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
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
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
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
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
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
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
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
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
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
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
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
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
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# The effects of catechin isolated from green tea GMB-4 on NADPH and nitric oxide levels in endothelial cells exposed to high glucose

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## ABSTRACT

**Aim:** This study aimed to investigate whether a catechin isolated from GMB-4 green tea is able to increase the reducing equivalent system and nitric oxide (NO) level in endothelial cells exposed to high glucose (HG) level. **Materials and Methods:** Endothelial cells were obtained from human umbilical vascular tissues. **At** confluent, human endothelial cells were divided into five groups, which included control (untreated), endothelial cells exposed to HG (30 mM), endothelial cells exposed to HG in the presence of green tea catechin (HG + C) at the following three doses: 0.03; 0.3; and 3 mg/ml. **Analysis of NADP<sup>+</sup>, NADPH, and NO levels were performed colorimetrically.** **Results:** This decrease in NADPH was significantly ( $P < 0.05$ ) attenuated by both the 0.3 and 3 mg/ml treatments of catechin. HG level significantly decreased NO compared with untreated cells. **This increase in NO was significantly attenuated by the 0.3 mg/ml dose of the catechin.** **Conclusion:** **in conclusion, catechin isolated from GMB-4 green tea prohibits the decrease in NADPH and NO in endothelial cells induced by HG.** **Therefore this may provide a natural therapy for attenuating the endothelial dysfunction found in diabetes mellitus.**

**KEY WORDS:** Endothelial dysfunction, green tea, high glucose level, oxidative status

## INTRODUCTION

Reactive oxygen species (ROS) are causal factors for oxidative damage to various cells and induce organ dysfunction after high glucose (HG) level injury. However, they are also involved in cellular signal transduction pathway [1]. NADPH oxidase, a complex formed by Nox and other cytosolic subunits, whose only function is ROS production of molecular oxygen, has been extensively investigated in many cell types, including vascular endothelial cells [2,3]. The amount and site of ROS production as well as antioxidant defense determined its cellular's effect. Low levels of oxidative stress appear to be physiological and beneficial cellular signal in reparative angiogenesis, while excess amount of ROS contributes to endothelial cells injury and dysfunction [4-6]. Exposure of endothelial cells to HG induces ROS production in vitro [7,8]. NADPH oxidase have been reported to induce ROS production in diabetic vascular, which significantly contributes to endothelial dysfunction [9-11]. Excessive ROS production also

induces a dysfunctional eNOS, call as eNOS uncoupling, caused by superoxide and nitric oxide (NO) production [9,12]. These product will reacts directly to form a more harmful molecule peroxynitrite (ONOO<sup>-</sup>), thereby reducing NO bioavailability [12,13].

Previous studies were carried out to find specific agents to inhibit the NADPH oxidase activity. Peptides such as Gp91ds-tat and PR39 were shown to possess decoy p47phox binding sites, which prevent interaction between p47phox and NOX proteins, then suppress NADPH oxidase activation [14,15]. Many traditional plant products are in use due to their therapeutic potential, including garlic, ginger and green tea [16]. The Gambung Research Institute for Tea and Quinine has successfully developed a green tea with higher levels of catechin (14-16%) than other tea plant, known as GMB4 clones [17]. Previous studies showed that catechin from GMB4 clones act as insulin resistance inhibitor in visceral fats and adipose tissue [18], significantly decrease C/EBP $\alpha$  on the culture of visceral preadipocytes rat [19], and decreases eNOS expression, increases PI3K expression, and decreases p38 MAPK activity in rat

fed high lipid diet [20]. As far we know, there is no previous studies explore this catechin on endothelial cells.<sup>[10]</sup> Therefore this study aimed to investigate whether green tea catechin able to modulates the level of NADPH as reducing equivalent and increase nitric oxide level in endothelial cells exposed to HG.

## <sup>[10]</sup> MATERIALS AND METHODS

### Endothelial Cells Isolation and Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from pregnant women at Gambiran Hospital, Kediri, East Java, Indonesia.<sup>[10]</sup> The criteria for pregnant women are a healthy pregnancy (hemoglobin level  $\geq 10$  g/dl) accompanied by a section cesarean delivery (38 weeks of gestation). Immediately postpartum, 10 cm of the umbilical cord was placed in the buffer (100 ml Hank's Balance Salt Solution, gentamycine (GENTA, MERCK, Germany), sodium hydrogen carbonate, 4 ml red phenol, 2 ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid solution, and deionized water) and kept cold until endothelial cells were isolated. Endothelial cell isolation was performed no 12 h after delivery. For the cell isolation, umbilical veins were cleaned with alcohol to remove tissue debris and blood clots [21].

A cannula was then inserted ( $\pm 1.5$  cm) at one end of the vein and secured tightly using suture. Using the cannula, veins were gently flushed with phosphate-buffered solution then closed off tightly at the distal to the cannula. Collagenase (5 mg/10 ml; 10 ml) was injected into the vein via 10 ml of syringe and incubated at 37°C approximately 8 min. The cell pellet was suspended in 4 ml 199 culture medium (SIGMA, M-5017, USA) supplemented with gentamycin, bicarbonate phenol red, 20% fetal bovine serum (GIBCO), and 20 ml newborn calf serum (SIGMA, N-4637, USA). This cell suspension was seeded into the culture wells that had been coated with gelatin. Cell were allowed to grow to confluency at 37°C 95%O<sub>2</sub> 5% CO<sub>2</sub> [21].

