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

by Wahyu Widowati

Iranian Journal of Basic Medical Sciences
ijbms.mums.ac.ir
IJBMS

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 Ratih 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 Utama, Jl. Babakan Jeruk 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

ARTICLE INFO

Article type:
Original article

Article history:
Received: Oct 12, 2013
Accepted: Jun 19, 2014

Keywords:
Antioxidant
Endothelial progenitor cells
Green tea
Oxidative stress
Reactive oxygen species

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-ac-LDL 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 (H_2O_2) 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 H_2O_2 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 ROS levels of EPCs induced by H_2O_2 at doses of 50, 100, 200 µM for about 84.24, 92.27, and 93.72% compared to controls, respectively.

Conclusion: GTE improves cell viability by reducing the intracellular ROS accumulation in H_2O_2-induced EPCs.

► *Please cite this paper as:*
Widowati W, Widyanto RM, Husin W, Ratnawati H, Laksmitawati DR, Setiawan B, Nugrahenny D, Bachtiar I. Green tea extract protects endothelial progenitor cells from oxidative insult through reduction of intracellular reactive oxygen species activity. Iran J Basic Med Sci 2014; 17:702-709.

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 H_2O_2-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 extract of green tea was stored at 4˚C.

Superoxide dismutase (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.

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{Sample LR-y intercept}}{\text{Slope}}\right) \text{X } 0{,}23\text{ml}/0{,}01\text{ml}\right] \text{X Sample dilution}$$

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 Gallocatechin equivalent (GCE) was calculated by the following formula:

$$C = \frac{c * V}{m}$$

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 value was obtained from the regression equation for EGCG: Y=0.0048X+0.036, with R^2=0.99 and for GC: Y=0.02X+0.057 with R^2=0.97.

ABTS-reducing activity

The antioxidant capacity green tea extract was measured using the 2,2'-Azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) diammonium salt free radical assay (29, 30, 31). $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ absorbance in the presence of the test sample relative to the $ABTS^{\bullet+}$ absorbance in the absence of the test sample (negative control). ABTS-reducing activity (%) was then continued to be calculated as median inhibitory concentration (IC_{50}).

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* (2006) (32). The FRAP reagent was prepared by adding 2,4,6-tripyridyl-s-triazine (TPTZ) and ferric chloride, forming the Fe^{3+}-TPTZ complex. Antioxidant reduced to Fe^{2+} 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

Sample	Concentrations of green tea extract (µg/ml)		
	500	125	31.25
GTE	1.24±0.06 (U/ml)	1.22±0.04 (U/ml)	1.09±0.03 (U/ml)

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

Sample	Phenolic content	
	µg EGCGE/mg GTE	µg GCE/mg GTE
GTE	139.17±0.833	29.20±0.200

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% CO_2 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-I; 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 hr. Cells were then fixed with 3% paraformaldehyde for 10 min. After washing, cells were incubated with FITC-UEA-I (1 mg/ml) at 37°C for 1 hr. 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 10^5) 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, 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 H_2O_2 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 10^4 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 H_2O_2 (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 10^4 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 mg/l) for 1 hr before treatment with various concentrations of H_2O_2 (50, 100, 200 µM) for a subsequent 24 hr (38). Cell viability was measured by CellTiter® assay (Promega). Cells treated without H_2O_2 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 3. The IC_{50} 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, 0.3.125, 0.156, 0.078, 0.038 µg/ml. Linear equation, coefficient of regression (R^2) of GTE and EGCG were calculated

Sample	The highest activity of ABTS reducing activity (%)	Linear equation	R^2	IC_{50} (µg/ml)
GTE	97.30	Y=17.248X+12.636	0.9197	2.17
EGCG	97.83	Y=9.950X+36.452	0.9597	2.14

Table 4. The FRAP activity. The standard curve was linear between 0.019 and 95 µg/ml $FeSO_4$. [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 $FeSO_4$ was Y=0.0064X+0.0445). The data are presented as mean±standard deviation

