ORIGINAL ARTICLE

Effect Of Green Tea Extract Gel (Camellia Sinensis) on the Number of Blood Vessels in Dental Pulp after Extracoronal Bleaching with Hydrogen Peroxide 40%

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ABSTRACT

Introduction: Free radicals produced by bleaching can cause oxidative stress which may impact on pulp inflammation, therefore antioxidants are needed to counteract these negative effects. Green tea is a herb that contains the antioxidant EGCG so it has great potential to neutralize the effects of those free radicals. The aim: This study aims to learn the effect of green tea (camellia sinensis) extract gel with a 10% concentration compared to that of 5% concentration on the number of blood vessels in the dental pulp after extracoronal bleaching with 40% hydrogen peroxide on the number of blood vessels in the dental pulp of Wistar rats. **Methods:** This study used 15 maxillary molar teeth of male Wistar rats which were divided into 3 treatment groups. Group 1 (control) was rinsed with distilled water, Group II used 5% green tea extract gel and Group III used 10% green tea extract gel, each for 3 minutes after 5 minutes of bleaching treatment on the occlusal surface of Wistar rat molar teeth. Wistar rats were sacrificed on the 5th day after treatment. The teeth were processed for histological microscopic evaluation and stained with hematoxylin-eosin, then observed using a light microscope with 400x magnification and the number of visible blood vessels in the dental pulp was counted. **Results:** One Way ANOVA test results showed that there was an effect of green tea extract gel concentration on the number of blood vessels in all groups ($p < 0.05$). Post Hoc test results with LSD showed that there was a significant difference in the mean number of blood vessels in all pairs of groups ($p < 0.05$). **Conclusion:** Green tea extract (camellia sinensis) of 10% concentration causes fewer blood vessels compared to application with green tea extract of 5% concentration and control groups.
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INTRODUCTION

Nowadays, the bleaching agent hydrogen peroxide with a 40% concentration is often used by clinicians in cases of extrinsic discoloration. Bleaching agents with higher concentrations can produce more peroxide radicals for bleaching, resulting in a faster whitening process. The higher the concentration of hydrogen peroxide, the more free radicals are produced to break the double bonds of the chromophore molecules into smaller ones, but also the higher the effects it causes in the form of tooth sensitivity, gingival irritation, throat irritation and nausea (1, 2).

Hydrogen peroxide applied to the tooth surface can penetrate the enamel and dentin and oxidize the organic components. After passing through the enamel and dentin, hydrogen peroxide in the form of reactive oxygen species (ROS) diffuses rapidly, reaches the pulp chamber, and causes the release of inflammatory mediators, which if continued can cause pulp (3, 4).

High level of reactive oxygen species (ROS) can cause oxidative stress that contributes to pulp inflammation (5). Cell damage caused by hydrogen peroxide penetration induces the synthesis and release of biochemical mediators. These mediators cause increased vascular permeability and vasodilation within the pulp cavity (6). Inflammatory reactions stimulate the secretion of cytokines that contribute to the recruitmet of immune cells. These immune cells transmigrate to reach injured tissue and attach to the vascular endothelium (7). The

accumulation of inflammatory cells causes pressure on the tissue resulting in hypoxia. Hypoxic conditions or lack of oxygen in the tissue will stimulate the formation of blood vessels through the process of angiogenesis process which is an important indicator in the wound healing process (8). Angiogenesis begins as a result of an imbalance between oxygen supply and consumption in the dental pulp and then reacts and produces blood vessels (9). The angiogenesis process will stop when the tissue's oxygen needs are met, so that formed blood vessels become regress (10). Physiological angiogenesis can maintain healthy vascular homeostasis, but long-term and prolonged angiogenesis is a factor in the development of cancer and chronic disease (11). Low ROS concentrations are necessary to initiate tissue repair processes. Antioxidants are needed to support this process by inhibiting oxidation reactions by binding free radicals and highly reactive molecules so that cell damage is inhibited (12).

Antioxidants mainly function to neutralize free radicals produced during metabolic processes including protecting tissues from inflammation (13). Green tea has beneficial effects due to its active ingredient polyphenol content, especially catechins. Catechin is a derivative of polyphenol that has high antioxidant properties (14). Catechins have been proven in vitro and in vivo to have strong antioxidant properties. In addition, green tea contains minerals and vitamins that further increase the antioxidant potential of this type (15).

