Green Tea Extract Protects Endothelial Progenitor Cells from Oxidative Damage Through Reduction of Intracellular Reactive Oxygen Species Activity

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A number studies have examined that tea consumption decreases cardiovascular risk, but the mechanisms remain undefined. Endothelial dysfunction has been correlated with coronary artery disease and circulating endothelial progenitor cells (EPCs) is contributed of this repair process. Endothelial dysfunction is associated with increased oxidative stress and may be reverse by antioxidant. Green tea is known as free radical scavenger which has a powerful antioxidant action. The aim of this study is to investigate whether green tea extract (GTE) can protect EPCs from oxidative stress through antioxidant protective mechanism. Total mononuclear cells (MNCs) were isolated from peripheral blood by Ficoll density gradient centrifugation. The cells were then plated on fibronectin-coated culture dishes. After being cultured for 7 d, EPCs were characterized as adherent cells double positive for DiLDL-uptake and lectin binding. Further characterizations were done by demonstrating the expression of CD34/45, CD133, and KDR. EPCs were then induced for oxidative stress using various concentrations of H_2O_2 (50, 100, 200 µM), and incubated with or without GTE (25 mg/L) and result showed that GTE ameliorated the cell viability of H_20_2-induced EPC at concentration 50, 100, 200 µM for about 28.72 ± 10.5%, 34.55 ± 7.64%, and 27.04 ± 3.42%, respectively, higher than that control. The level of intracellular reactive oxygen species (ROS) was quantified by fluorescence with 2',7'- dichlorofluorescein diacetate (DCFH-DA) using flow cytometry and showed that GTE decreased intracellular ROS level of H_20_2-induced EPC at concentration 50, 100, 200 µM for about 84.24 ± 8.59 %, 92.27 ± 1.08 %, and 93.72 ± 0.36%, respectively, compare to that control. The result showed that GTE may ameliorate cell viability by decreasing accumulation of intracellular ROS in H_20_2-induced EPCs.

Keywords: endothelial progenitor cell, oxidative stress, reactive oxygen species, green tea, antioxidant, endothelium, coronary diseases

Introduction

Tea, a product made from *Camellia sinensis*, is the second most widely consumed beverage in the world after water and well ahead of coffee, beer, wine, and carbonated soft drinks (Costa, et al., 2002; Macfarlane & Macfarlane, 2004; Rietveld & Wiseman, 2003). A number of studies have examined the relation between tea consumption and cardiovascular risk (Hertog, et al., 1993; Geleijnse, et al., 1999; Sesso, et al., 1999). The reduction of cardiovascular risk by tea consumption is suggested due to its flavonoid compound (Knekt et al., 1996; Hertgog et al., 1995). This suggestion is convincing by other studies that dietary intake of flavonoid from tea and other sources (onions, apples, red wine) is associated with reduced in cardiovascular risk (St Leger et al., 1979; Knekt et al., 1996; Yochum et al., 1999).

Green tea is a free radical scavenger and has abundant flavonoid, which has a powerful antioxidant action. Tea flavonoid contain of catechins (30 to 36% of dry weight) including Epigallocatechin-3-gallate (EGCG), which constitutes up to 63% of total catechins (Mannning & Roberts, 2003). The antioxidant activity of EGCG has been shown to be 25 to 100 times more potent than vitamins C and E (Doss, et al., 2005). One benefit of the dietary flavonoids is their antioxidant properties.

Flavonoids are well known for their free radical scavenger, such as reactive oxygen species (ROS) (Robak & Gryglewski, 1988). Other studies suggest that flavonoid may prevent LDL oxidation, a key early occurrence in atherosclerosis development (Diaz et al., 1997). Recent studies also suggest that flavonoid may favourably affect endothelial function (Fitzpatrick, et al., 1995; Andriambeloson, et al., 1997).