Concentration (µg/ml)	FRAP activity (µM Fe (II)/µg)	
	GTE	EGCG
25.000	98.35±1.73	186.16±14.64
12.500	60.76±0.56	163.08±19.40
6.250	34.85±0.86	83.77±3.48
3.125	15.74±0.48	46.19±1.45
1.563	14.42±0.40	31.72±0.41
0.782	11.36±0.71	14.14±0.52
0.391	10.38±0.47	11.92±0.15

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 10^5 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 H_2O_2 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 H_2O_2 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 Dil-ac-LDL uptake and lectin binding (adherent cells were stained with DAPI and percentage of the double-stained cells was calculated)

No	Number of cells	Number of double positive cells (Dil-ac-LDL uptake and lectin binding)	% of double-stained cells
1	30	22	73.33
2	30	25	83.33
3	31	23	74.19
4	30	26	86.67
5	24	22	91.67
6	29	22	75.86
7	26	13	50.00
8	40	20	50.00
9	31	16	51.56
Mean ± SD		70.74 ± 16.28	

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).

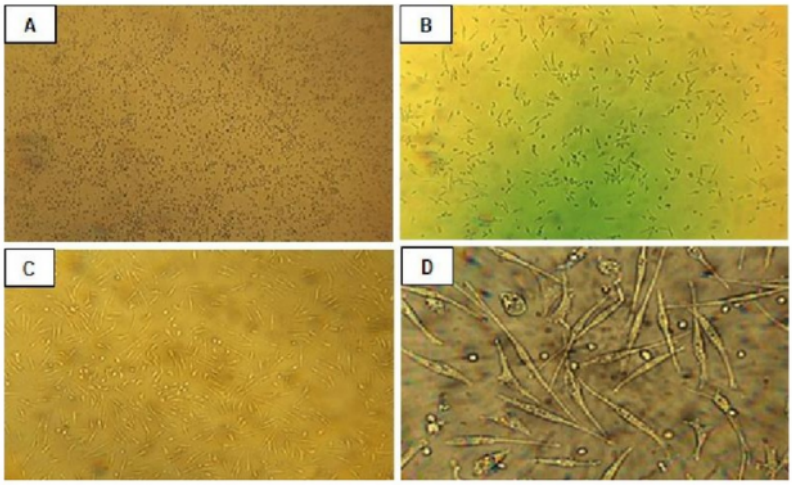

Figure 1. The EPCs morphology characterization. MNCs (A, 400x magnification) were cultured on VascGrow™ medium. Cells exhibited a spindle-shaped on day 4 (B, 400x magnification) and were being sharper in pattern on day 7 (C, 400x magnification; D, 800x magnification)

Table 6. The expression of EPCs markers (CD34/45, CD133, KDR) identified by flow cytometry. The data are presented as mean±standard deviation

No.	CD34/45 (%)	CD133 (%)	KDR (%)
1.	0.12	0.10	0.24
2.	0.10	0.16	0.26
3.	0.18	0.16	0.20
Mean	0.13±0.041	0.14±0.035	0.23±0.031

As a representative of antioxidant activity, ABTS-reducing activity of GTE was measured using EGCG as the control. The IC_{50} is the concentration of GTE or EGCG to scavenge 50% of the ABTS radical. ABTS-reducing activity of GTE was comparable with EGCG (Table 3).

FRAP activity of GTE and EGCG was measured using $FeSO_4$, TPTZ, and ferric chloride forming the Fe^{3+}-TPTZ complex. The presence of antioxidant will reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. Based on FRAP assay, EGCG showed more active antioxidant compared to GTE (Table 4).

EPCs characterization

The attached EPCs proliferated rapidly forming spindle-shaped cells within 4-7 days of culture (40). Beside cell morphology, functional assay was also be used to demonstrate that putative progenitors have endothelial cell potential, including uptake of DiI-ac-LDL (41) and binding of FITC-UEA-1 plant lectin (42). Cell surface markers analysis by flow cytometry has also been used to identify the EPCs.

In the present study, MNCs isolated and cultured for 7 days resulted in attached spindle-shaped endothelial cell-like morphology (Figure 1). EPCs were then characterized as adherent cells double positive for DiI-ac-LDL uptake and lectin binding (70.74±16.28%) (Table 5). Surface markers identification of EPCs demonstrated the expression of CD34/45 (0.13±0.041%), CD133 (0.14±0.035%), and KDR (0.23±0.031%) (Table 6).

