Green tea extract protects endothelial progenitor cells from oxidative insult through reduction of intracellular reactive oxygen species activity

Wahyu Widowati 1*, Rahma Micho Widyanto 2, Winsa Husin 1, Hana Ratnawati 1, Dian Ratri Laksmitawati 3, Bambang Setiawan 4, Dian Nugrahenny 5, Indra Bachtiar 6

1 Medical Research Center, Faculty of Medicine, Maranatha Christian University, Jl. Prof. Drg. Suria Sumantri 65, Bandung 40164, West Java, Indonesia
2 Biomolecular and Biomedical Research Center, Aretha Medika Irama, Jl. Babakan Jukung 2 no 9, Bandung, 40163, West Java, Indonesia
3 Faculty of Pharmacy, Pancasila University, Jl Jagakarsa, Jakarta, Indonesia
4 Department of Medical Chemistry and Biochemistry, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, South Kalimantan, Indonesia
5 Department of Pharmacology, Faculty of Medicine, Brawijaya University, Jl. Veteran, Malang, 65145, East Java, Indonesia
6 Stem Cell and Cancer Institute, Jl. A. Yani no.2 Pulo Mas, Jakarta, 13210, Indonesia

**Abstract**

Objective(s): Many studies have reported that tea consumption decreases cardiovascular risk, but the mechanisms remain unclear. Green tea is known to have potent antioxidant and free radical scavenging activities. This study aimed to investigate whether green tea extract (GTE) can protect endothelial progenitors cells (EPCs) against oxidative stress through antioxidant mechanisms.

Materials and Methods: Mononuclear cells (MNCs) were isolated from peripheral blood by density gradient centrifugation with Ficoll. The cells were then plated on fibronectin-coated culture dishes. After 7 days of culture, EPCs were characterized as adherent cells double positive for DiI uptake and lectin binding. EPCs were further identified by assessing the expression of CD34/45, CD133, and KDR. EPCs were then treated with hydrogen peroxide (H₂O₂) at doses of 50, 100, 200 µM and incubated with or without GTE (25 µg/ml). The intracellular reactive oxygen species (ROS) levels were detected by flow cytometry using a 2',7'-dichlorofluorescein diacetate (DCF-DA) fluorescent probe.

Results: GTE ameliorated the cell viability of EPCs induced by H₂O₂ at doses of 50, 100, 200 µM for about 84.24, 92.27, and 93.72% compared to controls, respectively. GTE also decreased the intracellular ROS levels of EPCs induced by H₂O₂ at doses of 50, 100, 200 µM for about 25.47, 22.52, and 11.96% higher than controls, respectively. GTE also decreased the intracellular reactive oxygen species (ROS) levels compared to controls.

Conclusion: GTE improves cell viability by reducing the intracellular ROS accumulation in H₂O₂-induced EPCs.

**Introduction**

Endothelial dysfunction plays an important role in pathogenesis of atherosclerosis caused by cardiovascular risk factors. It has been observed in patients with established coronary artery disease or coronary risk factors (1). Endothelial progenitor cells (EPCs), a kind of stem cells forming a new vessel, play critical roles in maintaining the vessel tone and repairing the endothelial cells injury (2-5). Recent studies indicated that the number of circulating EPCs reduces in patients with atherosclerosis risk factors leading to the development of atherosclerosis lesions (6, 7).

Endothelial dysfunction is closely related with increased oxidative stress and may be reversed by antioxidant treatment (2, 8). Previous studies suggested that flavonoids may improve endothelial function (8, 9). Flavonoids have powerful antioxidant properties and they exert their effects by scavenging free radicals (10, 11). Previous studies also suggested that tea flavonoid prevents LDL (low density lipoprotein) oxidation, inflammatory, endothelial dysfunction that is crucial to the development of atherosclerotic plaque (8, 12).

