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To cite this article: I D A Susilawati *et al* 2019 *IOP Conf. Ser.: Earth Environ. Sci.* **293** 012022

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# Coffee reduced the production of neutrophil superoxide radical *in vitro*

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**Abstract.** Stimulated neutrophil is the main source of oxidants/free radicals, that might contribute to the state of oxidative stress. Coffee has been well known to have a high antioxidant capacity, therefore it was plausible to hypothesize that coffee might reduce oxidants/radicals produced by neutrophil. This study aimed to analyze effect of coffee on superoxide radical produced by neutrophil *in vitro*. This study was conducted experimentally using the post test only control group design. Neutrophil was isolated from healthy human peripheral blood. Superoxide radical production was analyzed by NBT (nitro blue tetrazolium) assay. Results showed that coffee significantly ( $P < 0.05$ ) reduced the number of neutrophils that produce superoxide radicals. In conclusion, coffee demonstrated antioxidant capacity against neutrophil superoxide radicals.

**Keywords:** Antioxidant, NBT (nitro blue tetrazolium), oxidant, oxidative stress, ROS (reactive oxygen species).

## 1. Introduction

Neutrophil is the main white blood cell that play important role in human innate immune system, mainly during inflammation process [1]. In doing so, neutrophil phagocyte and destroy injurious agents by oxidative and enzymatic mechanism [2] Although neutrophil activity is intended to destroy injurious agents, however, its mechanism produce oxidative and toxic milieu that can cause damage to the molecules surrounding tissue. Therefore excessive activation of neutrophil will follow by excessive production oxidants (mainly reactive oxygen species, ROS), and when this condition is not balanced by sufficient antioxidant capacity, it will cause oxidative stress. This mechanism is thought to be the molecular basis role of neutrophil in various acute pathological event such as cerebral and myocardial infarction [3, 4].

Initial response of neutrophil against injurious agents is respiratory burst (increasing oxygen uptake). This will follow by activation of membrane NADPH oxidase or NOX that responsible for production of superoxide radicals ( $O_2^{\bullet-}$ ) and subsequent chain reaction and production of any other ROS such as hydrogen peroxide, peroxynitrite, hydroxyl radicals, hypochloric acids. These ROS can cause oxidative modification of molecule surrounding tissue. In addition, over production of ROS can cause neutrophil degranulation and release of its toxic oxidative molecule into surrounding tissue. Therefore it is very important to reduce over oxidative activity of neutrophil.



Coffee was alleged to be able to modulate oxidative activity of neutrophil, since it has a high antioxidant capacity [5, 6]. This study aimed to analyze effect of coffee on superoxide radical produced by neutrophil *in vitro*.

## 2. Material and methods

### 2.1. Materials

Ground Robusta coffee [*Coffea canephora* (Pierre ex A.Froehner)] was purchased from Coffee State Plantation PTPN XII, East Java, Indonesia. Whole blood of healthy adult human donor (no history of systemic or chronic diseases). Materials for neutrophil isolation i.e. Ficoll-Hypaque gradient 1077 and 119 were purchases from Sigma; Dextran 500 from Merk; NBT from Scy Teck; RBC lysing buffer, Safranin (counter stain), Hank's Buffered Salt Solution (HBSS) from Sigma. Stimulated antigen was medium culture of *Streptococcus mutans* (wild type).

### 2.2. Coffee solution

Coffee was prepared by diluting 6 g ground coffee into 200 mL boiled water (90 °C), steered for 1 min and let it cooled down until room temperature. Coffee solution then centrifuged for 3 000 rpm (1 rpm = 1/60 Hz) at room temperature for 10 min., the supernatant filtrate was separated and then stored until used for further experiments.

### 2.3. Neutrophil cell suspension

Neutrophil was isolated from freshly collected venous peripheral whole blood of healthy adult human donor. Neutrophil isolation was done by method double ficoll gradient density (according to manual procedure from Sigma). A total amount of 12 mL whole blood was drawn and immediately pour into heparized tube and homogenized by gently shaking. Heparinized blood was then diluted threefold in HBSS and layered on histopaque 1077 and 119, and centrifuged for 1 900 rpm, at room temperature for 30 min. Polymorphonuclear cell layer was separated, remaining red blood cells were lysed by RBC lysing buffer. Cell pellet were then subjected for three times rinses, and resuspension in 500 mL HBSS, and neutrophils suspension were ready for assay.