Catechins are complex compounds in tea that are composed of epicatechin gallate (ECG), epicatechin (EC), epigallocatechin gallate (EGCG), epigallocatechin (EGC), and gallocatechin (GC). The dominating components are epigallocatechin gallate and epigallocatechin (14). Epigallocatechin gallate (EGCG) is the main catechin contained in green tea (16).

One of the antiangiogenic substances is EGCG (Epigallocatechin-3-gallate), which is the main active substance in green tea. Epigallocatechin-3-gallate (EGCG) as the main polyphenol in green tea, functions as an antioxidant to neutralize ROS, anti-inflammatory, etc. Therefore, EGCG has potential functions for application as an antiangiogenic agent. Elimination of oxidative stress as a result of high ROS concentration is a promising approach from green tea to reduce inflammation and reduce the number of blood vessels resulting from tissue ischemia symptoms (17).

This study aims to learn the effect of green tea (camellia sinensis) extract gel with a 10% concentration compared to that of 5% concentration on the number of blood vessels in the dental pulp after extracoronal bleaching with 40% hydrogen peroxide on the number of blood vessels in the dental pulp of Wistar rats.

MATERIALS AND METHODS

This research is a laboratory research. This study used 15 maxillary first molar teeth of male Wistar rats. This research has been approved by the Dental Research Ethics Commission of the Faculty of Dentistry UGM (Number35/UN1/KEP/FKG-RSGM/EC/2023).

Preparation of 5% and 10% green tea extract gel

The green tea extraction process is carried out using the maceration method. Green tea is dried in the oven at 50-60°C for 120 minutes. The leaves are then crushed with a grinder. The maceration process uses a 70% ethanol solvent, which is left for 24 hours, with stirring for the first 3 hours. The maceration filtrate is then collected, and the ethanol is removed using a rotary evaporator to obtain green tea extract with concentrations 100%. This extraction process was carried out in the Unit III Laboratory of the UGM Faculty of Pharmacy. Gelling agent CMC-Na was developed in 10 ml of water at a temperature of 80°C. Add green tea extract with a concentration of 5% and 10%, as well as glycerin. Next, propylene glycol and methylparaben were added. Add water to the mixture and stir until homogeneous.

Experiment with the wistar rat's teeth

Wistar rats were anesthetized using ketamine-xylazine at a dose of 40 mg/kg body weight intramuscularly. Apply 0.01 ml of 40% hydrogen peroxide (Opalescence Boost PF 40%, Ultradent, United States) on the occlusal surface of the rat's teeth and leave for 5 minutes then rinse with 5 ml of warm distilled water at 50oC, by running the distilled water 1 ml every minute for 5 minutes on the occlusal surface of the tooth. Fifteen maxillary molar teeth of male Wistar rats divided into 3 treatment groups. Group I (control) was rinsed with warm distilled water, group II used 5% green tea extract gel and group III used 10% green tea extract gel, each for 3 minutes and 0.1 g applied on the occlusal surface of Wistar rat molar teeth. Wistar rats were sacrificed and jaw samples were taken on the 5th day after treatment with a lethal dose of ketamine-xylazine. After the rat died, the rat's teeth and upper jaw were removed and then fixed in 10% formalin buffer for 24 hours.

Preparation of hematoxylin-eosin (H&E) staining

Teeth and maxillary samples were soaked in 10% EDTA solution for 1 month to decalcify the teeth. The decalcified samples were cut to obtain right and left molar samples. Samples were soaked in 70%, 80%, 95%, and 100% alcohol for 1.5 hours using an automatic tissue processor (Sakura Tissue-Tek II, Japan). Subsequently, the samples were soaked in xylol I, II, and III solutions for 1.5 hours. Afterward, the samples were embedded in liquid paraffin at a temperature of 57oC - 59oC for 2 hours and allowed to harden for 20 minutes to make paraffin blocks. The samples in paraffin blocks

were then sliced with a microtome (Sakura Accu-cut SRM Microtome, Japan) with a thickness of 5 µm from the mesiodistal direction. These sliced samples were placed in a paraffin water bath (Sakura PS-M, Japan) at a temperature of 50o C. Sample slices were taken with an object glass (Sail Brand, China). The sample attached to the glass object was set on a slide warmer (Sakura PS-53, Japan) at a temperature of 40o C - 45o C for 15 minutes. Deparaffinization was carried out using xylol for 3 minutes. Samples were dehydrated with 100%, 95%, 80%, and 70% alcohol for 2 minutes each. Following this, they were subjected to hematoxylin and eosin staining and then mounted using Canadian balsam, covered with a cover slip (Menzel Glaser, Germany).

