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Effectiveness evaluation of chlorhexidine and hydrogen peroxide combination toward anaerobic gingival sulcus bacteria

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Abstract*—Anaerobic bacteria in the gingival sulcus is the critical etiological factor for initiation and development of periodontal disease such as gingivitis and periodontitis. In addition to scaling and other regular treatments, supporting treatment such as giving irrigating solution using chlorhexidine and hydrogen peroxide to the gingival sulcus was commonly performed by many dental practitioners. Several pharmaceutical companies also provide some modified irrigating solutions for better efficacy and convenience. However, there is still insufficient evidence for each modification. The purpose of this study is to confirm the effectiveness of utilizing combined irrigation solution of chlorhexidine and hydrogen peroxide toward bacterial growth inhibitory capacity. This research was conducted as a laboratory experiment in vitro. The isolated bacteria from human gingival sulcus was taken as the bacterial sample. The experiment was performed by using disc diffusion method on blood agar plate media which was then followed by measuring the bacterial inhibitory zone using sliding caliper. Irrigating solutions for the treatment consisted of 0.2% chlorhexidine and 0.2% chlorhexidine combined with 3% hydrogen peroxide. Homogeneity test by Levene showed that the data were normally distributed. Therefore, Anova variant analysis test was performed. The statistical analysis results showed that the obtained F value was higher than F table with significant p value of < 0.05. These results suggested that there was a significant bacterial growth inhibitory capacity among the groups. The post-hoc analysis indicated that using 0.2% chlorhexidine solution gave higher mean-difference. A Standalone of 0.2% chlorhexidine solution was more effective for anaerobic gingival sulcus bacteria growth inhibition.

Keywords*—The bacterial inhibitory zone, anaerobic gingival sulcus bacteria, 0.2% chlorhexidine, 3% hydrogen peroxide.

I. NTRODUCTION

The most commonly occurred periodontal disease is gingival inflammation such as gingivitis and periodontitis. Untreated gingival inflammation could lead to further periodontal inflammation or periodontitis [1]. Periodontitis is defined as an inflammatory disease in periodontal tissues that supports some specified-microorganisms or certain groups of specific bacteria, which are needed to progressively destroy periodontal ligament and alveolar bone with pocket locking, recession, or accordingly [2].

Domination and also the increased number of plaque bacteria could be the main etiological factor for the initiation and development of periodontal disease [3]. The area of gingival sulcus in periodontal tissue is harboring the highest percentage of the population of gram-negative anaerobic bacteria [4]. Among several types of bacteria, commonly found gram-negative anaerobic bacteria around the gingival sulcus area such as *Porphorymonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans* belong to a group of microorganisms that are potentially involved in the occurrence of periodontal disease [5].

One of the regular periodontal treatments that can be widely performed is dental scaling. Dental scaling is the most common non-surgical treatment that has been known to be effective in reducing microorganisms in dental plaque. However, scaling has several limitations, such as difficulty in accessing deeper gingiva, narrower area and removing penetrated pathogenic microbes in the dentinal tubules [6]. This condition requires the utilization of antibacterial agents in a better penetrable form as the supporting approach in microorganisms control therapy in the periodontal diseases [7].

Chlorhexidine has been known to have a strong antimicrobial effect as a microorganism control agent [8]. Chlorhexidine effectively inhibits the growth of both gram-positive and gram-negative bacteria, depending on its' working concentration [7]. Chlorhexidine is also a broad-spectrum antimicrobial agent with less side effects and less toxic which is commonly used as an active compound in oral rinsing solution [9]. Since it harbors a lot of caution while the bacterial membrane is negatively charged, it alters the bacterial membrane's permeability and promotes bacterial cytoplasm leakage towards bacterial death [5]. Another compound that

could be utilized as an oral microorganism control agent is hydrogen peroxide which is not only good against bacteria but also against virus and fungi [6]. Antimicrobial effects of hydrogen peroxide come from generating the hydro-toxic radicals. Hydro-toxic radicals which belong to the strong oxidant species that could be easily generated by the macromolecules such as lipids membrane and DNA so that they could promote bacterial death, especially toward anaerobic bacteria [10].

Independent or sequential utilization of each active agent has been well known to be effective. However, we found lack of evidence for the utilization of both compound combined. Therefore, this study was aimed to evaluate the effectiveness of both active agent's combination in a single preparation using a 0.2% chlorhexidine di-gluconate solution and 3% hydrogen peroxide.

II. MATERIAL AND METHODS

A. Material

Our study was designed under the laboratory experimental procedure utilizing clinical isolated bacteria as the representative condition and strain specific bacteria as a control group. This research was conducted at the Microbiology Laboratory of the Faculty of Veterinary of *Universitas Gadjah Mada* and Microbiology Laboratory and Pharmaceutical-Technology Laboratory of the Faculty of Medicine and Health Sciences, *Universitas Muhammadiyah Yogyakarta*. The clinical anaerobic bacteria were taken from human gingival sulcus area with gingivitis condition while the *Aggregatibacter actinomycetemcomitans* strain-specific bacteria were used as the control group. The research procedures had been reviewed and approved by the ethics committee of the Faculty of Medicine and Health Sciences under the certification number of 055/EP-FKIK-UMY/II/2018. Both groups were treated and cultured in similar condition using 5 mL of Tryptose Phosphate Broth media for 24 hours at 37°C under the anaerobic condition.