### 2.4. Superoxide radical assay

Superoxide was assayed by means of NBT assay as previously described [7, 8]. The parameter was the number of neutrophil that produced superoxide, indicated by neutrophil containing dark blue NBT formazan granula inside and in membrane of neutrophils. The procedures were as follow. Initially, 12 coverslips was prepared and then were placed into six well plastic chamber. Suspension of neutrophil were gently layer and spread on the surface of each coverslip (50 µL) and added by 500 mL HBSS and incubated 30 min at 37 °C, 5 % CO<sub>2</sub>. After twice rinsing, neutrophil in the wells number 1 to 6 were subjected as coffee group, and the other as control group. For coffee group, neutrophil were incubated with coffee suspension (100 µL), then stimulated by antigen *S. mutans* (50 µL), and added by NBT solution. Same procedure was done for control group, however it did not incubated with coffee but HBSS. Incubation were done for 30 min. At the of experiment, cells were rinsed and fixed with (methanol) and stain with safranin. And afterword, coverslip was mount on microscopic slide. Positive neutrophil containing superoxide radical demonstrated dark blue deposit, while cell cytoplasm demonstrated red color. This description was identified using light microscope magnification 400 times. The number of neutrophil containing superoxide radicals were counted per 100 cells.

## 3. Results

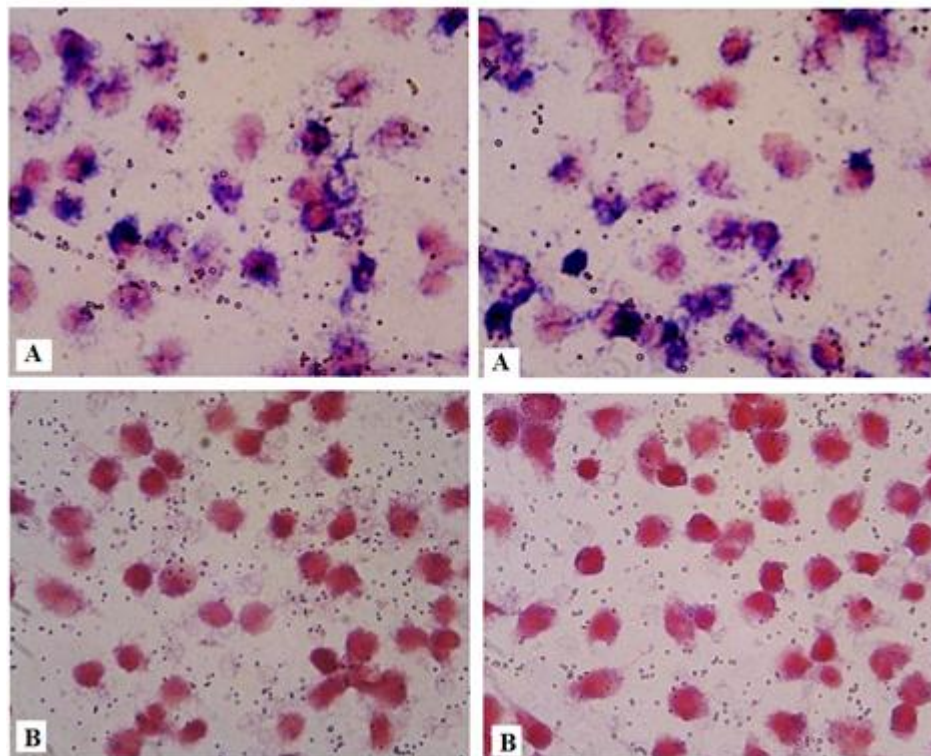
The number of stimulated neutrophil that produced superoxide radicals was significantly ( $P < 0.5$ ) lower in coffee group compared to control group (not incubated with coffee) (table 1). Using NBT assay, cells that produced superoxide radicals could be identified by the formation of dark-blue deposit

formasan. Application of counter-stain (safranin) colored the cytoplasm to become red. Neutrophil in coffee group demonstrated predominantly red color with a minimum formasan deposit (figure 1).