EPCs cytotoxicity assay

The cell viability was measured using colorimetric method for determining the cytotoxicity assay. CellTiter® solution (Promega) was used in this study.

Table 8. The effect of GTE pretreatment (25 µg/ml) on H_2O_2-induced EPCs
(The cells viability was estimated by CellTiter® assay after treatment with 50, 100, 200 µM H_2O_2 for 24 hr. The data are presented as mean±standard deviation)

Samples	Cell viability (%) over negative control (Mean±standard deviation)
H_2O_2 0 µM (Control)	100±3.31 ^{d}
H_2O_2 50 µM	63.21±9.31 ^{d}
H_2O_2 100 µM	42.66±4.98 ^{b}
H_2O_2 200 µM	32.29±1.51 ^{e}
H_2O_2 50 µM + GTE 25 µg/ml	88.69±2.61 ^{d}
H_2O_2 100 µM + GTE 25 µg/ml	65.16±3.38 ^{c}
H_2O_2 200 µM + GTE 25 µg/ml	44.25±6.86 ^{b}

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 H_2O_2 or H_2O_2 + 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 methoxy-phenyl)-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 H_2O_2 is shown in Table 7. After treatment with GTE at concentrations of 50 and 100 µg/ml 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 H_2O_2 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 H_2O_2 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 H_2O_2-induced EPCs at concentrations of 50, 100, and 200

Table 7. The effect of GTE and H_2O_2 on cytotoxicity of EPCs
(EPCs were cultured on 96-well tissue culture plates (5 x 10^4 cells/well) and treated with GTE or H_2O_2 at a wide range of doses for 24 hr After treatment, cells were prepared for CellTiter® analysis of cells cytotoxicity)

Samples	Cell viability (%) over negative control (Mean±standard deviation)	Samples	Cell viability (%) over negative control (Mean±standard deviation)
Green tea extract samples		H_2O_2 samples	
GTE 0 µg/ml	100.00±4.48 ^{d}	H_2O_2 0 µM	100.00±4.26 ^{d}
GTE 3.13 µg/ml	95.70±11.03 ^{cd}	H_2O_2 12.5 µM	104.49±9.20 ^{d}
GTE 6.25 µg/ml	101.75±6.49 ^{d}	H_2O_2 25 µM	103.82±5.34 ^{d}
GTE 12.5 µg/ml	84.51±16.38 ^{c}	H_2O_2 50 µM	79.06±8.35 ^{c}
GTE 25 µg/ml	101.19±6.28 ^{d}	H_2O_2 100 µM	49.67±3.95 ^{b}
GTE 50 µg/ml	60.15±5.50 ^{b}	H_2O_2 200 µM	25.44±0.99 ^{a}
GTE 100 µg/ml	20.06±1.07 ^{a}	H_2O_2 400 µM	15.87±2.83 ^{a}
DMSO 0.5%	105.29±3.71 ^{d}		

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 GTE/DMSO for the first coloumn and H_2O_2 for the second coloumn) based on Duncan's *post-hoc* comparisons ($P < 0.05$)

Table 9. The fluorescence intensity of ROS in H_2O_2-induced EPCs
(The cells were incubated with 10 µM DCF-DA for 30 min and exposed to several doses of H_2O_2 (50, 100, and 200 µM). The basal level of ROS with no exposure to H_2O_2 was obtained by gating with the ROS level of control unstained-DCF-DA cells. High concentrations of H_2O_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_2O_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_2O_2). H_2O_2 exhibited an increase in ROS level)

Samples (H_2O_2)	ROS level (%) over negative control	Samples (H_2O_2+GTE)	ROS level (%) over negative control
H_2O_2 0 µM (Control)	100.00±0.00 [a]	H_2O_2 500/100/200 µM	100.00±0.00 [c]
H_2O_2 50 µM	61.75±11.17 [b]	H_2O_2 50 µM + GTE 25 µg/ml	15.76±1.92 [b]
H_2O_2 100 µM	77.38±11.79 [b]	H_2O_2 100 µM + GTE 25 µg/ml	7.73±1.07 [a]
H_2O_2 200 µM	80.59±7.88 [b]	H_2O_2 200 µM + GTE 25 µg/ml	6.28±0.36 [a]