Tea from *Camellia sinensis* is the second most widely consumed beverage in the world after water (13-15). Many studies have reported the relation between tea consumption and cardiovascular risk (15-17), and suggested that the risk reduction is due to flavonoid compounds in tea (8, 9, 18, 19). Other studies also indicated that dietary flavonoid from tea and other sources (such as red wine, apples, onions, chocolate, blueberries, and strawberries) is related

*Corresponding author: Wahyu Widowati, Medical Research Center, Faculty of Medicine, Maranatha Christian University, Jl. Prof. Drg. Suria Sumantri 65, Bandung 40164, West Java, Indonesia. email: wahyu_w60@yahoo.com
with reduced cardiovascular risk (20-23). Green tea has abundant flavonoids, including catechins (30-36% of dry weight), and epigallocatechin-3-gallate (EGCG) constitutes up to 63% of total catechins in tea (24). The antioxidant activity of EGCG has been shown to be 25-100 times more potent than vitamins C and E (25). We hypothesized that green tea extract (GTE) is able to protect EPCs from oxidative stress through antioxidant mechanism, thereby contributes to the protective effect on endothelial cells. To test this hypothesis, we assessed the protective effects and ROS-inhibiting effects of GTE on H2O2-induced oxidative damage in human EPCs.

Materials and Methods

Preparation and extraction of green tea

Dried green tea leaves was obtained from PT. Perkebunan Nusantara (PTPN) VIII, Bandung, west Java Indonesia. Green tea was planted and harvested from Cisaruni plantation, Garut, West Java. The dried green tea leaves contained water level 7.15%; protein 22.00%; fiber 14.33%; ash 5.13%; crude lipid 1.33%; carbohydrate 57.31%. The green tea plant were identified by staff of herbarium, Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The green tea plant was identified as Camellia sinensis L. Kuntze or Thea sinensis (L.), Camellia theifera Griff. The preparation and extraction of green tea were performed according maceration extraction method (12, 26, 27). One kilogram of dried green tea leaves was extracted with distilled methanol 96% by maceration method for 5 days filtered and collected until the colorless methanol filtrate. The collected methanol filtrate was evaporated using rotatory evaporator to produce methanol extract of green tea 173.9 g or 17.39%. The methanol filtrate was stored at 4°C for 5 days filtered and the absorbance value was measured at 760 nm. The total phenolic content expressed as Epigallocatechin Gallate equivalent (EGCG) was calculated by the following formula:

\[ C = \frac{c \times V}{m} \]

where C: total content of phenolic compounds, μg/mg green tea extract, in EGCGE and in GCE; c: the concentration of EGCG or GC established from the calibration curve, μg/ml; V: the volume of extract (ml); m: the weight of green tea extract (mg).

Total phenol content

Total phenol content was assayed according to the Folin–Ciocalteu method. Samples (15 μl) were introduced into microplate; 75 μl of Folin-Ciocalteu’s reagent (2.0 M) and 60 μl of sodium carbonate (7.5%) were added. The samples were mixed and incubated at 45°C for 15 min (28). Subsequently, absorbance value was measured at 760 nm. The total phenolic content expressed as Epigallocatechin Gallate equivalent (EGCGE) and Gallic acid equivalent (GAE) was calculated using the following formula:

SOD assay

The SOD assay was done using a SOD assay kit (Cayman) comprising assay buffer, sample buffer, radical detector, SOD standard, and xanthine oxidase. SOD standards were prepared by introducing 200 μl diluted radical detector and 10 μl SOD standard (7-level standard) per well. Green tea extract was dissolved in DMSO in concentrations of 500, 125, and 31.25 μg/ml (27). The sample well contained 200 μl diluted radical detector and 10 μl sample. All wells were added 20 μl diluted xanthine oxidase. The mixtures were shaken carefully for few seconds, incubated for 20 min at room temperature, SOD activity was measured on a microplate reader at 450 nm (Cayman). The SOD value was calculated using the equation from the linear regression of standard curve substituting linear rate (LR) for each sample.

Superoxide dismutase (SOD) assay

The antioxidant capacity green tea extract was measured using the 2,2’-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•⁺) diammonium salt free radical assay (29, 30, 31). ABTS•⁺ was produced by reacting 14 mM ABTS and equal volume of 4.9 mM potassium persulfate achieved final concentration of 7 mM ABTS in 2.45 mM potassium persulfate. The mixture was incubated in the dark room temperature for 16 h. The ABTS•⁺ solution was diluted with 5.5 mM PBS (pH 7.4) and measured with microplate reader at 745 nm, resulting the absorbance of 0.70±0.02. Briefly 2 μl sample was added to 198 μl of ABTS•⁺ solution, incubated for 6 min at 30°C, and the absorbance was measured at 745 nm. The percentage inhibition of ABTS radical (%) was expressed by the ratio of the reducing of ABTS•⁺ absorbance in the presence of the test sample relative to the ABTS•⁺ absorbance in the absence of the test sample (negative control). ABTS-reducing activity (%) was then continued to be calculated as median inhibitory concentration (IC₅₀).

