Effect of Extract Gel Green Tea *(Camelia sinensis)* on Neutrophil of Post Extracoronal Bleaching Rat's Teeth

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ABSTRACT

Background: *Free radicals produced by hydrogen peroxide after teeth bleaching which diffuse through the enamel and dentinal tubules can trigger inflammation of the pulp. To overcome the negative side effect, exogenous antioxidants have been proposed. This research was conducted to ascertain the impact of natural antioxidants, specifically green tea at 5% and 10% which was applied on rat molars teeth that were bleached using 40% hydrogen peroxide.*

Method: *Fifteen molar teeth of male Wistar rats were bleached using 40% hydrogen peroxide on their occlusal surface. Furthermore, the control group (Group I) rinsed using warm distilled water, while in Group II and Group III, the teeth were rinsed using 5% and 10% green tea extract gel. Wistar rats were sacrificed on the fifth day after treatment, followed by haematoxylin-eosin staining. Histological examinations were observed under an illumination microscope using 400x magnification and the number of the neutrophil were counted.*

Result: *The number of neutrophils was significantly impacted by the concentration of green tea extract gel, according to the results of a one-way ANOVA test (p 0.05). The findings of the post hoc test indicated that every pair of groups analyzed had significant differences (p<0.05).*

Conclusion: *Group III which was treated using10% green tea extract gel has the lowest number of neutrophils compared to Group II which was treated using 5% green tea extract gel and the control group.*

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INTRODUCTION

Bleaching is whitening teeth treatment to overcome certain grade of discoloration that many people are interested in because the procedure is not invasive and does not require a long time to see results.Tooth discoloration leads people to seek teeth bleaching to restore their teeth to be a brighter and cleaner look since it will improve their appearance and boost their confidence. This is because white teeth are associated with good oral hygiene and are considered aesthetically pleasing in many cultures. There are 2 main types of vital tooth bleaching, namely in office bleaching and at home bleaching. In office bleaching conducted by applying 25%-40% hydrogen peroxide.[1] Applying 40% of hydrogen peroxide extra coronally provide more effective and shows quicker result compare to at home options. On the contrary, at-home bleaching uses a very low concentration of whitening chemical (10–20% carbamide peroxide) in a specially designed mouthguard that must be used every day for two to six weeks.[2]

Apart from the positive effect of being able to whiten teeth with satisfactory result, bleaching material also have several detrimental side effects, particularly if administering a high concentrations of hydrogen peroxide or using it for extended period. Frequently reported side effects of teeth whitening include minor gingival irritation and increased tooth sensitivity. The concentration of the peroxide bleach component and the duration of therapy affected how severe the side effect was.[3] Another side effect of using bleaching materials is a reduction in hardness of enamel and restoration materials. In addition, irritating substances can proceed into the pulp after entering through the dentinal tubules and email. The process of oxidative replacement with oxidants is how hydrogen peroxide works as a tooth-whitening agent.[4]

By employing chemicals to oxidize organic pigments inside the tooth structure, bleaching whitens teeth by producing Reactive Oxygen Species (ROS). [5] Perhydroxyl free radicals (HOO•) and On (O•) react with the double bonds of chromogen molecules in enamel and dentin, so that they are broken down into simpler molecules. If this process continues, a maximum point will be reached, called the saturation point. At this point, tooth structure damage and mineral loss begin to occur (Vilhena et al., 2019).[6] Deeper hydrogen peroxide penetration, however, may cause irreversible damage to tooth structures and pulp cells by penetrating not just the enamel and dentin but also the dental pulp.7. Pulp cells subjected to a certain bleaching gel concentration experienced oxidative stress, the severity of which increased with the amount of time the bleaching gel and enamel came into contact.[8] Neutrophils are the first inflammatory cell defenses to appear in the body minutes after the injury occurs and turns on in reaction to phagocyte stimulus or binds to chemotactic mediators, antigen complexes antibodies with special receptors on cell membranes and system complement constituents. Neutrophil cells have a brief lifespan in the site of injury before they pass away, reaching their maximum concentration in around 24 hours.[9]

