The Effect of Coffee Brew on the Production of Superoxide Radical by Monocytes Exposedto Dental Bleaching Material

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ABSTRACT

Aims: This study aimed to analyze whether robusta coffee brew (RCB) can inhibit the production of free radicals (superoxide radicals) by chronic inflammatory cells (monocytes) exposed to dental bleaching material carbamide peroxide (CP).
Methods: This experimental ex vivo study used the post-test-only control group design. The object was theex vivo human monocytes isolated from peripheral blood veinsusing the gradient centrifugation method. The dental bleaching material was 10% CP.The coffee brew was prepared by steeping the roasted robusta coffee ground into 200 mL of boiled distilled water. Two concentrations of coffee brew were studied i.e. 3 g/200 mL and 6 g/200 mL.There were threeexperimental groups, i.e.,1) monocyte + CP (without RCB), 2) monocyte + 3 g/200 mLRCB + CP, and3) monocyte + 6 g/200 mLRCB + CP. Production of superoxide radical was analyzed using the Nitroblue-tetrazolium (NBT) assay, which was indicated by the production of formazan (purple spots) by monocytes. The parameter was the number of monocytes that produced superoxide radicals (monocytes that expressed purple spots), which was identified under the light microscope. Data were analyzed using ANOVA and Least Significance Difference (LSD).
Results: Coffee groups demonstrated a significantly ($P<0.5$) lower number of monocytes that produced superoxide radicals.
Conclusion: Robusta coffee brew demonstrated an antioxidant effect againstsuperoxide radicals produced by monocytes exposed to dental bleaching material carbamide peroxide. The antioxidant activity of coffee brew might suggest its potentialas an agent for protecting gingiva from the irritating effect of dental bleaching material.

Keywords: [Antioxidant, inflammatory cell, gradient centrifugation, Nitroblue-tetrazolium, robusta coffee)

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1. INTRODUCTION

The application ofdental bleaching materialcarbamide peroxide (CP) produces free radicals that potentiate irritating gingival tissue, leading to chronic gingivitis. Carbamide peroxide10% is commonly used as at-home bleaching material [1]. Although CP 10 % has been approved as a safe and effective ingredient for dental bleaching, however, CP can cause an irritating effect on gingiva [2, 3, 4]. In this case, a small amount of CP can contact the marginal gingiva, potentially causing cell alterations [5] and stimulating chronic inflammatory response [6, 7].

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The chronic inflammatory response mainly involves monocytic inflammatory cells known as the mononuclear phagocytic system (MPS). These phagocytic cells circulate in the blood and migrate through capillaries into tissue where they crawl between cells in search,

phagocytize,and destroy foreign substances or antigens. This is one important part of the body's innate immune system[8].

During phagocytosis, monocytes activatethe membrane-bound enzymeNicotinamide Adenine Dinucleotide Phosphateoxidase (NADPH oxidase), which works to activate the respiratory burst. This monocyte respiratory burst produces Reactive Oxygen Species (ROS), namely superoxide radical ($O_2^{\bullet}$). Superoxide radicals will take electrons from nearby molecules to achieve chemical stability, while molecules that lose electrons become new free radicals, and attack any other molecules, leading to induce chain reactions. Thus, the production of superoxide radicals induces the production of any other ROS such as hydrogen peroxide (H_2O_2), and hydroxyl free radicals ($OH^{\bullet}$), the most reactive oxygen species. Excessive production of ROS in the body without adequate antioxidant production can result in oxidative stress [9, 10, 11, 12].It plays a role in the pathogenesis of various inflammatory diseases, such as gingivitis and periodontitis [13].

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Oxidative stress can be reduced by antioxidant substances. Coffee is one of the major sources of antioxidants [14, 15]. Antioxidants are electron-donor compounds that can counteract or prevent the formation of free radicals [11, 13]. Robusta coffee has been reported to have an effect as an antioxidant derived from the content of polyphenols, chlorogenic acid [14, 15], and flavonoids [10, 16]. This study aimed to study the effect of Robusta coffee brew (RCB)onthe production of free radicals (superoxide radicals) by chronic inflammatory cells (monocytes) exposed to dental bleaching material carbamide peroxide (CP).