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power assay (FRAP) of each standard solution and sample were measured according to a modified protocol developed by Mishra et al (32). The FRAP reagent was prepared by adding 2,4,6-tripyridyl-s-triazine (TPTZ) and ferric chloride, forming the Fe3⁺-TPTZ complex. Antioxidant reduced to Fe2⁺-TPTZ at low pH was measured at 595 nm. The standard curve was linear between 0.019 and 95 μg/ml FeSO4. Results were expressed in μM Fe (II)/μg extract and compared with EGCG (33).
Table 1. The mean of SOD activity (U/ml) of GTE. SOD activity was measured in triplicate for each concentration of GTE. Linear equation, coefficient of regression (R^2) of SOD standard, and SOD activity of GTE were calculated. The data are presented as mean±standard deviation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentrations of green tea extract (µg/ml)</th>
<th>SOD (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTE</td>
<td>500</td>
<td>1.24±0.06</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>1.23±0.04</td>
</tr>
<tr>
<td></td>
<td>31.25</td>
<td>1.09±0.03</td>
</tr>
</tbody>
</table>

Table 2. The mean of phenolic content of GTE and GC was expressed as ECGE and GCE. The phenolic content was measured in triplicate for each sample. Linear equation, coefficient of regression (R^2) of ECGG and GC were calculated. The data are presented as mean±standard deviation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phenolic content</th>
<th>ECGE (µg/mg GTE)</th>
<th>GCE (µg/mg GTE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTE</td>
<td></td>
<td>139.17±0.833</td>
<td>29.20±0.200</td>
</tr>
<tr>
<td>EGCG</td>
<td></td>
<td>314.00±4.000</td>
<td>62.00±2.000</td>
</tr>
<tr>
<td>GCE</td>
<td></td>
<td>19.20±0.200</td>
<td>3.80±0.100</td>
</tr>
</tbody>
</table>

Isolation and cultivation of EPCs

EPCs were cultured according to the previously described method of Chen et al. (2004) (34). Mononuclear cells (MNCs) were isolated from peripheral blood of healthy young human volunteers by Ficoll-Paque plus (GE Healthcare) using density gradient centrifugation. MNCs were then plated on culture dishes coated with human fibronectin (Roche) and cultured in VascGrow™ medium (Stem Cell and Cancer Institute) at 37°C in a 5% CO₂ incubator. After 4 days of culture, medium were changed and the culture was maintained through day 7. Informed consent was obtained from all volunteers and all procedures performed in this study were approved by Research Ethics Committee of Faculty of Medicine, Maranatha Christian University and Immanuel Hospital, Bandung, Indonesia.

EPCs characterization

EPCs were characterized as adherent cells after 7 days of culture. Direct fluorescent staining was used to detect dual binding of Fluorescein isothiocyanate-conjugated Ulex europaeus agglutinin I (FITC-UEA-1; Sigma) and 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine-labeled acetylated LDL (DiI-ac-LDL; Invitrogen). To detect the uptake of DiI-ac-LDL, adherent cells were incubated with DiI-ac-LDL (1 mg/ml) at 37°C for 4 h. Cells were then fixed with 3% parafomaldehyde for 10 min. After washing cells, they were incubated with FITC-UEA-1 (1 mg/ml) at 37°C for 1 h. Cell nuclei were also stained with 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen). Finally, cells were observed using an inverted fluorescence microscope (Axiovert 40 CFL, Zeiss). Cells that were double positive for DiI-ac-LDL and lectin were defined as EPCs (35).