B. Methods

Bacterial growth inhibitory capacity was observed using the disc diffusion method through the appeared-inhibitory zone. Bacterial suspension that met 10^8 CFU/mL was taken using a micropipette, then was plated on the surface of the TSA blood media in 6 dishes. After 24 hours of incubation, each dish was divided into 3 parts which would be treated using paper discs and well-construct for clinically isolated bacteria and *Aggregatibacter actinomycetemcomitans* respectively. Paper discs and well-constructs with the diameter of 6 mm were immersed in a 0.2% chlorhexidine di-gluconate test solution combined with 3% hydrogen peroxide, 0.2% chlorhexidine and sterile distilled water as a negative control for ± 1 hour using a separate sterile dish. After that, the conditioned-paper disc was inserted for the isolated clinical bacteria and the treating solution was poured into the prepared-well construct for the *Aggregatibacter actinomycetemcomitans*.

All the dishes were incubated in the jar containing anaerogen for ± 24 hours at 37°C. In this study, the inhibitory (radial) zone was measured by using a sliding caliper with a 0.01 mm precision level which was carried out after 24 hours of incubation at 37°C. The obtained numerical data were then analyzed by Shapiro-Wilk normality test and One-Way ANOVA analysis.

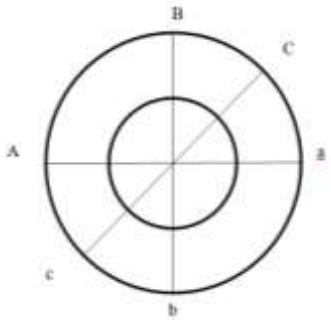

Fig. 1. Inhibitory Zone Measurement (*A-a*, *B-b*, *C-c*: representative plate diameter)

$$\text{Inhibitory zone: } \frac{\{(A\text{-}a)+(B\text{-}b)+(C\text{-}c)\}}{3}$$

III. RESULTS AND DISCUSSION

Fig. 2. Inhibitory Zone from Isolated Sulcus Bacteria Culture

Fig. 3. Inhibitory zone from *Aggregatibacter actinomycetemcomitant*

TABLE I. INHIBITORY ZONE USING CLINICALLY ISOLATED BACTERIAL CULTURE FROM GINGIVAL SULCUS IN GINGIVITIS CONDITION.

	Inhibitory zone		
Plate	***Chlorhexidine 0.2% + Hydrogen Peroxide 3%***	***Chlorhexidine 0.2%***	***Negative control (Aquades)***
1	10.43mm	13.14mm	6.00mm
2	10.33mm	13.54mm	6.00mm
3	10.43mm	13.76mm	6.00mm
4	10.57mm	13.70mm	6.00mm
5	10.57mm	13.76mm	6.00mm
6	10.97mm	13.60mm	6.00mm
Mean	10.55mm	13.58mm	6.00mm
SD	±0.22mm	±0.23mm	±0mm

[a.] Data were obtained from a single solution (chlorhexidine 0.2%) which showed in average that radical zone is greater than the combination solution (chlorhexidine 0.2% + hydrogen peroxide 3%) toward the **anaerobic gingival sulcus bacteria**.

TABLE II. *ANOVA* ANALYSIS RESULT OF THE CLINICALLY ISOLATED BACTERIA AMONG EXPERIMENTAL GROUPS

	Sum Of Square	Df	Mean Square	F	Sig
Between Groups	174.821	2	87.411	2477.936	0.000
Within Groups	0.529	15	0.035		
Total	175.35	17			

Table II showed that the biggest inhibitory zone from the clinically isolated bacteria could be observed from the bacterial culture plate which was treated by the chlorhexidine alone and better than the chlorhexidine combined with hydrogen peroxide. The normality test of the data indicated that the data were normally distributed. Therefore, the variant analysis of the data was analyzed using *ANOVA* as shown in table IV which indicated that the F value was higher than F table that meant the H_0 was rejected with a high significant difference among the groups ($p<0.05$).