There are 3 types of endogenous antioxidant proteins which are the first line to protect tissue from oxidative damage caused by free radicals, namely superoxide dismutase, catalase and glutathione peroxidase. Endogenous antioxidants are a large system for maintaining redox balance in the body but requires the help of Mg, Zn and Cu to work.[10] If antioxidants system cannot take out these free radicals in a short time, tissue damage happened due to this free radical's action.[11] Consequently, it is crucial to employ antioxidants during the bleaching process in order to counteract any remaining oxidants in the oral environment. Green tea contain approximately 4000 bioactive compounds and a third of them consist of polyphenols. One kind of polyphenol is epigallocatechin gallate (EGCG) that is often found in green tea and has antioxidant, anti-inflammatory and

anti-carcinogenic properties. [12] This substance has the ability to remove ROS. [13] Research using a 5% concentration of green tea solution for 10 minutes, showed an increase in the attachment of composite resin to dentin due to neutralization of free radicals. A 10% concentration of green tea solution was more beneficial than a lower concentration. This is indicated by the higher enamel-resin shear bond strength when the specimen is exposed to a higher concentration. [14]

RESEARCH METHOD

After receiving ethical clearance letter No. 35/UN1/ KEP/ FKG-RSGM/EC/2023 from the Ethics and Advocacy Unit of the Faculty of Dentistry-Prof Soedomo Dental Hospital Universitas Gadjah Mada, this study was carried out. The antioxidants used in this research came from green tea leaves taken from plantations and extracted using the maceration method. The green tea leaves are washed under running water, cut into small pieces, air-dried and then dried in the oven at 45C for 2 hours. Next, the dried tea leaves are ground until they become powder. The tea powder is put into a macerator, 70% ethanol solvent is added and stirred until homogeneous. The macerate was filtered and evaporated using a rotary evaporator to obtain an extract, and continued with making a topical gel. Fifteen male Wistar rat maxillary molar teeth were used in this study. The criteria for Wistar rats used were healthy rats aged 2.5-3 months. Three groups of five maxillary molar teeth each were created from the specimens.

Samples treatment

First of all, Wistar rats were anesthetized using ketamine xylazine intramuscularly. After placing gingival dam, the occlusal area of the teeth was applied with 0.01 milliliters of 40% H2O2 (Ultradent, USA; Opalescence Boost PF 40%) for five minutes followed by cleaning them using an aspirator tip. All Wistar rat were divided into 3 groups. Group I treated with warm distilled water only after bleaching, group II treated with 5% green tea extract gel after bleaching, while Group III treated with 10% green tea extract gel after bleaching.

Five days after treatment, the Wistar rats were sacrificed using the lethal dose of intramuscular anesthesia with ketamine-xylazine, then the jaw and the molar teeth were collected and fixed with 10% buffered formalin for 24 hours. Fifteen maxillary molars of male Wistar rats were divided into 3 groups. Group I following a 40% hydrogen peroxide bleaching, the teeth were rinsed with warm distilled water 50C. Group II after bleaching was applied with 0,1 gram of 5% green tea extract gel using microbrush for 3 minutes. Group III after bleaching was applied with 0,1 gram of 10% green tea extract gel using microbrush for 3 minutes.

Haematoxylin-eosin (H&E) staining

First of all, the jaw and the teeth of Wistar rat was decalcified using EDTA solution for 35 days, and then washed with 70%, 80%, 95%, and 100% ethanol for 90 minutes in the automatic tissue processor (Sakura Tissue-Tek II, Japan). Furthermore, the tissue washed using xylene I, II, and III for 90 minutes, soaked in liquid paraffin at 57º-59º C for two hours, and proceed to make a paraffin block, The paraffin blocks were sliced using microtome with a thickness of 5 µm. All of them were soaked in the water bath at 50º. (Sakura PS-M, Japan). Furthermore, the slides were put onto the slide warmer at 40-4º (Sakura PS-53, Japan) for 15 minutes to

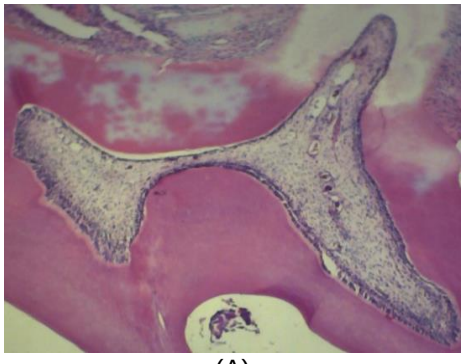
evaporate the remaining air. Deparaffinization was carried out with xylol for 3 minutes then rinsed with flowing air. The specimens were rehydrated with 100%, 95%, 80% and 70% alcohol respectively for 2 minutes. The remaining alcohol was removed by running the specimen sections under running water followed by hematoxylin eosin staining.