ROS are form of unpaired electron, such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical (HO'), and also non-free radical species such as hidrogen peroxide (H_20_2) (Halliwell, 1995). In the cardiovascular system, ROS are recognized as important signaling molecules. Long-term exposure to ROS, can damage diverse macromolecules, including proteins, carbohydrates, lipids, and DNA. These damaging actions cause in vascular injury and result in endothelial dysfunction (Bauer & Bauer, 1999)

Endothelial dysfunction has been correlated with coronary artery disease and observed in patients with established coronary artery disease or coronary risk factors (Drexler, 1997). Endothelial progenitor cells (EPCs), a kind of stem cell that makes a vessel in peripheral blood, play important roles in maintaining the vessel tone and have role to repair the endothelial cell injury, an early stage of atherosclerosis caused by cardiovascular risk factor (Asahara, et al., 1997; Walter et al., 2002; Sata, 2003; Gill, et al., 2001). Recent a number of studies found that the number of circulating EPCs were reduced when there were more atherosclerosis risk factors causing the process of atherosclerosis (Vasa et al., 2001; Hill et al., 2003).

Endothelial dysfunction in atherosclerosis is close related with increased oxidative stress and might be reversed by antioxidant treatment (Diaz, et al., 1997). We hypothesized that green tea extract (GTE) protect EPC from oxidative stress through antioxidant protective mechanism, which contributes to protective effect on endothelial cells. To test this hypothesis, we assessed the protective effect and ROS-inhibiting effect of GTE on H_20_2-induced oxidative damage in human EPCs.

Methodology

Isolation and Cultivation of EPCs

EPCs were cultured according to the previously described method (Chen et al., 2004). Total mononuclear cells (MNCs) were isolated from peripheral blood of healthy young human volunteers by Ficoll-paque plus (GE Healthcare) using density gradient centrifugation method. MNCs were then plated on culture dishes coated with human fibronectin (Roche) and cultured using VascGrow^{TM} (Stem Cell and Cancer Institute). After 4 days in culture, new media were applied and the culture was maintained through day 7. Informed consent was provided from all volunteers. All of the procedures were done in accordance with ethical clearance board.

EPC Characterization

EPCs were characterized as adherent cells after 7 days in culture. Direct fluorescent staining was used to detect dual binding of Ulex europaeus agglutinin I conjugated with fluorescein isothiocyanate (FITC-UEA-I; Sigma) and 1, 1-dioctadecyl- 3, 3, 3, 3-tetramethylindo carbocyanine-labeled acetylated low density lipoprotein (DiI-acLDL;

Invitrogen). To detect the uptake Dil-acLDL, adherent cells were incubated with DiLDL (1mg/ml) at 37°C for four hours. Cells were then fixed with 3% paraformaldehyde for 10 minutes. After washing, cells were incubated with FITC-UEA-I (1 mg/ml) at 37°C for one hour. Cells also were nucleus-stained using DAPI (Invitrogen). After staining, cells were then observed using inverted fluorescent microscope (Axiovert 40 CFL, Zeiss). Cells that were double positive for DiLDL and lectin were defined as EPCs (Kalka et al., 2000; Vasa et al., 2001).

Further identified of EPCs were done using Fluorescence-activated cell sorting (FACS). Adherent cells were detached using 2 mM ethylene diamine tetra acetate (EDTA). 1 x 10^5 cells were pre-incubated for 15 minutes at room temperature with FcR Blocking (Miltenyi Biotech). Cells were then incubated at 4º C with FITC-conjugated anti-CD45/phycoerythrin -conjugated anti-CD34 (BD Biosciences) and phycoerythrin -conjugated anti-CD133 (Miltenyi Biotech) for 15 minutes, and PE-conjugated VEGF R2/KDR (R&D System) for another 40 minutes. Isotype-identical antibodies served as negative controls. Quantitative FACS was performed on a FACSCalibur *Flow Cytometer* (BD Biosciences).

EPC Cytotoxicity Assay

EPC cytotoxicity was done to determine the maximal tolerance concentration of GTE on EPC culture and to determine the optimal oxide damage concentration of H_20_2 for the following experiments. Cytotoxicity of EPC was determined by CellTiter® (Promega) based on quantitative colorimetric assay. After 7 days in culture and identification of EPC, cells were then digested with Trypsin-EDTA and were cultured at a density of 5 x 10^4 cells/mL on 96-well tissue culture plates using serum-free medium and cultured 24 h before treatment (Bickford et al., 2006; Chen, et al., 2004; Gu, et al., 2006). Cells were then treated with different concentration of GTE (3.13-100 mg/L) and H_20_2 (12.5 – 400 µM) for 24 h. EPCs were supplemented with 20 µL of CellTiter® each well and incubated for another 4 hours. OD value was measured at 490 nm using microplate reader (Bio-Rad)

Assessment Protective Effect of GTE on Oxidative Damage in EPC

After 7 days in culture, EPCs were then digested with Trypsin-EDTA and were cultured at a density of 5 x 10^4 cells/mL on 96-well plates using serum-free medium and cultured 24 h before treatment. Culture medium was replaced with fresh medium containing various concentration of H_20_2 (50, 100, 200 µM). GTE (25 mg/L) was added 1 h before treatment with H_20_2 for a subsequent 24 h (Jie, et al., 2006). Cell viability was measured by CellTiter® assay (Promega). Control was done by treated cells without H_20_2. The value of different absorbance was expressed as a percentage of control.