Further identification of EPCs was performed using fluorescence-activated cell sorting (FACS) analysis. Adherent cells were detached using trypsin-ethylenediamine tetra-acetic acid (EDTA). Cells (1 x 10⁵) were pre-incubated with FcR blocking reagent (Miltenyi Biotech) for 15 min at room temperature. Cells were then incubated at 4°C with FITC-conjugated anti-CD45/phycoerythrin (PE)-conjugated anti-CD34 (BD Biosciences) and PE-conjugated anti-CD133 (Miltenyi Biotech) for 15 min. Cells were then incubated with FcR blocking reagent (Miltenyi Biotech) for 15 min at room temperature. Cells were then incubated with FcR blocking reagent (Miltenyi Biotech) for 15 min at room temperature. Cells were then incubated with FcR blocking reagent (Miltenyi Biotech) for 15 min at room temperature. Cells were then incubated with FcR blocking reagent (Miltenyi Biotech) for 15 min at room temperature. Cells were then incubated with FcR blocking reagent (Miltenyi Biotech) for 15 min at room temperature.

Table 3. The IC₅₀ of ABTS-reducing activity of GTE and EGCG. ABTS-reducing activity (%) was calculated in triplicate for each concentration of GTE and EGCG. Seven concentrations of sample included: 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.038 µg/ml. Linear equation, coefficient of regression (R^2) of GTE and EGCG were calculated.

<table>
<thead>
<tr>
<th>Sample</th>
<th>The highest activity of ABTS-reducing activity (%)</th>
<th>Linear equation</th>
<th>R²</th>
<th>IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTE</td>
<td>97.30</td>
<td>Y=17.24X+12.636</td>
<td>0.9197</td>
<td>2.17</td>
</tr>
<tr>
<td>EGCG</td>
<td>97.83</td>
<td>Y=9.95X+36.452</td>
<td>0.9597</td>
<td>2.14</td>
</tr>
</tbody>
</table>

CD133 (Miltenyi Biotech) for 15 min, and PE-conjugated vascular endothelial growth factor receptor 2 (VEGFR2)/KDR (R&D System) for another 40 min. Isotype-identical antibodies served as negative controls. Quantitative FACS was performed on a FACSCalibur Flow Cytometer (BD Biosciences).

EPCs cytotoxicity assay

EPCs cytotoxicity was performed to determine the maximum tolerance concentration of GTE on EPCs culture and to determine the optimal oxidative damage concentration of H₂O₂ for the following experiments. Cytotoxicity of EPCs was determined by CellTiter® (Promega) based on quantitative colorimetric assay. After 7 days of culture, cells were then digested with trypsin-EDTA and cultured at a density of 5 x 10⁴ cells/ml on 96-well tissue culture plates using serum-free medium for 24 hr before treatment (28, 36, 37). Cells were then treated with various concentrations of GTE (3.13-100 µg/ml) and H₂O₂ (12.5-400 µM) for 24 hr. EPCs were supplemented with 20 µL of CellTiter® (Promega) each well and incubated for another 4 hr. Optical density values were measured at 490 nm using microplate reader (Bio-Rad).

Assessment protective effect of GTE on oxidative damage in EPCs

After 7 days of culture, cells were digested with trypsin-EDTA and 5 x 10⁴ cells were cultured on 96-well tissue culture plates using serum-free medium for 24 hr before treatment. Cells were then treated with GTE (25 µg/l) for 1 hr before treatment with various concentrations of H₂O₂ (50, 100, 200 µM) for a subsequent 24 hr (38). Cell viability was measured by CellTiter® assay (Promega). Cells treated without H₂O₂ served as controls. Optical density values were measured at 490 nm using microplate reader (Bio-Rad). The values were expressed as a percentage of control.

Measurement of intracellular reactive oxygen species

The intracellular ROS levels were detected by flow cytometry using a DCF-DA fluorescent probe (Invitrogen) according to the method of Jie et al. (38)
Table 4. The FRAP activity. The standard curve was linear between 0.019 and 95 μg/ml FeSO₄. The FRAP activity was calculated in triplicate for each concentration of GTE and EGCG. Seven concentrations of sample included 25.000, 12.500, 6.250, 3.125, 1.563, 0.782, 0.391 μg/ml. Results of FRAP activity were expressed in μM Fe ([II])/μg sample. Linear equation of FeSO₄ was $Y=0.0064X+0.0445$.