2. MATERIAL AND METHODS

2.1 Materials.

Robusta coffee ground was purchased from the factory of the Coffee State Plantation *PTPN XII*, East Java, Indonesia. Reagents for monocyte isolation, Phosphate Buffer Saline (PBS), and Hanks Balanced Salt Solution (HBSS) were purchased from Sigma, RPMI media from Roswell Park Memorial Institute, Lymphoprep (Fisher Scientific Inc.), Penicillin-streptomycin (Sigma), Fungizone (Gibco). The dental bleaching material was Whiteness perfect carbamide peroxide 10 % (FGM). Nitro Blue Tetrazolium (NBT) was purchased from Scytek. Syringe filter0.2µm and 0.45 µm were purchased from Whatman.

2.2 Preparation of robusta coffee brew (RCB)

The coffee brew was made by steepingroasted robusta ground coffee into 200 mL of boiled distilled water (90°C), according to the method by Susilawati et.al with some modifications [17]. It wasmade in two concentrations i.e, 1) RCB 3 g/200 mL,2) RCB 6 g/200 mL.The procedure was as follows, 200 mL distilled water in a beaker glass was placed on a hotplate stirrer at a temperature of 90°C, the coffee ground was then poured and steered with the speed of 60 rpm for 1 min. This coffee brew was left until the temperature reached around 25°C, andthen filtered using a syringe filter0.2µm. The filtrate of the coffee brew was kept in the refrigerator (4°C) until before being used for the experiment.

2.3 Preparation of dental bleaching material

Carbamide peroxide 10 % was taken as much as 10 mg by 214 Adam PW analyticalbalance, put in Eppendorf tubes, and dissolved with 50 mL of PBS [6]. After that, it was placed on a vertex to get a homogeneous solution, and filtered with a syringe filter of 0.45 µm.

2.4 Preparation of Monocytes.

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Monocyte was isolated from healthy human peripheral blood, according to the procedure recommended by Fisher Scientific Inc. (lymphoprepbrochure), with some modifications. An amount of 6 mL blood wastaken intravenously in the human cubital fossa, then put in the two heparin tubes (3 mL) and shaken. Blood samples in each tube were then poured into falcon tubes (15 mL) using a micropipette, diluted using Hanks Balanced Salt Solution (HBSS) with a ratio of 1:1, and then mixed until homogenous. Blood diluent was then layered on 3 mL lymphoprep solution and centrifuged (900 rpm for 20 min at 20ºC).Centrifugation resulted in four layers (plasma, mononuclear, lymphoprep, polymorphonuclear erythrocytes). The second layer containing mononuclear cells was carefully taken using a micropipette, then inserted into one other falcon tube and rinsed (twice) using RPMI media containing 5 μL fungizone, and 20 μL penicillin-streptomycin. The pellet of the monocyte was re-suspended in 1200μL HBSS and ready for further assay.

2.5 Experimental ProcedureandNBT Assay.

The first step was monocyte isolation and preparation. A total of three plastic six-well plate cultures were prepared and given a sterile coverslip inside the bottom of each well. The amount of100μLmonocyte suspension was taken (using a micropipette)and put on the coverslip. The plastic well plate culture was then incubated for 5 minat 37ºC and added 1 mL RPMI media to each well, and incubated for 30 min at 37ºC. The plastic well plate culture was taken from the incubator shaker, and then observed under an inverted microscope, by gently rocking it to see the attachment of the cell. Cells in each well were washed with RPMI media three times carefully to removethe unattached cells (lymphocytes), then observed again under an inverted microscope to ensurethe homogeneity of monocytes. Afterward, the RPMI media was replaced with HBSS; then, the cell was ready to be treated.

The next step was coffee treatment and NBT assay. Group coffee 1 was treated with 3 g RCB, and coffee 2 with 6g RCB, and the control group was not treated withRCB (without coffee). (3) All of the groups were then exposed to 20 μL of carbamide peroxide 10 % and were given 250 μLNBT test solution. The summary of the experimental groupswas as follows: 1) monocyte + CP (without RCB) + NBT, 2) monocyte + 3 g RCB + CP + NBT, and 3) monocyte + 6 g RCB + CP + NBT.Allof the wells were incubated for one hour in a shaker incubator at 37ºC. After incubation, the glass cover on the well plate culture was taken, and the monocytes attached were washed twice using HBSS, aerated, and then fixed with methanol. Furthermore, they were subjected to the counter-stainSafranin solutionand ready for examination microscopically using the light microscope magnification 400 and 1000.