Data and statistical analysis

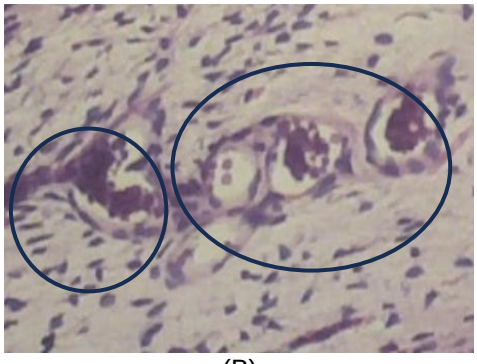
Research findings on how the quantity of neutrophils is affected by 5% and 10% concentrations of green tea gel extract are shown as ratio data**.** The results of the perception similarity test between observers showed very reliable between observers A and B (0.925) and between observers B and C (0.974). A one-way ANOVA test was used because the data was homogeneous and regularly distributed. The mean differences between the pairs of groups under comparison were then ascertained by testing the data using post hoc analysis. A significance level of $p<0.05$ was established.

RESULTS

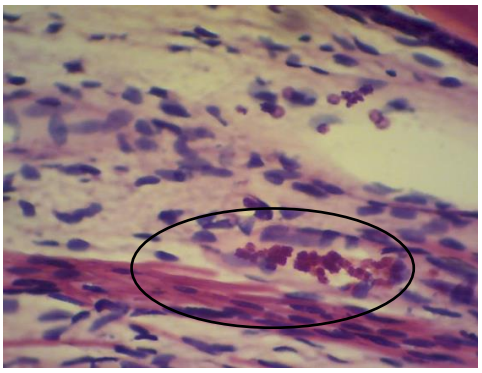
Observation under a light microscope shown that the appearance of neutrophils is purplish blue with a cell nucleus, varies in shape and sometimes resembles with the letter U or a horseshoe, and has light pink cytoplasm. The diameter of neutrophils ranges from 12-15µ. Neutrophils have multi lobes, namely 2-5 lobes/segment so they are often called polymorphonuclear cells (PMN). Figure 1 shows the histological picture following the use of a bleaching solution that contains 40% hydrogen peroxide, followed by rinsing with warm distilled water on the number of neutrophil cells with a magnification of 400x in the control group (Fig. 1B), the group rinsed with 5% green tea extract gel after bleaching (Fig.1C) and the group rinsed with 10% green tea extract gel after bleaching (Fig.1D). A count of neutrophil cells was conducted in nearby areas of the pulp roof in 3 fields of view starting from the mesial to the distal part of the tooth.



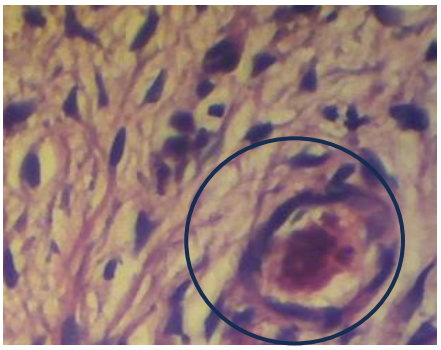
(A)



(B)



(C)



(D)

Figure 1. (A) microscope examination with 40x magnification on rat molar tooth pulp with 3 different fields of view. Neutrophil cells appearance in the control group (B), Neutrophil cells appearance in group II treated with 5% green tea extract after bleaching (C), Neutrophil cells appearance in group II treated with 10% green tea extract gel after bleaching (D).

The result showed that the highest mean of the number of the neutrophil can be seen in Group III which was treated using 10% green tea extract gel after bleaching. The lowest one was shown in Group I which was treated using warm distilled water only (Figure 2).