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>FRAP activity (μM Fe (II)/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GTE</td>
</tr>
<tr>
<td>25.000</td>
<td>98.1±0.13</td>
</tr>
<tr>
<td>12.500</td>
<td>60.7±0.56</td>
</tr>
<tr>
<td>6.250</td>
<td>34.8±0.86</td>
</tr>
<tr>
<td>3.125</td>
<td>15.7±0.48</td>
</tr>
<tr>
<td>1.563</td>
<td>14.4±0.40</td>
</tr>
<tr>
<td>0.782</td>
<td>11.3±0.71</td>
</tr>
<tr>
<td>0.391</td>
<td>10.3±0.47</td>
</tr>
</tbody>
</table>

Measurement of intracellular reactive oxygen species

The intracellular ROS levels were detected by flow cytometry using a DCF-DA fluorescent probe (Invitrogen) according to the method of Jie et al (38) and Stolzing & Scutt (39) with modification. After 7 days of culture, EPCs were digested with trypsin-EDTA and 1 x 10⁵ cells were incubated with 10 μM DCF-DA at 37°C for 30 min. Next, the excess probes were washed out with phosphate-buffered saline (PBS)+KCl, and then incubated with GTE (25 μg/ml) for 30 min. Cells were then incubated with H₂O₂ at final concentrations of 50, 100, and 200 μM for another hour. Finally, the intracellular ROS levels were measured using FACSCalibur Flow Cytometer (BD Biosciences). Cells treated with H₂O₂ without GTE pre-treatment served as controls. The measured fluorescence values were expressed as a percentage of control.

Table 5. The EPCs functional characterization of DiI-ac-LDL uptake and lectin binding (adherent cells were stained with DAPI and percentage of the double-stained cells was calculated)

<table>
<thead>
<tr>
<th>No</th>
<th>Number of cells</th>
<th>Number of double positive cells (DiI-ac-LDL uptake and lectin binding)</th>
<th>% of double-stained cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>22</td>
<td>73.33</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>25</td>
<td>83.33</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>23</td>
<td>74.19</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>26</td>
<td>86.67</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>22</td>
<td>91.67</td>
</tr>
<tr>
<td>6</td>
<td>29</td>
<td>22</td>
<td>75.86</td>
</tr>
<tr>
<td>7</td>
<td>26</td>
<td>13</td>
<td>50.00</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>20</td>
<td>50.00</td>
</tr>
<tr>
<td>9</td>
<td>31</td>
<td>16</td>
<td>51.56</td>
</tr>
</tbody>
</table>

Statistical analysis

Data are presented as mean, the differences between groups were analyzed using one-way analysis of variance (ANOVA) with SPSS 20.0 statistical package. Only probability values of $P<0.05$ were considered statistically significant and later subjected to Duncan’s post hoc test.

Results

Antioxidant activity of GTE

The antioxidant activities of GTE were examined including SOD activity, total phenolic content, ABTS-reducing activity, and FRAP activity. SOD activity was measured in concentrations of 500, 125, and 31.25 μg/ml. GTE showed high antioxidant activity and similar activity among concentrations (Table 1).

Total phenolic content of GTE was measured using the EGCG and GC standard, the phenolic major in green tea. It was demonstrated that compared to the GC, the content of EGCG was higher in GTE (Table 2).

![Figure 1](image)
The data are presented as mean ± standard deviation. Different superscripts in the same column (a, b, c, d) indicate significant differences among the means of groups (concentrations of H2O2 or H2O2 + GTE) based on Duncan’s post-hoc comparisons (P < 0.05).

The solution contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS]. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product due to conversion by dehydrogenase enzymes in metabolically active cells (27, 43).

The result of cytotoxicity effect from GTE and H2O2 is shown in Table 7. After treatment with GTE at concentrations of 50 and 100 µM for 24 hr, the viability of cells decreased about 40 and 80%, respectively, relative to the negative controls. Treatment with GTE at concentration of 3.13-25 µg/ml had relatively no effect on cells cytotoxicity.

The cells treated with H2O2 at concentrations of 12.5-25 µM demonstrated no toxicity. The viability of EPCs decreased for about 21, 55, 75, and 84% relative to the negative controls at the concentrations of 50, 100, 200, and 400 µM, respectively. GTE concentration of 25 µg/ml and H2O2 concentrations of 50, 100, and 200 µM had been chosen for independent and dependent concentrations of the following experiments.