2.6Radical superoxide identification.

Production of superoxideradicals was demonstrated by monocytes expressingpurple-formazan particles (positive NBT cells). The percentage of positive NBT cells was determined by randomly calculating the number of positive NBT cells in four fields of view (each slide). Three observers carried out the examination using a light microscope magnification400 times. The antiradical activity of RCB could be seen from the number of cells that do not produce superoxide radicals[17].

2.7 Statistical analysis

Data were analyzed with the normality test Shapiro Wilkand homogeneity test using the Levene test. One-way ANOVA was used to determine the difference between the overall treatment and LSD to detect a significant difference between groups.

3. RESULTS

The number of monocytes thatproduced superoxide radicals was significantly (*P<0.05*) lower in the coffee groups than in the control group (Table 1). Coffee brews of 6 gr/200 mLaffected

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the reduced significance superoxide radicals than 3 gr/200 mL.The results of the microscopic examination demonstrated the expression of superoxide radicals (purple spots of formazan) by monocytes(Figure 1).

Table 1. The effect of robusta coffee brew on the production of superoxide radicals by monocytes exposed to carbamide peroxide

Groups	The number of monocytes that produced superoxide radicals (X ±SD)
Monocytes+ 10% CP (without coffee, control)	89±2.50
Monocytes + RCB 3 gr/200 mL + 10% CP	61 ± 7.23*
Monocytes + RCB6 gr/200 mL+ 10% CP	43 ±5.10*

* Significantly different (*P<0.05*) compared to control group (without coffee)
RCB: Robusta Coffee Brew; CP: carbamide peroxide

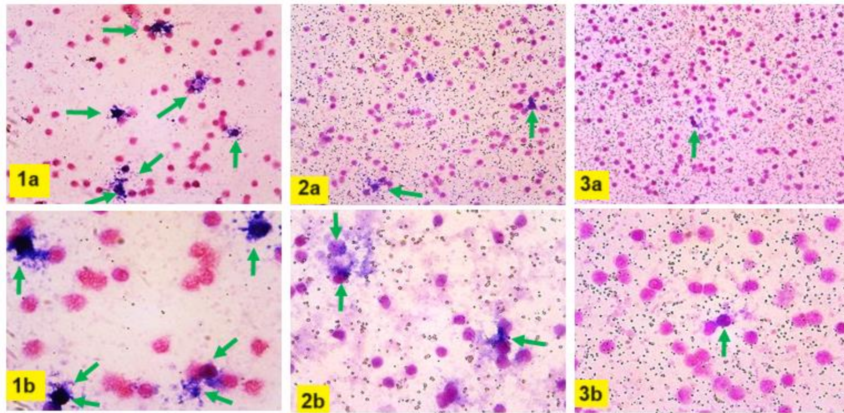

Figure 1.The effect of robusta coffee brew on the production of superoxide radicals by monocytes exposed to carbamide peroxide. The green arrow indicated monocytes that produce superoxide radicals (purple-formazan). Microscopic feature (a) 400x magnification and (b) 1000x magnification. 1) Control Group: monocytes+ 10% CP (without coffee); 2) Coffee Group 1:monocytes + RCB 3 gr/200 mL + 10% CP;3) Coffee Group 2:monocytes + RCB6 gr/200 mL+ 10% CP. RCB= robusta coffee brew; CP= carbamide peroxide.