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 H_2O_2 for the first coloumn and H_2O_2 + GTE for the second coloumn) based on Duncan's post-hoc comparisons (P<0.05)

µM increased the cells viability of EPCs induced by H_2O_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% (44.25-32.295) relative to the positive controls (H_2O_2-induced EPCs), respectively (Table 8). These data showed that GTE protects the cells from oxidative damage and ameliorates the H_2O_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_2O_2 was about 7-16 % compared to that in controls (unstained-DCF-DA cells). After treatment with different doses of H_2O_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_2O_2-induced EPCs at concentrations of 50, 100, 200 µM, respectively. The effect of H_2O_2 concentrations on ROS levels in EPCs (high concentrations of H_2O_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_2O_2-induced cells.

Discussion

The antioxidant assay of GTE and EGCG 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 EGCG constitutes up to 63% of total catechins (24). Based on ABTS-reducing activity, GTE and EGCG 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 EGCG exhibited higher ABTS reducing activity at 97.30-97.83%. The FRAP activity showed that EGCG 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 VEGFR2/KDR, von Willebrand factor (VWF), VE-cadherin, DiI-ac-LDL uptake, and lectin binding (35).

Previous studies showed that intracellular steady-state concentrations of H_2O_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_2O_2 inducing growth arrest and cell death (47). Cellular responses elicited by H_2O_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_2O_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_2O_2 to EPCs due to intracellular ROS level, increased the phosphorylation of p38 MAPK, JNK, and NF-κB, 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_2O_2-induced EPCs. GTE

has a potent antioxidant property, and one of the active compounds is catechin. The cathecin, 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_2O_2-induced EPCs. The mechanism of ROS reduction maybe due to scavenging activity or upregulated intracellular antioxidant. This study confirmed previous studies that H_2O_2 may impair EPCs proliferation and induce EPCs apoptosis. Catechin may increase the capacity of EPCs for the resistance to apoptosis induced by H_2O_2 (53).

Conclusion

H_2O_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_2O_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