Protective effect of GTE on oxidative damage in EPCs

Pre-treatment with 25 µg/ml of GTE on H2O2-induced EPCs at concentrations of 50, 100, and 200 µM decreased cytotoxicity for about 12, 50, and 74%, respectively.

The data are presented as mean ± standard deviation. Different superscripts in the same column (a, b, c) indicate significant differences among the means of groups (concentrations of GTE or H2O2 for the first column and GTE or H2O2 for the second column) based on Duncan’s post-hoc comparisons (P < 0.05).
Table 9. The fluorescence intensity of ROS in H$_2$O$_2$-induced EPCs
(The cells were incubated with 10 μM DCF-DA for 30 min and exposed to several doses of H$_2$O$_2$ (50, 100, and 200 μM). The basal level of ROS with no exposure to H$_2$O$_2$ was obtained by gating with the ROS level of control unstained-DCF-DA cells. High concentrations of H$_2$O$_2$ increased ROS levels in EPCs but not statistically significant. Parallel samples were treated with 25 μg/ml of GTE and the ROS level in cells decreased relative to the controls (H$_2$O$_2$-induced cells) at the concentrations of 50, 100, and 200 μM. The measured ROS levels were expressed as a percentage of negative controls (cells not induced by H$_2$O$_2$). H$_2$O$_2$ exhibited an increase in ROS level)

<table>
<thead>
<tr>
<th>Samples (H$_2$O$_2$)</th>
<th>ROS level (%) over negative control</th>
<th>Samples (H$_2$O$_2$+GTE)</th>
<th>ROS level (%) over negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$:0 μM (Control)</td>
<td>100.00±0.00 $^a$</td>
<td>H$_2$O$_2$:50/100/200 μM</td>
<td>100.00±0.00 $^a$</td>
</tr>
<tr>
<td>H$_2$O$_2$:50 μM</td>
<td>61.75±11.17 $^b$</td>
<td>H$_2$O$_2$:50 μM + GTE 25 μg/ml</td>
<td>15.76±1.92 $^b$</td>
</tr>
<tr>
<td>H$_2$O$_2$:100 μM</td>
<td>77.38±11.79 $^b$</td>
<td>H$_2$O$_2$:100 μM + GTE 25 μg/ml</td>
<td>7.73±1.07 $^a$</td>
</tr>
<tr>
<td>H$_2$O$_2$:200 μM</td>
<td>80.59±7.88 $^b$</td>
<td>H$_2$O$_2$:200 μM + GTE 25 μg/ml</td>
<td>6.28±0.36 $^a$</td>
</tr>
</tbody>
</table>

The data are presented as mean±standard deviation. Different superscripts in the same column (+,-) indicate significant differences among the means of groups (concentrations of H$_2$O$_2$ for the first column and H$_2$O$_2$ + GTE for the second column) based on Duncan's post-hoc comparisons ($P<0.05$)

μM increased the cells viability of EPCs induced by H$_2$O$_2$ at doses of 50, 100, 200 μM for about 25.47% (88.69-63.21%), 22.52% (65.16-42.66%), and 11.96% (44.25-32.92%) relative to the positive controls (H$_2$O$_2$-induced EPCs), respectively (Table 8). These data showed that GTE protects the cells from oxidative damage and ameliorates the H$_2$O$_2$-induced loss of EPCs viability.

Intracellular reactive oxygen species levels in EPCs

The level of fluorescence intensity is an indicator of ROS production. The basal level of ROS with no exposure to H$_2$O$_2$ was about 7-16 % compared to that in controls (unstained-DCF-DA cells). After treatment with different doses of H$_2$O$_2$ (50, 100, 200 μM) for 1 hr, the level of ROS in EPCs increased for about 7-34 % compared to that in negative controls (untreated cells). When the cells were treated with 25 μg/ml of GTE, ROS levels decreased dose-dependently for about 84.24% (100-15.76%), 92.27% (100-7.73%), and 93.72% (100-6.28%) (Table 9). Compared to the H$_2$O$_2$-induced EPCs at concentrations of 50, 100, 200 μM, respectively. The effect of H$_2$O$_2$ concentrations on ROS levels in EPCs (high concentrations of H$_2$O$_2$ induced an increase in EPCs ROS levels) is shown in Table 9. These results indicated that GTE treatment reduces the accumulation of ROS in H$_2$O$_2$-induced cells.