### Raw Extraction

A total of 25.1 g of green tea samples clones GMB - 4 is brewed with 500 mL of distilled water at temperature 90°C, then filtered with plastic tea strainer, which combined with cloth flannel and Buchner funnel. The process is repeated twice (using a sample that has been brewed in advance) to obtain a sample extract as much as 1.5 L.

### Catechin Isolation

Extract samples were eluted into the column, the elution results then accommodated. After that, the column was eluted with 300 mL of 10% ethanol. The column was eluted with 10% ethanol 2.100 mL again, elution were done gradually with each elution of 100 mL, the elution were accommodated with a different container, then dried in a vacuum oven (fraction 10% ethanol). Subsequently, the column was eluted with 95% ethanol and 300 ml of elution results will accommodated. The column was eluted with 95% ethanol, elution was carried out gradually with each elution of 100 mL, and accommodated with containers different, then evaporate (95% ethanol fraction).<sup>[10]</sup> The eluent that used for thin layer chromatography analysis fraction

was 10% ethanol is ethyl acetate, while the eluent to 95% ethanol fraction was methanol:chloroform at ratio 1:9.

### High-performance Liquid Chromatography (HPLC) analysis

Analysis of catechins by HPLC using standard methods and additional external standard. Eluent to catechins using HPLC analysis was made with aquabidest: acetonitrile: methanol: glacial acetic acid = 79.5:18:2:0.5; and for eliminating the dissolved gas is used ultrasonic bath. EGCG standard solution prepared as a comparison. Furthermore, analysis of catechins from ethanol fraction 10% done dissolving 1.3 mg of sample in 3 mL of eluent, while the fraction of ethanol 95 % made with dissolving 2.06 mg of the sample in 1 mL of eluent. Measurements were made using wavelength of 280 nm.

### Endothelial Cells Treatment

Human umbilical vascular endothelial cells were cultured in 5-well dish (at 90% confluence) and grown in serum free medium for 4 h. After confluent, cells were exposed to three doses of the catechin (0.03 mg/ml, 0.3 mg/ml, 3 mg/ml) simultaneously with 30 mM glucose treatment. This catechin was isolated from GMB-4 green tea species. The negative control group of the HUVEC was not exposed to glucose (untreated group) and the positive control HUVEC was exposed to 30 mM glucose HG but not exposed to green tea catechins. We performed five replications each group.

### Analysis of NO

NO levels were detected with a colorimetric method using NO Assay Kit KGE001 (R and D System, USA).

### Analysis of NADPH

NADPH levels were assayed with a colorimetric method using NADPH Assay Kit ab65349. (Abcam, USA).

### Ethics

This study was approved by the Health Research Ethics Committee of the Faculty of Medicine Brawijaya University.

### Statistical Analysis

Data are presented as mean  $\pm$  standard deviation and differences between groups were analyzed using one-way ANOVA with SPSS 17.0 Statistical Package. The *post-hoc* test was used if the ANOVA was significant.  $P < 0.05$  was considered as statistically significant.

## RESULTS

Standard EGCG solution shows the presence of high peaks and large areas at a retention time was 5.153. Furthermore, the analysis of freeze dried extract, obtained seven major peaks, which indicate caffeine compound, C, EC, GC, EGC, EGCG and ECG.<sup>[10]</sup> Fraction of 10% ethanol shows four main peaks at retention time

of 1.663; 2.367; 2.950; and 4.890. This indicates that there are four components of catechins that the C, EC, GC, and ECG. Meanwhile, 95% ethanolic fraction found two main peaks at the time retention 5.167 and 9.82; which indicated EGCG and ECG.

Table 1 presents the levels of NADPH, NADP and NADPH/NADP<sup>+</sup> ratio in the cell culture media for each endothelial cell experimental group. The level of NADPH was significantly lower in the EC+HG group compared with the untreated control group ( $P < 0.05$ ). Out of the 0.03 mg/ml, 0.3 mg/ml, 3 mg/ml doses of green tea catechin, only the two highest doses significantly prevented HG-induced decrease in NADPH level. There was significant no difference between the effects of these two highest doses ( $P > 0.05$ ). In addition, the levels of NADP<sup>+</sup> and NADPH/NADP<sup>+</sup> were not significant differences between groups ( $P > 0.05$ ).

Figure 1 presents the NO levels in the culture media from each endothelial cell experimental group. The NO levels were significantly lower in the HG group compared to the untreated group ( $P < 0.05$ ). These decreased levels of NO in the HG group were significantly elevated by 0.3 mg/ml administration of green tea catechins. Indeed, administration of 0.3 mg/ml extract to the HG-treated endothelial cells reduced NO levels to those comparable to the untreated group.