Measurement of Intracellular Reactive Oxygen Species.

Quantification of intracellular ROS level was done by fluorescence with 2',7'-dichlorofluorescein diacetate (DCF-DA; Invitrogen) using modification methods from Stolzing & Scutt (2006) and Jie et al. (2006). After 7 days in culture, EPCs were then digested with Trypsin-EDTA and 1 x 10^5 cells were incubated with 10 µM DCF-DA for 30 min at 37 °C. After the incubation, the excess probes were washed out with PBS+KCl, then incubated with GTE (25 mg/L) for 30 min. Cells were then incubated with H_2O_2 for final concentration 50, 100, 200 µM for another hour. The intracellular ROS levels were measured using FACSCalibur *Flow Cytometer* (BD Biosciences). Control was done by treated cells with H_2O_2 without GTE pre-treatment. The measured fluorescence values were expressed as a percentage of control cells.

Statistical analysis

Data were presented as mean ± standard deviation. Estimation of overall significance was statitiscally analized with one-way ANOVA and Duncan PostHoc test using SPSS V. 15.0. A probability level of 5% ($p < 0.05$) was considered significant.

Results and Discussions

EPC Characterization

When cultured in in vitro system, EPCs will attach to the fibronectin-coated dish and proliferate rapidly forming spindle-shaped cells within 4–7 days of culture (Hristov & Weber, 2004). Beside cell morphology, functional assay also be used to demonstrate that putative progenitors have endothelial cell potential including uptake of DiI-labeled acetylated-low density lipoprotein (Ac-LDL) (Voyta, et al., 1984) and binding of fluorescently labeled *Ulex europaeus agglutinin 1* (UEA-1) plant lectin (Suzuki, et al., 1990). Cell surface markers assay has been used for convincing the EPC identification.

In the present study, MNCs isolated and cultured for 7 days resulted in an attached cell with spindle-shaped morphology (Fig 1). EPCs were characterized as adherent cells double positive for DiLDL uptake and lectin binding (Fig 2). Further characterization were demonstrating the expression of CD34/45 (0.13 ± 0.041%), CD133 (0.14± 0.035%), and KDR (0.23±0.031%) (Fig 3)

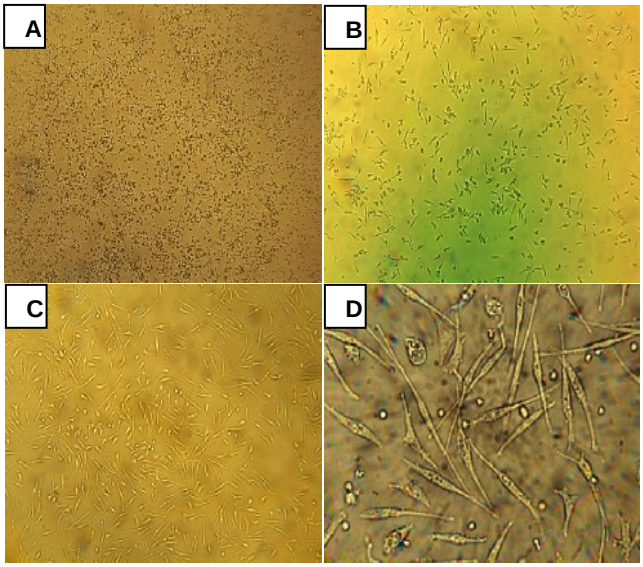

Figure 1. EPC morphology charac- terization. MNCs (A, 400 X) were cultured on VascGrow™ medium and start to exhibited a spindle-shaped on day 4 (B, 400 X) and more sharp in pattern on day 7 (C, 400X; D, 800X)