Observation of the number of blood vessels

The preparations were observed using a light microscope (Nikon YS 100, Japan) with 400x magnification, equipped with a microscope camera (OptiLab, Indonesia). Blood vessels appeared in the form of red circles with a clear colour, and the nuclei of the endothelial cells were purple. The boundary of the observation area on the coronal side is the pulp horn, and on the apical side is the orifice. The observation area is divided into 3 fields of view. The number of blood vessels in each visual field is accumulated and then divided by the number of visual fields.

Statistical analysis

The data that obtained in the research is ratio data, that is the number of blood vessels. The data is in the form of the average result, which is calculated by dividing the blood vessels number by the number of visual fields observed by three different observers. The reliability of the average results was assessed prior to testing for normality. The data were analyzed using Shapiro-Wilk normality test. Furthermore, the data underwent a test for homogeneity of variance using Levene's test. If the data is found to be normally distributed and exhibits homogeneity, a one-way ANOVA test is conducted. The one-way ANOVA test is to show significant results, and was carried out using the Post Hoc Least Significance Different (LSD) test. The confidence level to be used is 95% ($\alpha = 0.05$).

RESULT

The results of the research show data of the mean number of blood vessels in Wistar rats' dental pulp. The study included three groups. Group I, is a group of the upper jaw molar teeth of Wistar rats that underwent bleaching using 40% hydrogen peroxide, followed by rinsing with warm distilled water at a temperature of 50oC. Group II, is the post-bleaching group treated with 40% hydrogen peroxide then rinsed with warm distilled water, and followed by application of 5% concentration green tea extract gel. Group III, is the post-bleaching group treated with 40% hydrogen peroxide, then rinsed with warm distilled water and followed by application of 10% concentration green tea extract gel.

Figure 1. Shows the group of the upper jaw molar teeth of Wistar rats that underwent bleaching using 40% hydrogen peroxide, followed by rinsing with warm distilled water at a temperature of 50oC, without application of green tea extract gel. Figure 2. Shows post-bleaching group treated with 40% hydrogen peroxide then rinsed with warm distilled water, and followed by application of 5% concentration green tea extract gel. Figure 3. Shows post-bleaching group treated with 40% hydrogen peroxide, then rinsed with warm distilled water and followed by application of 10% concentration green tea extract gel. Figure 4. Shows the highest mean number of blood vessels seen in Group I which was rinsed with warm distilled water, that is 11.02. The lowest mean number of blood vessels was seen in Group III, that is 1.33.

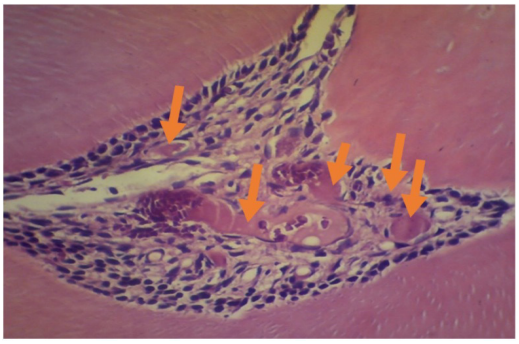

Figure 1. The effect of 40% hydrogen peroxide application on the number of blood vessels after bleaching with 400x magnification in the control group,

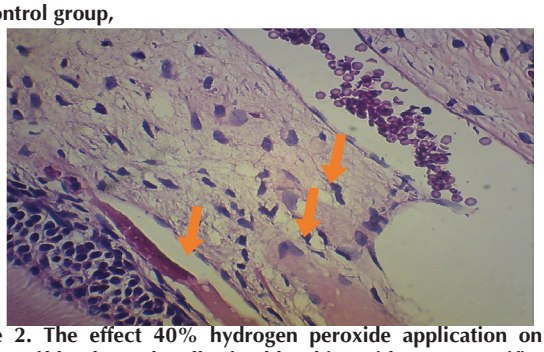

Figure 2. The effect 40% hydrogen peroxide application on the number of blood vessels cells after bleaching with 400x magnification in group II.