4. DISCUSSION

Home bleaching is a tooth whitening technique performed by patients themselves under the supervision of a dentist using 10-20% carbamide peroxide[15,18]. This technique is relatively easy; the patient applies dental material whitening using a special tray without gingival tissue isolation. This differsfrom the in-office bleaching technique in which gingival tissue isisolated with a rubber dam or protective gel to prevent material penetration into soft tissue [18, 19].Carbamide peroxide 10 to 35% has been suggested as a safe option for bleaching vital teeth [20]. Bleaching effects are primarily based on the effects of carbamide peroxide, releasing about 33% of its content as hydrogen peroxide (H_2O_2) acts as a powerful oxidizing agent (ROS). It can give rise to agents known to be effective bleaching agents [3, 21, 22]. Despite the positive effect (teeth whitening) of bleaching, it may have some side effects,

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such as dentin sensitivity and irritation of the oral soft tissues in contact with the bleaching gel [1, 7], in addition to the possibility of cell mutation [23].

The present study showed that the dental bleaching material carbamide peroxide could inducethe production of a high amount of superoxide radical by monocytes. ROS are fundamental for monocytes to eliminate foreign substances; however, excessive production of ROS can cause detrimental effects. Dental bleaching material is a foreign material for the human tissue and, therefore, could induce the activation of phagocytes such as monocytes [28, 29].The initial response of monocytes is the activation of NADPH oxidase, which will trigger a respiratory burst,formingsuperoxide radicals. It is the first ROS produced by monocytes that can trigger the production of other ROS[9]. The content of the active ingredient H_2O_2 in bleaching dental material can further induce the formation of free radicals in the event of an oxidation reaction. The presence of $H_2O_2$and superoxide radicalswill form hydroxyl radicals, the most reactive ROS [21, 30].

The present study found that the number of monocytes that produce superoxide radicals in the coffee group was lower than in the control group, which was not treated by coffee brew.This study highlights the potency of coffee as an antioxidant, which can mute the production of superoxide radicals and terminate free radicals chain reactions. The superoxide radical is the center of the redox reaction in phagocytes; it is easily converted to other ROS and causes excessive production of ROS,leading to oxidative stress. Inhibition of superoxide radicals would reduce the production of other ROS and oxidative stress,leading to protection against cellular oxidative damage[17]. The present study showed that the amount of intact monocytes in the coffee group wasgreater than in the control group, which was not treated with coffee.

Coffee contains antioxidant compoundsthat arecapable of donatingelectrons and neutralizing oxidants or free radicals. Several compounds in coffeeare well-known to have antioxidant properties, including caffeine, phenolics, and melanoidin. The antioxidant activity of caffeine was shown by the affinity to scavenge ROS such as superoxide radicals, and hydroxyl radicals, and the potencyto decompose hydrogen peroxide [15]. Phenolics, mainly chlorogenic acids (CGAs),are known to be the main antioxidant component in coffee. Chlorogenic acidsare powerful hydroxyradical scavengers. Melanoidin, a high molecular weight compound, brown colored, is produced during the Maillard reaction in coffee roasting. Other phenolics, such as flavonoid compounds contained in robusta coffee, also have the ability as antioxidants. Flavonoids can scavenge peroxyl radicals. It is an effective inhibitor of lipid peroxidation and chelates redox-active metals, thus preventing the catalytic breakdown of hydrogen peroxide [10, 11]. Melanoidin has an affinity to scavenge hydroxyl and proxy radicals, breaking the radical chain reaction by donating hydrogen and also chelating pro-oxidant transition metal ions [15].

Results in the present study showed that the percentage of monocytes that produce superoxide radicals in the coffeegroup 6 g/200 mLsuppressed the productionof superoxide radicals more than in the group of 3 g/200 mL. Presumably, it was due to the more antioxidant content. However, it does not meanthat the greater the dose, the better the antioxidant power. Further studies are needed to analyze the concentration of antioxidant substancesin the coffee brew and whether ahigher dose can demonstratebetter antioxidant activity.

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5. CONCLUSION

Robusta coffee brew demonstrated antioxidant activity against superoxide radicals produced by monocytes exposed to dental bleaching material carbamide peroxide. The antioxidant activity of coffee brew might suggest its potential as an agent for protecting gingiva from the irritating effect of dental bleaching material.

ETHICAL APPROVAL

All of the experimental procedures have been examined and approved by The Ethical Committee of the Medical Research Faculty of Dentistry University of Jember, Indonesia (No. 022/UN.25.8/KEPK/DL/2018).

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