1. Montorsi P, Ravagnani PM, Galli S, Ali SG, Briganti A, Salonia A, *et al.* The triad of endothelial dysfunction, cardiovascular disease, and erectile dysfunction: clinical implications. European Urology Supplements 2009; 8:58-66.
2. Versari D, Daghini E, Virdis A, Ghiadoni L, taddei S. Endothelial dysfunction as a target for prevention of cardiovascular disease. Diabetes Care 2009; 32:S314-S321.
3. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, *et al.* Statin therapy accelerates reendothelialization: A novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 2002; 105:3017–3024.
4. Sata M. Molecular strategies to treat vascular diseases: Circulating vascular progenitor cell as a potential target for prophylactic treatment of atherosclerosis. Circ J 2003; 67:983–991.
5. Siddique A, Shantsila E, Lip GYH, Varma C. Endothelial progenitor cells: what use for the Cardiologist. J Angiogenesis Res 2010; 2:6.
6. Lenk K, Uhlemann M, Schuler G, Adams V. Role of endothelial progenitor cells in the beneficial effects of physical exercise on atherosclerosis and coronary artery disease. J Appl Physiol 2011; 111:321–328.
7. Mavromatis K, Aznaouridis K, Mheid IA, Veledar E, Dhawan S, Murrow JR, *et al.* Circulating proangiogenic cell activity is associated with cardiovascular disease risk. J Biomol Screening 2012; 17:1163-1170.
8. Hodgson JM, Croft KD. Tea flavonoids and cardiovascular health. Mol Aspects Med 2010; 31:495-502.
9. Hodgson JM. Tea flavonoids and cardiovascular disease. Asia Pac J Clin Nutr 2008; 17:288-290.
10. Amic D, Davidovic-Amic D, Beslo D, Rastija V, Lucic B, Trinajstic N. SAR and QSAR of the antioxidant activity of flavonoids. Curr Med Chem 2007; 14:827-845.
11. Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. BMC Complementary and Altern Med 2012;12:1-12.
12. Widowati W, Ratnawati H, Mozefis T, Pujimulyani D, Yelliantty Y. Hypolipidemic and antioxidant effects of black tea extract and quercetin in atherosclerotic rats. Int J Med Pharm Sci Eng 2013; 7:373-380.
13. Costa LM, Gouveia ST, Nobrega JA. Comparison of heating extraction procedures for Al, Ca, Mg and Mn in tea samples. Ann Sci 2002; 18:313–318.
14. Macfarlane A, Macfarlane I. The empire of tea: the remarkable history of the plant that took over the world. Newark: Audible Studios; 2010.
15. Rietveld A, Wiseman S. Antioxidant effects of tea: Evidence from human clinical trials. J Nutr 2003; 133:3285–3292.
16. Arab L, Khan F, Lam H. Tea consumption and cardiovascular disease risk[1,2,3]. Am J Clin 2013; 98:1651S-1659S.
17. Kokubo Y, Iso H, Saito I, Yamagishi K, Yatsuya H, Ishihara J, *et al.* The impact of green tea and coffee consumption on the reduced risk of stroke incidence in japanese population. The Japan Public Health Center-Based Study Cohort. Stroke 2013; 44:1369-1373.
18. McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R, Dwyer JT. Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults [1,2,3,4]. Am J Clin Nutr 2012; 95:454–464.
19. Stangl V, Lorenz M, Stangl K. The role of tea and tea flavonoids in cardiovascular health. Mol Nutr Food Res 2006; 50:218-228.
20. Heiss C, Keen CL, Kelm M. Flavanols and cardiovascular disease prevention. European Heart J 2010; 31:2583–2592.
21. Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, *et al.* Flavonoid intake and cardiovascular disease mortality : a prospective study in postmenopausal women [1,2,3,4]. Am J Clin Nutr 2007; 85:895–909.
22. Boyer J, Liu RH. Apple phytochemicals and their health benefits. Nutrition J 2004; 3:5.
23. Terao J, Kawai Y, Murota K. Vegetable flavonoids and cardiovascular disease. Asia Pac J Clin Nutr 2008; 17:291-293.
24. Manning J, Roberts JC. Analysis of catechin content of commercial green tea products. J Herbal Pharmacother 2003; 3:19-32.
25. Doss MX, Potta SP, Hescheler J, Sachinidis A. Trapping of growth factors by catechins: a possible therapeutical target for prevention of proliferative diseases. J Nutr Biochem 2005; 16:259-266.
26. Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction : A Review. Internationale Pharmaceutica Sciencia 2011; 1:98-106.
27. Widowati W, Wijaya L, Wargasetia TL, Bachtiar I, Yellianty, Laksmitawati DR. Antioxidant, anticancer, and apoptosis-inducing effects of Piper extracts in HeLa cells. J Exp Integr Med 2013; 3:225-230.
28. Widowati W, Herlina T, Ratnawati H, Mozef T, Risdian C. Antioxidant and platelet aggregation inhibitor activities