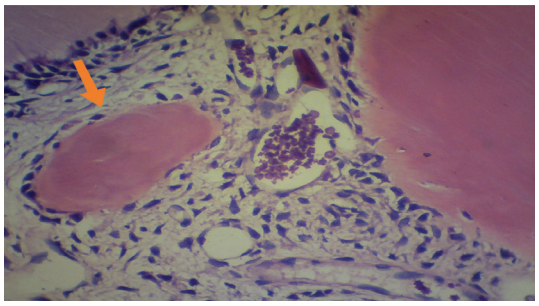

Figure 3. The effect 40% hydrogen peroxide application on the number of blood vessels cells after bleaching with 400x magnification in group III.

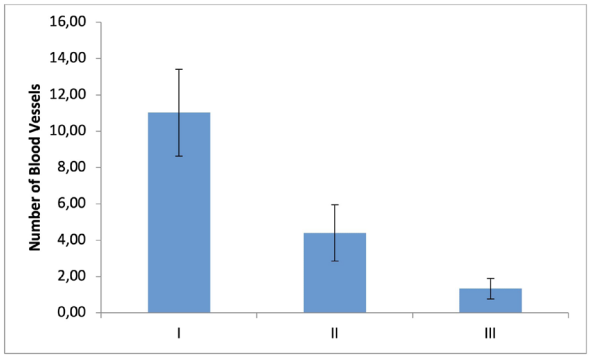

Figure 4. Mean of blood vessels count assessment after application of 5% and 10% green tea extract after extra coronal bleaching with 40% hydrogen peroxide.

The data obtained were observed using a light microscope at 400x magnification. The histological image obtained shows that blood vessels appear as irregular purplish red circles with cell nuclei in the shade of purple, with or without the presence of blood cells and inflammatory cells. The boundary of observation on the coronal side is the pulp horn, and on the apical side is the orifice (19). The calculation of the number of blood vessels was performed in the coronal pulp region from the mesial to distal direction of the tooth.

The data is normally distributed and has a homogeneous variance, so parametric testing using one-way ANOVA is appropriate. One-way ANOVA parametric test was conducted to determine the effect of application of 10% and 5% green tea extract gel on the mean number of blood vessels.

Based on the one-way ANOVA test results (Table I), it is seen that there is an influence of the green tea extract gel concentration on the mean number of blood vessels ($p<0.005$). To determine the difference in the mean number of blood vessels between the pairs of groups being compared, a Post Hoc Least Significant Difference (LSD) test was carried out.

Table I. One-way ANOVA was conducted on the mean number of blood vessels in the dental pulp of rats after extracoronal bleaching with 40% hydrogen peroxide, rinsed with warm distilled water and application of 5% and 10% concentration green tea extract gel.

Variable		Number of quadrants	db	Average of quadrants	F	p
Number of blood vessels	Among the groups	245.181	2	122.590	43.579	0.0001*
	Within the groups	33.757	12	2.813		
	Total	278.938	14			

Note: (*) significant difference if $p<0.05$

The results of the LSD post hoc test showed that there is a significant differences in the mean number of blood vessels in all pairs of compared groups (Table II).

Table 2. The results of the Post Hoc LSD test for the mean number of blood vessels in the dental pulp of rats after extracoronal bleaching with 40% hydrogen peroxide, with the application of 5% and 10% green tea extract gel, and warm distilled water.

Group	Group	Difference in mean	p.
Group I	Group II	6.62	0,000*
	Group III	9.68	0,000*
Group II	Group I	-6.62	0,000*
	Group III	3.06	0,014*
Group III	Group I	-9.68	0,000*
	Group II	-3.06	0,014*

Note: (*) significant difference with p value <0.05

DISCUSSION

This research was conducted to see the effect of 5% and 10% concentration green tea extract gel on the mean number of blood vessels in the dental pulp of Wistar rats. The dental pulp consists of nerves, lymphatic tissue, connective tissue and blood vessels with a very small size so it is referred to as the microcirculatory system. Blood vessels in the microcirculatory system have a diameter of <100 µm, and vary according to the tooth and its location within the tooth, with high blood flow rate, approximately 40-50 ml/minute/100 g, consisting of arterioles, capillaries and venules (17).