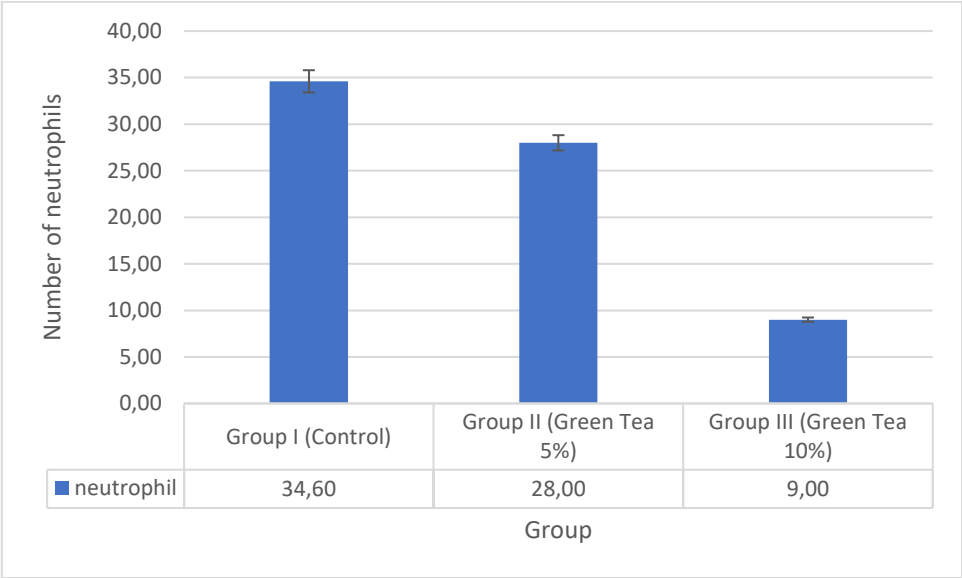


Figure 2. Mean and standard deviation of the number of neutrophils.

The number of neutrophils was significantly impacted by the concentration of green tea extract gel, according to a one-way ANOVA test ($p < 0.05$) (Table 1).

Table 1. One Way ANOVA test on the effect green tea extract gel concentration on the number of neutrophils.

	df	Mean Square	F	p
Between Groups	2	883.118	246.561	0.00*
Within Groups	12	3.582		
Total	14			

Description *= p value <0.05 (Significant)

Group II, which received 5% green tea extract gel, differed significantly from Group I, according to the post hoc test. Group III treated with 10% green tea extract gel, a significant different between Group II and control treated

with distilled water only and a significant different between Group III and Group I. In short, Table 2 indicates that all pairs of groups compared had significant differences ($p<0.05$).

Table 3. Post Hoc Test (LSD) on the effect of green tea extract gel concentration on the number of neutrophils.

Group Comparison of Sample	Mean difference	p
Control - 5% green tea	6,60000	0.000*
Control - 10% green tea	25,59800	0.000*
5% green tea - 10% green tea	18,99800	0.000*

Description* = p value <0,05 (Significant)

DISCUSSION

The bleaching agent used in this research was 40% hydrogen peroxide, it is a strong oxidant that works by breaking down the double chains of chromophores so that the tooth color is whiter. On the other hand, it also produces free radical[15]. The free radical imbalances with the antioxidant produced by the body can result in pulp inflammation. Numerous types of antioxidan was recognized as a material that has ability to neutralize the side effect oxidant. This study was conducted to determine the effect of natural antioxidants, namely 5% and 10% green tea extract gel, which was applied on rat molars teeth that were bleached using 40% hydrogen peroxide on the number of neutrophil cells.

The ANOVA test findings demonstrated that the number of neutrophils was significantly impacted by the concentration of the green tea extract. Higher concentration of green tea contains more active substance so that different concentration affects the number of neutrophils detected. The results of the LSD post hoc test showed that there were significant differences in all pairs of groups compared. The LSD test results indicated a significant mean difference between Group I and Group II and between Group I and Group III.

Group I, which was only rinsed with warm distilled water after bleaching, showed a more severe inflammatory condition than Group II, characterized by the highest number of neutrophils compared to the other two groups (Figure 1B). In group II, after rinsing with warm water followed by application of 5% green tea extract gel, less inflammation was detected compare to group I, indicated by the less number of neutrophils compare to the one in group I. Group III showed less inflammation was detected compare to group II and showing the best result in controlling inflammation.