of black tea (*Camellia sinensis* L.) extract and fractions. Med Plants 2011; 3:21-26.
29. Jang A, Liu X-D, Shin M-H, Lee B-D, Lee S-K, Lee J-H, *et al.* Antioxidative potential of raw breast meat from broiler chicks fed a dietary medicinal herb extract mix. Poultry Sci 2008; 87:2382–2389.
30. Thring TSA, Hili P, Naughton DP. Anti-collagenase, anti-elastase and antioxidant activities of extracts from 21 plants. BMC Complement Altern Med 2009; 9:27.
31. Etoundi CB, Kuaté D, Ngondi JL, Oben J. Anti-amylase, anti-lipase and antioxidant effects of aqueous extracts of some Cameroonian spices. J Nat Products 2010; 3:165-171.
32. Mishra A, Bapat MM, Tilak JC, Devasagayam TPA. Antioxidant activity of *Garcinia indica* (kokam) and its syrup. Curr Sci 2006; 91:90-93.
33. Adedapo AA, Jimoh FO, Afolayan AJ, Masika PJ. Antioxidant activities and phenolic contents of the methanol extracts of the stems of *Acokanthera oppositifolia* and *Adenia gummifera*. BMC Complement and Altern Med 2008; 8:54.
34. Chen J, Wang X, Zhu J, Shang Y, Guo X, Sun J. Effects of *Ginkgo biloba* extract on number and activity of endothelial progenitor cells from peripheral blood. J Cardiovasc Pharmacol 2004; 43:347–352.
35. Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, *et al.* Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001; 103:2885-2890.
36. Bickford PC, Tan J, Shytle RD, Sanberg CD, Elbadri N, Sanberg R. Nutraceuticals synergistically promote proliferation on human stem cells. Stem Cells and Dev 2006; 15:118-123.
37. Gu J, Wang CQ, Fan HH, Ding HY, Xie XL, Xu YM, *et al.* Effect of resveratrol on endothelial progenitor cells and their contributions to reendothelialization in intima-injured rats. J Cardiovasc Pharmacol 2006; 47:711-721.
38. Jie G, Lin Z, Zhang L, Lv H, He P, Zhao B. Free radical scavenging effect of Pu-erh tea extracts and their protective effect on oxidative damage in human fibroblast cells. J Agric Food Chem 2006; 54:8058-8064.
39. Stolzing A, Scutt A. Effect of reduced culture temperature on antioxidant defences of mesenchymal stem cells. Free Rad Bio & Med 2006; 41:326-338.
40. Hristov M, Weber C. Endothelial progenitor cells: Characterization, pathophysiology, and possible clinical relevance. J Cell Mol Med 2004; 8:498-508.
41. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. Arterioscler Thromb Vasc Biol 2008; 28:1584-1595.
42. Suzuki K, Sakata N, Kitani A, Hara M, Hirose T, Hirose W, *et al.* Characterization of human monocytic cell line, U937, in taking up acetylated low-density lipoprotein and cholesteryl ester accumulation A flow cytometric and HPLC study. Biochim Biophys Acta 1990; 1042:210–216.
43. Malich G, Markovic B, Winder C. The sensitivity and specificity of the MTS tetrazolium assay for detecting the *in vitro* cytotoxicity of 20 chemicals using human cell lines. Toxicology 1997; 124:179-192.
44. Huang WY, Lin YR, Ho RF, Liu HY, Lin YS. Effects of water solutions on extracting green tea leaves. 2013; 2013:368350.
45. Miguel F, Augusto AC, Gurgueira SA. Effect of acute vs chronic H_2O_2-induced oxidative stress on antioxidant enzyme activities. Free Radic Res 2009; 43:340-347.
46. Stone JR, Yang S. Hydrogen peroxide: a signaling messenger. Antioxid Redox Signal 2006; 8:243-270.
47. Gülden M, Jess A, Kammann J, Maser E, Seibert H. Cytotoxic potency of H_2O_2 in cell cultures: Impact of cell concentration and exposure time. Free Rad Biol Med 2010; 49:1298–1305.
48. Temple MD, Perrone GG, Dawes IW. Complex cellular responses to reactive oxygen species. Trends Cell Biol 2005; 15:319–326.
49. Finkel T, Holbrook NJ. Oxidants, oxidative stress and biology of ageing. Nature 2000; 408:239–247.
50. Lee SH, Kim JY, Yoo SY, Kwon SM. Cytoprotective effect of dieckol on human endothelial progenitor cells (hEPCs) from oxidative stress-induced apoptosis. Free Radic Res 2013; 47:526-534.
51. Dernbach E, Urbich C, Brandes RP, Hofmann WK, Zeiher AM, Dimmeler S. Antioxidative stress-associated genes in circulating progenitor cells: evidence for enhanced resistance against oxidative stress. Blood 2004; 104:3591–3597.
52. He T, Peterson TE, Holmuhamedov EL, Terzic A, Caplice NM, Oberley LW, Katusic ZS. Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase. Arterioscler Thromb Vasc Biol 2004; 24:2021–2027.
53. Cao Y, Xu ZC, He XJ, Dang XQ, Yi ZW, Zeng XQ. Protective effects of cathecin on apoptosis of endothelial progenitor cells induced by H_2O_2 in rats. Zhongguo Dang Dai Er Ke Za Zhi 2009; 11:61-64.

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

ORIGINALITY REPORT

14%	14%	0%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	repository.petra.ac.id Internet Source	14%

Exclude quotes	On	Exclude matches	Off
Exclude bibliography	On		