## DISCUSSION

In this study, the exposure of HG level in endothelial cells decreases the level of NADPH compared with the untreated control group ( $P < 0.05$ ). This finding indicated that the HG level affects endothelial cells redox potential. Our finding confirming previous studies that hyperglycemia is associated with metabolic disturbances affecting cellular redox potential, particularly the NADPH/NADP<sup>+</sup> [22]. HG level exposure may induce metabolic insufficiency to support the increased

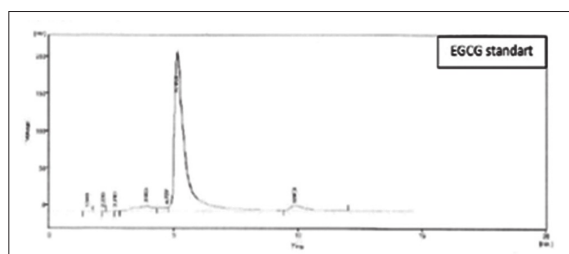


Figure 1: Level of nitric oxide endothelial cells induced by high glucose (HG). <sup>a</sup> $P < 0.05$  in comparison with control group; <sup>b</sup> $P < 0.05$  in comparison with HG group; NO: Nitric oxide,  $\mu$ M: Micromolar

Table 1: Level of NADP and NADPH endothelial cells induced by HG

Level	Control	HG	HG + catechin		
			0.03 mg/ml	0.3 mg/ml	3 mg/ml
NADPH ( $\times 10^{-9}$ M)	15.47 $\pm$ 1.58	5.16 $\pm$ 4.40 <sup>a</sup>	10.05 $\pm$ 8.95	23.08 $\pm$ 2.62 <sup>abc</sup>	18.22 $\pm$ 2.33 <sup>b</sup>
NADP <sup>+</sup> ( $\times 10^{-9}$ M)	13.60 $\pm$ 2.89	8.91 $\pm$ 1.78	13.53 $\pm$ 8.66	20.44 $\pm$ 8.06	21.68 $\pm$ 1.99
NADPH/NADP <sup>+</sup>	1.18 $\pm$ 0.31	0.57 $\pm$ 0.43	0.73 $\pm$ 0.70	1.32 $\pm$ 0.66	0.84 $\pm$ 0.13

Values are presented as mean  $\pm$  SD, <sup>a</sup> $P < 0.05$ ; in comparison with control group; <sup>b</sup> $P < 0.05$ ; in comparison with HG group, <sup>c</sup> $P < 0.05$ ; in comparison with first dose administered group, HG: high glucose, NADP: nicotinamide adenin dinucleotide phosphate, NADPH: reduced nicotinamide adenin dinucleotide phosphate; mg/ml: Miligram/mililiter; pmol: picomolar, SD: Standard deviation

metabolic demand for pentose phosphate pathway-generated NADPH [23]. There are 4 enzyme sources of cytosolic NADPH, 6-phosphogluconate dehydrogenase, malic enzyme and cytosolic NADP<sup>+</sup>-dependent isocitrate dehydrogenase, and glucose 6-phosphate dehydrogenase (G6PD). G6PD regarded as the major enzyme to generate cytosolic NADPH [24,25]. Out of the 0.03 mg/ml, 0.3 mg/ml, 3 mg/ml doses of green tea catechin, only the two highest doses significantly prevented HG-induced decrease in NADPH level. There was no significant difference between the effects of these two highest doses ( $P > 0.05$ ).

Previous in silico study showed that galled catechins, but not ungalled catechins, were NADP<sup>+</sup>-competitive inhibitors of G6PD and other enzymes that employ NADP<sup>+</sup> as a coenzyme, such as IDH and G6PD [26]. In other side, catechins (and other flavonoids) isolated from green tea inhibit mammalian glucose transporters including GLUT1 located in the endothelial cells [27]. This effect was avoided by simultaneously treatment of catechin and glucose. Furthermore, catechin were potent radical scavengers and provided protection against intracellular oxidative stress induced by hydrogen peroxide [28].

The NO levels were significantly lower in the HG group compared to the untreated group ( $P < 0.05$ ). These decreased levels of NO in the HG group were significantly elevated by 0.3 mg/ml administration of green tea catechins. This study confirming previous studies that treated with catechin increases the NO<sub>2</sub><sup>-</sup>/NO<sub>3</sub><sup>-</sup> production and not induces NO scavenging [29,30]. The mechanisms of NO production may be due to availability tetrahydrobiopterin as a co-factor for NO production. The presence of NADPH act as reducing equivalent for endogenous antioxidant defense. The integrity of endogenous antioxidant will inhibit oxidation of tetrahydrobiopterin, resulting in high NO bioavailability. Unfortunately, highest dose induces losing of NO bioavailability may be due to pro-oxidant effect of catechin. Catechin gallates has been found to produce H<sub>2</sub>O<sub>2</sub> *in vitro* and in cell culture systems, where catechin reacts with dissolved oxygen in aqueous solution, and generates H<sub>2</sub>O<sub>2</sub> [31,32].

## CONCLUSION

Catechin isolated from GMB-4 green tea suppresses HG-induced reducing NADPH and NO, suggesting that this catechin may be a potential candidate for the treatment and prevention of diabetic vascular complications.

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