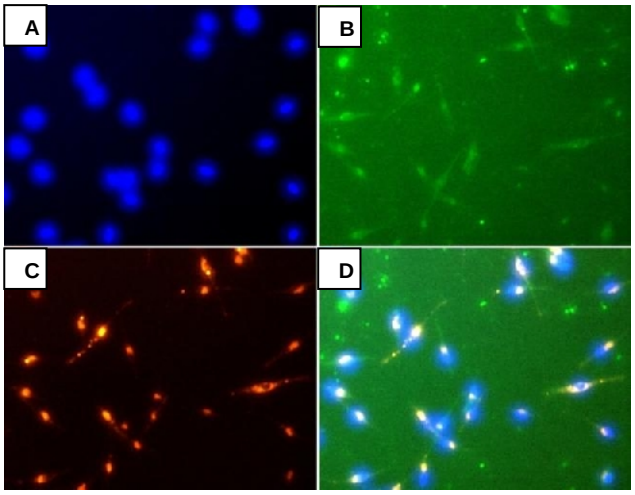

Figure 2. EPC functional characterization. Adherent cells were stained with DAPI (A), binding lectin (B) and taking up Dil-acLDL (C). Panel (D) was obtained by merging (A), (B) and (C). A-D were assessed under inverted fluorescent microscope, magnification 800X.

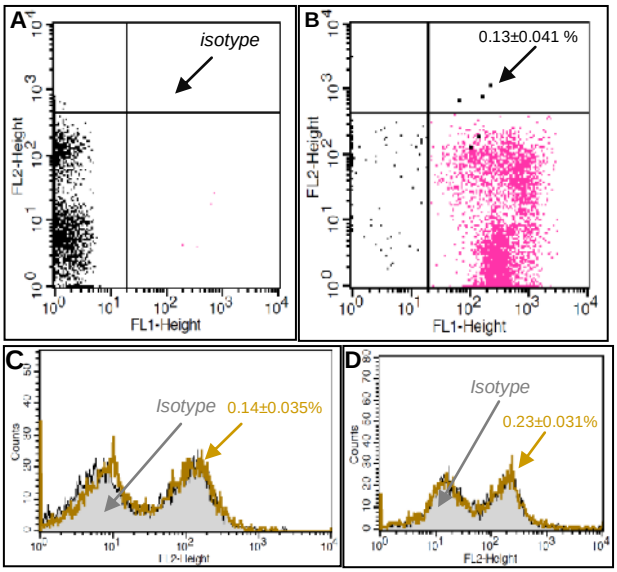

Figure 3. EPC marker characterization. Cells were demonstrating the expression of CD34/45 (A, isotype; B, marker CD34/45), CD133 (C), and KDR (D).

EPC Cytotoxicity Assay

The cell viability was measured using colorimetric method for determining the cytotoxicity assay. CellTiter® solution (Promega) has been used in this study. 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 (Berridge & Tan, 1993).

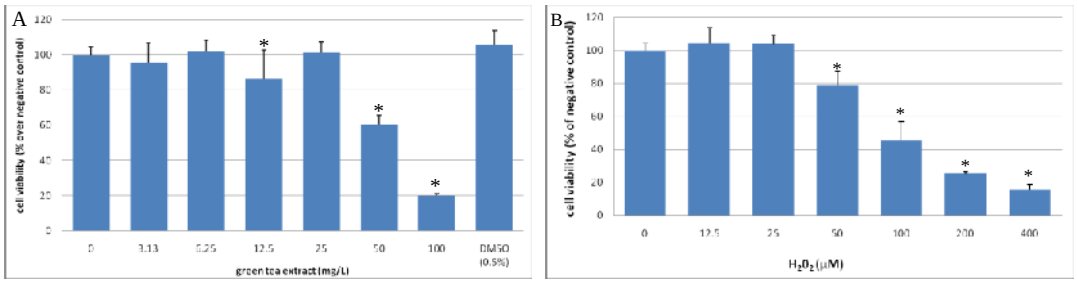

Figure 4. Effect of green te extract (A), and H_20_2 (B) on cytotoxicity of EPC. EPC were cultured on 96-well tissue culture plates (5 X 10^4 cells/well) and treated with GTE or H_20_2 at a wide range of doses for 24 hours. After treatment, cells were prepared for CellTiter® analysis of cell cytotoxicity as described in Methods. Data were expressed as mean (percentage over negative control) ± Standard Deviation (n = 3). *p < 0.05 when compared with that of the negative control (untreated cells).

