paired sample t-test to compare the antibacterial activity of OzOO and CHX against *P. gingivalis* and *A. actinomycetemcomitans*

Bacteria	Pair	Mean	SD	SE	95% Confidence Interval of the Difference		t	df	Sig (2-tailed)
					Lower	Upper			
P. gingivalis	OzOO - CHX	10.6	4.19	0.94	8.61	12.53	11.30	19	.000
A. actinomycetmocomitans	OzOO - CHX	7.5	2.12	0.50	6.43	8.53	14.97	17	.000

CHAPTER FOUR

DISCUSSION

Comparing the diameters of the growth inhibition zone for CHX and OzOO against *P. gingivalis* and *A. actinomycetemcomitans*, the antibacterial activity of OzOO was greater than that of CHX with a statistical significance ($p<0.001$). Based on the results, this study indicates that OzOO may be an alternative to CHX as an antiseptic agent.

Two important findings should be considered in this study. At first, the shape of the growth inhibition zone of OzOO against both microbials was not always a full circle. The possible reason is the uneven and/or incomplete distribution of OzOO into the sterile disk during the disk impregnation period resulting in uneven distribution of OzOO into microbial suspensions during the incubation period. In addition, when semi solid-liquid OzOO was warmed up in the heating bath circulator to be converted into liquid for disk impregnation, the amount of peroxide can be potentially lost. The second noticeable finding was the fully and/or partially blurred growth inhibition zone for OzOO and CHX against *A. actinomycetemcomitans*, being interpreted as the partial antibacterial activity. It is difficult to compare the degree of partial antibacterial activity, because the degree of blurring was different. The degree of blurring for OzOO varied between petri dishes. It was also different from the degree of blurring for CHX. The growth inhibition zone for CHX was a clear circle with a blurred edge. The possible reasons of the blurred growth inhibition zone are the potential regrowth of *A. actinomycetemcomitans* during the incubation period and non-purified *A. actinomycetemcomitans* itself.

There are various methods to evaluate the antibacterial activity; diffusion method, dilution method, thin-layer chromatography bioautography, time-kill test, ATP bioluminescence assay and flow cytofluorometric method (22, 23). Disk diffusion susceptibility test, one of the diffusion methods, was used in this in vitro study. Disk diffusion susceptibility test assesses the antimicrobial activity by measuring the diameters of the growth inhibition zone surrounding each disk with a caliper in millimeters. This test is widely used because of its simplicity, easy interpretation, flexibility in disk selection and low cost, though it is lacking mechanization or automatization. For the interpretation of the test, the result can be analyzed in two ways, both qualitatively and quantitatively. By the CLSI guideline (Clinical and Laboratory Standards Institute, formerly National committee for Clinical Laboratory Standards, NCCLS), there are 3 categories, susceptible, intermediate or resistant based on the standard diameter of each antibiotic for the qualitative interpretation (24). However, the qualitative interpretation from the result of this in vitro study was difficult because CLSI guideline doesn't give any information about OzOO and CHX, used in this study. Minimal inhibitory concentration (MIC) is evaluated for the quantitative interpretation. However, determining the exact MIC is not appropriate through disk diffusion susceptibility test, because it is difficult to quantify the amount of diffused antimicrobial agent. Therefore, in this in vitro study, only the diameter of the growth inhibition zone was compared to evaluate the antibacterial activity.

In the consensus report from the World Workshop of Periodontology, *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia* are considered to play the

major role in developing periodontal disease (2). As one of the red complex bacteria, *P. gingivalis*, Gram-negative anaerobic and non-motile rod, is elevated in the active lesions of destructive periodontal disease (2). *A. actinomycetemcomitans*, Gram-negative facultative anaerobic and non-motile rod, is an important virulence factor in the pathogenesis of aggressive periodontitis with highly leukotoxic effects (25). Related to these bacterial species, periodontal disease, also being affected by other co-factors, is developed and destructing peridontium.

To control periodontal disease, non-surgical treatment is usually performed to eliminate oral pathogens mechanically. Based on the series of studies, SRP favorably improved pocket depth (PD) and clinical attachment level (CAL) for both moderate and advanced periodontitis (26, 27). Microbiologically, SRP also reduced the amount of various bacterial species related to periodontal disease though they were still remained to some extent (28, 29). Therefore, 0.2% CHX mouthrinse was used following SRP for treating periodontitis and, it improved both PD and CAL in all initially different PD groups (14). Plus, CHX itself was also effective to reduce plaque index and gingival index in addition to the oral hygiene, toothbrushing and interdental cleansing (10).

A new OzOO for tooth and gum support (PurO3™) is now available in the field of dentistry as an alternative to CHX with fewer side effects. OzOO is basically produced through the chemical reaction between ozone and olive oil. When ozone, activated oxygen, is funneled into a tube, an electrical charge is generated. During this process, ozone is slowly infused into olive oil and OzOO is created. For the physiochemical property, there is a chemical reaction, called ozonolysis, between

ozone and unsaturated fatty acid of olive oil, especially alkene (carbon-to-carbon double bonds) in fatty acid (19). In PurO3™, 224 meq/Kg of hydrogen peroxide is formed as a result of ozonolysis. In another study showing significant growth inhibition against *P. gingivalis* and *S. aureus* by OzOO (Novox®, MoSS, Italy), 560-590 meq/Kg of hydrogen peroxide was formed by ozonolysis in OzOO (21).

Regarding to the safety of ozone as an alternative antiseptic, it is important to understand the potential toxic effects of ozone. According to studies evaluating the cytotoxic effects of gaseous and aqueous ozone, aqueous ozone proved the highest level of biocompatibility on oral epithelial cells, gingival fibroblasts and periodontal cells (30, 31).

CHAPTER FIVE

CONCLUSION

Within the limits of this study, it is concluded that the antibacterial activity of OzOO against *P. gingivalis* and *A. actinomycetemcomitans* is superior to that of CHX. For the clinical use of OzOO as an alternative to CHX, other research such as in vivo and clinical study should be designed and planned.

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