TABLE III. MEASUREMENT RESULTS OF 0.2% CHLORHEXIDINE RADICAL ZONE COMBINED WITH 3% HYDROGEN PEROXIDE, 0.2% CHLORHEXIDINE AND DISTILLED STERILE WATER FOR *AGGREGATIBACTER ACTINOMYCETEMCOMITANS*

Inhibitory zone			
Plate	***Chlorhexidine 0.2% + Hydrogen Peroxide 3%***	***Chlorhexidine 0.2%***	***Negative control (Aquades)***
1	9.23 mm	13.00 mm	6.60 mm
2	8.96 mm	13.56 mm	7.20 mm
3	9.23 mm	12.36 mm	6.63 mm
4	8.53 mm	13.23 mm	7.16 mm
5	8.56 mm	12.56 mm	6.00 mm
6	9.30 mm	12.20 mm	6.60 mm
Mean	8.96 mm	12.81 mm	6.69 mm
SD	±0.35	±0.53	±0.44

[b.] data were obtained from a single solution (chlorhexidine 0.2%) that showed an average radical zone was greater than the combination solution (chlorhexidine 0.2% + hydrogen peroxide 3%) toward ***Aggregatibacter actinomycetemcomitans***.

TABLE IV. *ANOVA* ANALYSIS RESULT OF STRAIN SPECIFIC *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* AMONG EXPERIMENTAL GROUPS

	Sum Of Square	Df	Mean Square	F	Sig
Between Groups	114.860	2	57.430	287.720	0.000
Within Groups	2.994	15	0.200		
Total	117.854	17			

Table IV showed that the biggest inhibitory zone from *Aggregatibacter actinomycetemcomitants* could be observed from the bacterial culture plate which was treated by the chlorhexidine alone and better than the chlorhexidine combined with hydrogen peroxide. The normality test of the data indicated that the data were normally distributed. Therefore, the variant analysis of the data which were analyzed using *ANOVA* as shown in table IV indicated that the F value was higher than F table which meant the H_0 was rejected with a high significant difference among the groups ($p<0.05$).

IV. DISCUSSION AND CONCLUSION

Our study showed that there was a significant difference in inhibitory growth capacity between both irrigating solutions compared to the control sterile distilled water towards both clinically isolated bacteria and *Aggregatibacter actinomycetemcomitans*.

Unexpectedly, we observed that the utilization of both combined solutions reduced the original effectiveness of the chlorhexidine itself. Even when the combined solution from the treated plate showed the reduced effect of chlorhexidine toward the bacterial culture, it still exhibited higher growth inhibitory capacity compared to the control group. The findings were different from the previous findings by Jhingta [4], which suggested the increased effectiveness in utilizing chlorhexidine combined with hydrogen peroxide. The contradictory results of our study to the previous report could be affected by the different experimental design and conditions, in which the previous clinical approach by Jhingta [4] was performed through sequential application procedures. Meanwhile, in our study, laboratory approach was conducted through a single application procedure of combined solution. Similar results were also suggested by Ramesh [11], which showed the effectiveness of utilizing hydrogen peroxide as an adjunctive agent after chlorhexidine. Interestingly, all of the previous studies applied the sequential procedures rather than applying the combination as a single mixture at once. Some controversies around the application procedures and also toxicity effect of chlorhexidine and hydrogen peroxide utilization in combination have also been discussed by Mirhadi [8]. In general, the consideration of cytotoxicity towards the host cells is an essential issue to be clarified, since different concentration may exhibit different cytotoxicity level to human fibroblast cells or the host cells themselves [12]. The studies showed that hydrogen peroxide addition could enhance antibacterial potency against facultative anaerobic *E. faecalis* during root canal irrigation by reducing the chlorhexidine's toxicity [8], since chlorhexidine alone may also disrupt the tissue healing process and induce epithelial desquamation [13].

Our data also demonstrated that the reduction in effectiveness by utilizing combined solution was observed not only in the total bacteria population from clinically gingivitis patient but also from the single *Aggregatibacter actinomycetemcomitants* as an anaerobic periodontal pathogen representative bacterium in similar pattern.

The reduction in growth inhibitory capacity which was observed through the formation of radical zone on the TSA bacterial culture plate system might be influenced by the existence of H_2O_2 scavenging activity from the cytoplasmic membrane fraction of *Aggregatibacter actinomycetemcomitants* due to quinol peroxidase activity in the anaerobic active state of respiratory chain reaction [14], which neutralized the bactericidal effect of H_2O_2 that could be detected through bubbling forms in the plate surface around the treated well and the surrounding of the treated-disc. Combination between chlorhexidine and hydrogen peroxide as a microbial control agent is originally conducted to enhance the beneficial effect of both mechanism of actions in which chlorhexidine allows H_2O_2 to easily penetrate the cell after

altering the cell membrane permeability [15], and then targeting the intracellular macromolecules components including DNA [16]. Under the similar amount of irrigating solution agent, it could be postulated that generating H_2O by using peroxidase activity could reduce chlorhexidine concentration. Thus, it led to the reduction of combined-chlorhexidine bacteriostatic capacity compared to the standalone solution.

The findings of our study along with the limitations confirmed the effectiveness of both chlorhexidine and its combination with hydrogen peroxide utilization as a supporting irrigation agent for periodontal therapy toward anaerobic bacteria. However, giving a combination solution together with hydrogen peroxide was not better than chlorhexidine alone. It even tend to reduce its effectiveness toward an anaerobic periodontal bacterial growth inhibitory capacity.

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