The result of cytotoxicity effect from GTE and H_20_2 is shown in Figure 4 (A and B). After treatment for 24h, the cells that were treated with GTE at concentration 3.13-25 mg/L had relatively no effect on cell cytotoxicity. But after treated with GTE at concentrations 50, and 100 mg/L, the viability of cells start to decrease about 40 dan 80 %, respectively, relative to the negative control.

The cells that were treated with H_20_2 gave no oxide damage effect to the cells at concentrations of 12.5-25 µM and start to decreased the viability of EPC for about 21, 55, 75, and 84% relative to the negative control for the concentration of 50, 100, 200, and 400 µM, respectively. Concentration 25 mg/L of GTE and 50, 100, and 200 µM of H_20_2 have been choose for the following experiments for independent and dependent concentrations, respectively.

Protective Effect of GTE on Oxidative Damage in EPC

ROS have important role on oxidative stress which can damage diverse macromolecules and result in decreasing the cell viability. Thus the oxidative stress occurrence might be pressured by antioxidant treatment (Diaz, et al., 1997). EGCG, the most abundant component in green tea, has a potent antioxidant property and shown to be 25 to 100 times more potent than vitamins C and E (Doss, et al., 2005). ECGC actions are very diverse and include direct free radical scavenging, antioxidant, anticancer, antibacterial, and antiviral activities (Cabrera, et al., 2006). Several studies also have demonstrated that EGCG can protect heart, kidney, and brain from oxidative injury (Fu & Ko, 2006; Hirai, et al., 2007; Itoh, et al., 2005). EGCG provide protective effect from oxidative stress through a variety mechanisms (Chung, et al., 2003; Guo, et al., 1999).

The protective effect of GTE on H_20_2-induced EPC is shown in Figure 5. Pre-treatment with 25 mg/L GTE on H_20_2-induced EPC at concentrations 50, 100, 200 µM increased the viability cells about 28.72%, 34.55%, and 27.04%, respectively, relative to the control (treatment only with 50, 100, 200 µM H_20_2). These data showed that GTE protects the cells from oxidative damage and were ameliorated the H_20_2-induced loss of EPC cell viability.

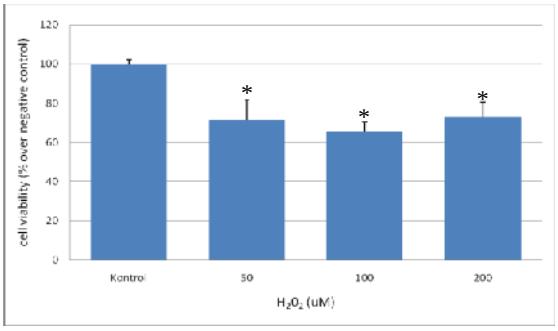

Figure 5. Effect of GTE on H_20_2-induced EPC. Viability of cell was estimated by CellTiter® assay after treatment with 50, 100, 200 µM H_20_2 only and control (50, 100, 200 µM H_20_2 with 25 mg/L GTE for 24 hours. Data were expressed as mean (percentage over negative control) ± Standard Deviation (n = 3). *p < 0.05 when compared with that of the negative control (treated only with H_20_2).

Measurement of Intracellular Reactive Oxygen Species.

In this study, intracellular ROS level measurement was done by fluorescence with 2',7'- dichlorofluorescein diacetate (DCF-DA; Invitrogen). More than decades, DCF-DA has been employed for several studies dealing with the effect of ROS in cell culture (Saez, et al., 1987; Scott, et al., 1988; Murphy, et al., 1989). DCF-DA is crosses membranes of viable cells and is enzymatically hydrolyzed by intracellular esterases to 2',7'- dichlorofluorescein (DCFH) without fluorescence. DCFH is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS within the cells. DCF remains trapped within the cell and can be measured to represent the intracellular ROS level (Lebel et al., 1992; Jie, et al., 2006).