The main function of microcirculation is the delivery of oxygen and nutrients to tissues and the removal of carbon dioxide and waste products. Tissue vitality is highly dependent on adequate microcirculation, and an imbalance microcirculation can lead to pathological processes and subsequently cause tissue dysfunction (18).

The presence of a large number of blood vessels in the tissue increases the supply of oxygen to more, as a response to overcome hypoxic conditions because the function of blood vessels in the microcirculation system is to send oxygen, send nutrients to the tissue and remove carbon dioxide and waste products (17). Tissue vitality is highly dependent on the adequacy of microcirculation. Microcirculation imbalance can lead to pathological processes and then cause tissue dysfunction (18). Excessive and continuous growth of blood vessels can strengthen the level of infiltration of inflammatory cells so that it can reduce tissue damage. Hydrogen peroxide which penetrates the enamel, dentin and then enters the pulp causes damage to the pulp ranging from an inflammatory response to necrosis (19). Increasing the concentration and contact time with enamel increases the effect of ROS on teeth that undergo bleaching treatment (20).

Based on the one-way ANOVA test, there was an effect of the concentration of green tea extract gel on the mean number of blood vessels between groups (Table 1). This is because the application of green tea extract gel with concentrations of 5% and 10% can reduce the amount of free radical residue on teeth after bleaching. Application of green tea extract gel with concentrations of 5% and 10% can reduce hydrogen peroxide irritation in tissues by donating electrons to free radicals and neutralizing them, thereby reducing the capacity of oxidants to damage and making free radicals more stable. This stability reduces irritation due to exposure to hydrogen peroxide so that inflammatory reactions as a form of physiological response to pulp injury are also reduced. The antioxidant effect of green tea is largely mediated by its polyphenols, especially catechins. Although green tea has various other active substances which also play a role in reducing inflammation, catechins generally provide the greatest effect. Antioxidants in green tea can inhibit cell damage, especially through their free radical scavenging properties. Green tea has been shown to play a role in blood vessels amount through its antioxidant effects and modulating blood vessels amount and inflammatory substances (21).

Post Hoc LSD test results between group I and group II showed that there was a significant difference in the mean number of blood vessels (Table 2). This is because the oxidants contained in group I cannot be neutralized by warmed distilled water. These results are found on a research by Hadi (22) explaining that histological findings 24 hours after the bleaching process with 40% hydrogen peroxide were applied twice and for 20 minutes showing tissue necrosis in the pulp chamber. Necrotic areas in the pulp of rat incisors were visible 24 hours after bleaching with 38% hydrogen peroxide (23). The occurrence of necrosis begins with significant cell damage due to the high amount of hydrogen peroxide in the pulp, then a more intense inflammatory response will occur (24) (20). Inflammation of the pulp to the point of necrosis is a result of disruption of angiogenesis in the dental pulp after bleaching. Very high levels of the number of blood vessels are characteristic of pulp inflammation. The pathogenesis of angiogenesis includes the formation of oxidative stress which causes tissue inflammation (25). The same results were also found in previous research on human teeth. Based on the results of clinical studies, it can be concluded that hydrogen peroxide gel applied to the enamel surface of human lower incisors causes severe pulp damage up to necrosis, regardless of how it is applied (26).

In group II the negative effects of hydrogen peroxide can be neutralized even though it is not optimal. This is because group II was rinsed using an antioxidant in the form of 5% green tea, as shown in Figure 1, which shows that the number of blood vessels in the control group was greater than in group II.

Antioxidants exert an inhibitory effect on then number of blood vessels. Green tea, containing EGCG as an antioxidant, demonstrates that its application after bleaching can effectively normalize excessive blood vessels in post-bleaching tissue inflammation (26).
These results are also supported by the opinions of (27) and (28) When antioxidants produced internally in the human body cannot overcome oxidants caused by external exposure, in this case, hydrogen peroxide is used for bleaching treatment, the damage can progress to the stage of necrosis. Green tea concentrations of 5% and 10% can be used as an antioxidant after bleaching with proven satisfactory results.