**Table 1.** The number of neutrophil that produced superoxide radicals.

Group	Neutrophil produced superoxide radicals per 100 cells ( $\bar{X} \pm SD$ )
Coffee	$34.50 \pm 4.87$
Control	$87.25 \pm 3.57$
t test	$P < 0.05^*$

\* significant

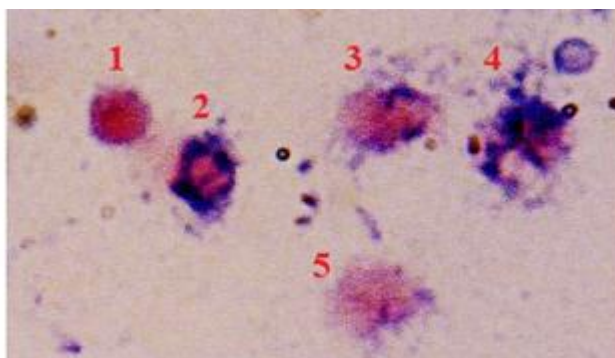


**Figure 1.** Coffee reduced neutrophil superoxide radicals production (NBT assay)

A. Control group, neutrophil produced superoxide radicals (dark blue deposit)

B. Coffee group, reduced production of superoxide radicals by neutrophil

Description of neutrophil to produce superoxide radicals was presented in figure 2. Stimulated neutrophil produced superoxide radicals started from cell membrane (1). Massive production of superoxide radicals was demonstrated by a heavy dark-blue deposit formasan around cell membrane (2), and it could cause neutrophil degranulation (3 and 4). Further degranulation followed by further secretion of intracellular components into extracellular milieu, and advanced degranulation caused neutrophil lysis (5).



**Figure 2.** Radical superoxide production by neutrophils. (1) light and (2) heavy darkblue formasan deposit around cell membrane, (3 and 4) neutrophil degranulation released superoxide radical extracellularly, and (5) lysis

#### 4. Discussion

Initial response of neutrophil against injurious agent or stimuli is respiratory burst (increase cellular oxygen consumption). During resting, neutrophils consume only little oxygen, because the main metabolism process to produce energy (*adenosine triphosphate*, ATP) use glycolysis pathway that does not need oxygen. If there are stimuli, however, neutrophils will activate the membrane enzyme system nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), and this triggers an increase in cellular oxygen consumption. The reaction between oxygen and NADPH oxidase produces superoxide radicals [9, 10].

Superoxide radical in this study was analyzed by means of the NBT method. The rationale, stimulated neutrophils will produce superoxide radicals which reduce NBT to form an insoluble formazan deposit, which can be detected by light microscope as a dark-blue granula in the cell cytoplasm. Results of this study showed that treatment using coffee solution reduced formazan production. It could be meant that coffee reduced superoxide production. In other words, coffee demonstrated antioxidant activity against superoxide radicals.

It is important to study further concerning mechanism of coffee antioxidant activity. Theoretically, antioxidant substances will donate their electron to radicals, so they are no longer become radicals. It could be shown that coffee scavenging superoxide radicals more quickly compared to NBT so that formazan production could be inhibited. Further study is also needed to study whether reduction of superoxide radicals is due to the modulation of coffee on NADPH oxidase.

Type of antioxidants in coffee is important to study as well. Ground coffee was prepared by roasting process that could affect the composition of antioxidant molecules. Perhaps this roasting process causes antioxidants of coffee specific compared to any other beverages. Also if it is compared with other sources of antioxidants such as fruits or vegetables.

Superoxide radical is a center of cellular oxidant. *In vivo*, production of superoxide will induce chain reaction to produce any other oxidants such as nitric oxide (NO), peroxynitrite (ONOO<sup>-</sup>). Superoxide also can be converted into hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by superoxide dismutase (SOD). Neutrophils contained myeloperoxidase that can convert H<sub>2</sub>O<sub>2</sub> into hypochlorous acid (HOCl) which is a strong oxidant. Hypochlorous acid can react with superoxide to produce strong toxic oxidant hydroxyl radicals. Therefore, acute stimulated neutrophils can produce oxidants and radicals in a large amount that trigger oxidative burst. And if it is not balanced by adequate antioxidant, it will cause oxidative stress that induces pathologic process and inflammatory diseases [3, 11, 12].

In conclusion, this study demonstrated that coffee reduced production of superoxide radicals *in vitro*. It could be meant that coffee might reduce potency of oxidative stress. In other words, coffee might protect our health against oxidative inflammatory diseases.