Representative dot blots of intracellular reactive oxygen species (ROS) levels in EPC is shown in Figure 6. The level of fluorescence intensity is an indicator of ROS production. The basal level of ROS with no exposure to H_20_2 was found about 7-16 % (Fig. 6B) compare to that control cells (unstained-DCFDA cells; Fig. 6A). After treatment with different doses of H_20_2 (50, 100, 200 µM) for 1 hour in EPC, the level of ROS in the cells increased for about 7-34 % (Fig. 6C,D,E) in comparison with that of negative control (untreated cells; Fig. 6B). When the cells were treated with 25 mg/L of GTE, the ROS levels were decreased dose-dependently for about 84.24 %, 92.27 %, and 93.72% compare to the control on H_20_2-induced EPC for the concentrations of 50, 100, 200 µM, respectively (Fig. 6F,G,H).

These results indicated that GTE treatment reduced the accumulation ROS level in H_20_2-induced cells. The graphic for fluoresence intensity of ROS level on EPC is shown in Figure 7.

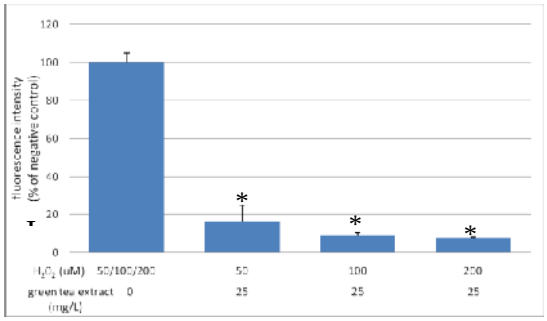

Figure 7. Fluorescence intensity of ROS from H_20_2-induced EPC. GTE showed its antioxidant capacity to reduce the ROS level in the cells. The cells were incubated with 10 µM DCF-DA for 30 min and exposure to several doses of H_20_2 with/without GTE treatment.

Data were expressed as mean (percentage over negative control) ± Standard Deviation (n = 3). *p < 0.05 when compared with that of the negative control (treated only with H_20_2).

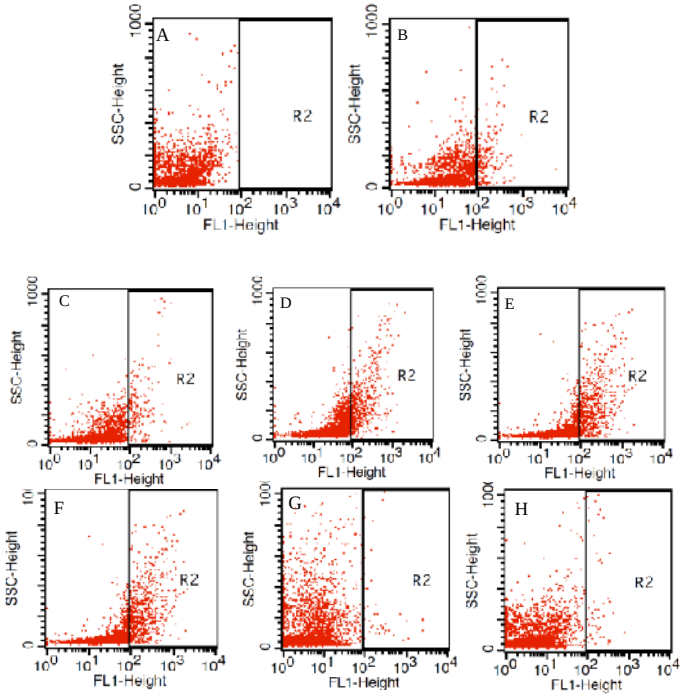

Figure 6. Intracellular ROS levels in EPC. The cells were incubated with 10 µM DCF-DA for 30 min and exposure to several doses of H_20_2 with/without GTE treatment. The basal level of ROS with no exposure to H_20_2 (B) was obtained by gating the ROS level with control unstained-DCFDA cells (A). The cells treated with of 50 µM (C), 100 µM (D), 200 µM (E) H_20_2 exhibited increasing in ROS level. Pararel samples were treated with 25 mg/L GTE and the ROS level in cells were decreased to the control (H_20_2-induced cells) for the concentrations H_20_2 of 50 µM (F), 100 µM (G), 200 µM (H).

Conclusion

In conclusion, GTE may protect EPC from oxidative damage by ameliorating the H_20_2-induced loss of EPC cell viability and decreasing its accumulation intracellular ROS

Acknowledgement

This work was funded by grant from Medicinal Faculty, Maranatha Christian University, Bandung and supported by Stem Cell and Cancer Institute (SCI), Jakarta.

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