In group I, the specimen was only rinsed using warm distilled water without any antioxidant intervention, so the number of neutrophils detected was greater than other group. This is because distilled water does not have the ability to neutralize free radicals produced by hydrogen peroxide, therefore demineralization occurred with ease. Demineralization caused by free radicals, a byproduct of hydrogen peroxide,led to the intertubular dentin being thinner and the dentin tubules becoming larger. Thinning of intertubular dentin occurs because intertubular dentin contains more collagen than minerals so it is susceptible to demineralization. Increasing the diameter of the dentinal tubules has an impact on increasing dentin permeability.[16] An increase in tubule diameter and dentin permeability makes it easier for free radicals, including perhydroxyl radicals, hydroxyl and reactive oxygen species, to reach the pulp. Reactive Oxygen Species are strong free radicals that contain oxygen. This type of free radical has several unpaired electrons and is included in the category of free radicals such as super oxidants and hydroxyls. The presence of excessive Reactive Oxygen Species can induce oxidative stress in pulp tissue.[17] An imbalance between endogenous antioxidants and ROS also trigger oxidative stress which causes injury to cells ranging from reversible lesions to cell death. Free radicals produced by

hydrogen peroxide as a bleaching agent can diffuse into the dentinal tubules and can even reach the pulp that can result in inflammation. [18] These results are in accordance with research which states that the results of examinations using X-ray Diffraction on teeth after bleaching using hydrogen peroxide and only rinsing with warm water showed that there was still hydrogen peroxide on the teeth.[19]

Groups II and III differed significantly, according to the Least Significant Difference test. This is due to 10% green tea extract contains L-theanine, polyphenols, and epigallocatecin gallate (EGCG) which is higher than the 5% green tea extract, so 10% green tea extract was more effective in controlling inflammation. This can be seen in Figure 1C, group III showed less neutrophils than in Group II. These results are in accordance with other researcher stated that higher concentrations of green tea extract has higher EGCG. The more EGCG means the more -OH groups that can neutralize free radicals so it is more effective in binding ROS.[13] In the group that applied with 10% green tea extract, the number of neutrophils appeared to be less than the other group. This situation is favorable since the proliferation phase can only begin if neutrophils had been eliminated. This statement in accordance with other researcher stated that at the beginning of inflammation, the presence of neutrophil cells in the lesion area are very necessary, but prolonged existence causes more damage and inhibit the recovery. This is because neutrophils also produce free radicals which causes oxidative stress. Therefore, in order for the proliferation phase to begin, neutrophils must be eliminated. Macrophages perform this function by triggering neutrophil phagocytosis and apoptosis.[20] The process of reparative tissue creation is interrupted if neutrophils stay in the area of damage because this inhibits the transition of proinflammatory M1 phenotype macrophages to reparative M2 phenotype macrophages.[21]

Because of its strong antioxidant qualities, epigalocatechin-3-gallate (EGCG) inhibits the synthesis of inducible nitric oxide synthase (iNOS) and lowers nitric oxide levels, causing inflammatory cells to undergo a shorter inflammatory response followed by a proliferation phase, which speeds up the repair process.[22] EGCG, the most prevalent catechin in green tea, makes up around 59% of the total catechin content. EGC, ECG, and EC are the next most common catechins, with 19%, 13.6%, and 6.4%, respectively.[23] Epigalocatechin-3-gallate is more effective at binding free radicals because it has eight -OH groups. Neutrophils that migrate toward target tissue were drastically reduced as a result of the more efficient elimination of free radicals; in other words, inflammatory and healing processes could proceed more quickly. EGCG has been demonstrated to affect a number of biological processes, such as inflammation and the inhibition of neutrophil migration via endothelial cells.[24] The antioxidant action of epigalocatechin-3-gallate was achieved by giving free radicals hydrogen ions. By either directly responding to stabilize ROS or indirectly by eliminating ROS, epigalocatechin-3-gallate (EGCG) reduces the quantity of ROS. By contributing the trihydroxyl and dihydroxyl groups of rings, polyphenols in green tea can also neutralize already-existing free radicals and stop new ones from forming.[25]

CONCLUSION

Group III which was treated using10% green tea extract gel has the lowest number of neutrophil compared to Group II which was treated using 5% green tea extract gel and the control group.

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