## Acknowledgement

This study was supported by grant from The Ministry of Research-Technology and Higher Education of Republic Indonesia (Grant number: 058/SP2H/LT/DRPM/2018). The authors also would like to thank to Mr. Erwan and Mrs. Azizah for helping us in laboratory works.

## References

- [1] Selders G S, Fetz A E, Radic M Z and Bowlin G L 2017 An overview of the role of neutrophil in innate immunity, inflammation and host biomaterial integration *Regen. Biomater.* **4**(1) 55–68  
<https://www.ncbi.nlm.nih.gov/pubmed/28149530>
- [2] Slauch. J M 2011 How does the oxidative burst of macrophages kill bacteria? Still an open question *Mol. Microbiol.* **80**(3) 580–83  
<https://www.ncbi.nlm.nih.gov/pubmed/21375590>
- [3] Vichova T and Motovska Z 2013 Oxidative stress: Predictive marker for coronary artery disease *Exp. Clin. Cardiol.* **18**(2) e88–e91  
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3718605/>
- [4] Rodrigo R, Fernández-Gajardo R, Gutiérrez R, Matamala J M, Carrasco R, Miranda-Merchak A and Feuerhake W 2013 Oxidative stress and pathophysiology of ischemic stroke: Novel therapeutic opportunities *CNS Neurol. Disord. Drug Targets.* 2013, Vol. 12,(2): 1-17  
<https://www.ncbi.nlm.nih.gov/pubmed/23469845>
- [5] Fukushima Y, et al. 2009 Coffee and green tea as a large source of antioxidant polyphenols in the Japanese population *J. Agric. Food Chem.* **57**(4) 1253–59  
<https://www.ncbi.nlm.nih.gov/pubmed/19187022>
- [6] Fukushima Y, et al. 2014 Coffee and beverages are the major contributors to polyphenol consumption from food and beverages in Japanese middle-aged women *J. Nutr. Sci.* **3**(e48) 1–10  
<https://www.ncbi.nlm.nih.gov/pubmed/26101616>
- [7] Susilawati I D A 2011 Induksi metabolit porphyromonas gingivalis pada produksi superoksida radikal netrofil [Induction of porphyromonas gingivalis metabolites on production of superoxide radicals by neutrophil] *Dentika Dental Journal* **16**(1) 9–13 [in Bahasa Indonesia]  
<https://jurnal.usu.ac.id/dentika/article/view/3301>
- [8] Dewi A A I P S, Siswoyo T and Susilawati I D A 2016 Antioxidant activity of hydrolyzed melinjo (*Gnetum Gnemon*) seeds protein against neutrophil superoxide radical in vitro *IJASEAT* **4**(4) 141–45  
[http://ijaseat.iraaj.in/paper\\_detail.php?paper\\_id=9947&name=Antioxidant\\_Activity\\_of\\_Hydrolyzed\\_Melinjo\\_\(Gnetum\\_Gnemon\)\\_Seeds\\_Protein\\_Against\\_Neutrophil\\_Superoxide\\_Radical\\_in\\_Vitro](http://ijaseat.iraaj.in/paper_detail.php?paper_id=9947&name=Antioxidant_Activity_of_Hydrolyzed_Melinjo_(Gnetum_Gnemon)_Seeds_Protein_Against_Neutrophil_Superoxide_Radical_in_Vitro)
- [9] Zalba G et al. 2007 Phagocytic NADPH oxidase-dependent superoxide production stimulates matrix metalloproteinase-9 *Arterioscler. Thromb. Vasc. Biol.* **27**(3) 587–93  
<https://www.ncbi.nlm.nih.gov/pubmed/17194891>
- [10] Hordijk, P L 2006 Regulation of NADPH oxidases *Circ. Res.* **98** 453–462  
<https://www.ahajournals.org/doi/abs/10.1161/01.res.0000204727.46710.5e>
- [11] Hajjar D P and Gotto A M 2013 Biological relevance of inflammation and oxidative stress in the pathogenesis of arterial diseases *Am. J. Pathol.* **182**(5) 1474–81  
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3644714/>
- [12] Sesti F, Tsitsilonis O E, Kotsinas A and Trougakos I P 2012 Oxidative Stress-mediated biomolecular damage and inflammation in tumorigenesis *In Vivo* **26**(3) 395–402  
<https://www.ncbi.nlm.nih.gov/pubmed/22523291>