Discussion

The antioxidant assay of GTE and EGGG showed that GTE had high antioxidant activities. It was demonstrated that GTE had high SOD activity at all concentrations. This result was consistent with previous study that black tea extract supplementation is able to increase SOD activity in hypercholesterolemic rat compared to control without black tea extract (12). It was also suggested that GTE contained high level of EGCG. This result was validated with previous study that GTE contains flavonoids, including catechins (30-36% of dry weight), and EGGG constitutes up to 63% of total catechins (24). Based on ABTS-reducing activity, GTE and EGGG had high activity with similar IC$_{50}$. This data was validated with previous study that green tea infusion is able to inhibit ABTS radical at 71.01-78.75% (44). Meanwhile, our study result showed that GTE and EGGG exhibited higher ABTS reducing activity at 97.30-97.83%. The FRAP activity showed that EGGG had higher antioxidant activity compared to GTE. Previous study suggested that GTE has high antioxidant and it consists of EGCG. The increase in polyphenol content in green tea will increase its antioxidant activity (44).

The result demonstrated that peripheral blood MNCs were able to differentiate into EPCs during 7-d incubation. EPCs are the precursors of mature endothelial cells and initially defined by the expression of CD34 or the more immature marker protein CD133 and the endothelial marker proteins such as VEGF2/KDR, von Willebrand factor (VWF), VE-cadherin, Dll-ac-LDL uptake, and lectin binding (35).

Previous studies showed that intracellular steady-state concentrations of H$_2$O$_2$ above 5 μM are able to cause oxidative stress (45, 46). Median cytotoxic concentrations (EC$_{50}$) decreased from 500 to 30 μM with increase in incubation time from 1 to 24 hr. Twenty-four-hour treatment was proved to be sufficient to determine incipient cytotoxic concentrations of H$_2$O$_2$ inducing growth arrest and cell death (47). Cellular responses elicited by H$_2$O$_2$ depend upon the severity of the damage, which is further influenced by the cell type and the magnitude of the dose of the exposure (48, 49). Besides, the antioxidant defense mechanisms also contributed. This study showed that H$_2$O$_2$, a categorized non free radical of ROS, is cytotoxic and decrease the EPCs viability start at dose 50 μM. The toxicity mechanisms of H$_2$O$_2$ to EPCs due to intracellular ROS level, increased the phosphorylation of p38 MAPK, JNK, and Nf-kB, and decreased the cellular levels of BCl-2 and AP-1 (50).

Compared with HUVECs and CAECs, EPCs exhibited ≥3- to 4-fold higher expression and activity of manganese superoxide dismutase (MnSOD) and glutathione peroxidase, but not copper zinc superoxide dismutase (CuZnSOD) or catalase (51, 52). Pretreatment with GTE (25 mg/l) was able to protect the viability of H$_2$O$_2$-induced EPCs. GTE
has a potent antioxidant property, and one of the active compounds is catechin. The catechin, the most abundant component in green tea, has a potent antioxidant property and is shown to be 25 to 100 times more potent than vitamins C and E (23). The GTE (25 mg/L) was able to significantly decrease ROS levels in H_{2}O_{2}-induced EPCs. The mechanism of ROS reduction may be due to scavenging activity or upregulated intracellular antioxidant. This study confirmed previous studies that H_{2}O_{2} may impair EPCs proliferation and induce EPCs apoptosis. Catechin may increase the capacity of EPCs for the resistance to apoptosis induced by H_{2}O_{2} (53).

**Conclusion**

H_{2}O_{2} may induce loss of EPCs viability. GTE may increase the capacity of EPCs for the resistance to loss of EPCs viability induced by H_{2}O_{2} through decreasing reactive oxygen species intracellular.

**Acknowledgment**

This work was funded by research grant from Ministry of Research and Technology, Indonesia (Ristek Insentif Kapasitas Produksi 2011), and Faculty of Medicine, Maranatha Christian University, Bandung, and also supported by Stem Cell and Cancer Institute (SCI), Jakarta, which facilitated the complete culture laboratory.

**Conflict of interests**

The authors declare no potential conflict of interests.

**References**