The phenomena observed in blood vessels as a response to the application of bleaching materials take the form of dilation and changes in blood vessel permeability. Blood vessel dilation is intended to facilitate increased blood flow, while changes in blood vessel permeability are meant to enable the migration of inflammatory cells from blood vessels to inflamed areas. The accumulation of inflammatory cells, resulting from exposure to free radicals, exerts pressure on the venules and capillaries. This pressure on the capillaries leads to reduced blood flow, creating hypoxic conditions in the dental pulp tissue. Hypoxic conditions, or oxygen deficiency in the blood, stimulate the angiogenesis (29).

One of the characteristics of the proliferative phase is angiogenesis. Angiogenesis occurs due to the expression of vascular endothelial growth factor. Vascular endothelial growth factor is induced by hydrogen peroxide to promote the angiogenesis process, which is the process of forming blood vessels (30).

The mechanism of angiogenesis that is mediated by inflammation, namely, the inflammatory response with the accumulation of inflammatory cells in the tissue creates pressure on the capillaries so that blood flow is reduced and the tissue experiences hypoxic conditions. In hypoxic conditions, pulp cells express hypoxia-inducible transcription factor -1 (Hif-1). Hif-1 will mediate proangiogenic growth factors such as vascular endothelial growth factor, platelet-derived growth factor, placental growth factor, fibroblast growth factor, and angiopoiten-1. Hypoxic conditions are the beginning of the angiogenesis. The response to tissue hypoxia is primarily mediated by hypoxia-inducible factor 1 (HIF-1). Angiogenesis is triggered by tissue hypoxia (31)(32).

Post Hoc test results with LSD in groups II and III showed a significant difference in means. Group III showed a lower mean number of blood vessels than groups II and I (Figure 1). This is because a higher concentration of antioxidants makes it possible to neutralize free radicals in greater quantities so that the negative effects of free radicals can be eliminated to a greater extent.

Antioxidants reduce the concentration of free radicals due to exposure to hydrogen peroxide by neutralizing free radicals and donating electrons to free radical ions (33). The mechanism of action of antioxidants is: 1) Blocking the production of free radicals, 2) Neutralizing free radicals, 3) Converting toxic free radicals into less toxic substances 4) Blocking the secondary production of toxic metabolites and inflammatory mediators 5) Blocking the propagation of secondary oxidant chains 6) Helping repair which tissue is affected. All these defense mechanisms work together to protect against oxidative stress (34). Green tea is a potent antioxidant. Important components in green tea that exhibit antioxidant properties include catechins. The four main catechins found in green tea are (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocationchin (EGC), and (-)-epigallocationchin-3-gallate (EGCG). Among these catechins, EGCG and EGC are the most abundant in green tea and have been the focus of most research (35). Catechins are more prevalent in green tea than in other types of tea. In vitro and in vivo studies have confirmed that catechins have strong antioxidant properties. Additionally, green tea contains minerals and vitamins that further enhance its antioxidant potential (36).

The results of this study are in accordance with the opinion of Berger (27) that 10% green tea is more effective than 5% green tea because green tea with a higher concentration has 60% catechin and 5% caffeine. Green tea catechins, like EGCG, have strong antioxidant activity due to three adjacent OH groups. The OH group on ring B removes free radicals more effectively. The greater the amount of EGCG antioxidant content, the more hydrogen peroxide is neutralized through electron transfer from EGCG. Therefore, the more hydrogen peroxide is neutralized, the lower the free radicals and negative effects of hydrogen peroxide on tooth tissue and pulp. The concentration of green tea has an effect on the defense of antioxidant function in neutralizing the large number of free radicals.

CONCLUSION

Based on the results of research regarding the effect of the applying 5% and 10% concentrations of green tea extract gel on the number of blood vessels in dental pulp after extracoronal bleaching with 40% hydrogen peroxide, it was concluded that the application of a 10% concentration of green tea extract gel resulted in a greater reduction in the number of blood vessels compared to the application of green tea extract gel with 5% concentration.

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