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Original Article

EFFECT OF FLAVONOIDS ON OXIDATIVE STRESS, APOPTOSIS, AND CELL MARKERS OF PERIPHERAL BLOOD-DERIVED ENDOTHELIAL PROGENITOR CELLS: AN *IN VITRO* STUDY

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ABSTRACT

Objective: Circulating EPCs (endothelial progenitor cells) play a role in neovascularization and vascular repair. Oxidative stress impairs endothelial progenitor. Flavonoid is a phytochemical compound for antioxidant activity. Flavonoid effects toward oxidative stress, apoptosis, and expression of the cell markers on EPCs are not fully understood. This study was aimed to elucidate the effects of quercetin, kaempferol, and myricetin toward oxidative stress, apoptosis, and cell markers of peripheral blood-derived-EPCs.

Methods: EPCs (endothelial progenitor cells) were isolated from peripheral blood mononuclear cells (PBMNCs) using cultivation under EPCs spesific media. Oxidative stress in EPCs was induced by H_2O_2 and then treated by quercetin, kaempferol, and myricetin. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, while intracellular reactive oxygen species (ROS), apoptosis and characterization of cells, which expressed CD133 and KDR, was measured using flow cytometry.

Results: Quercetin, kaempferol, and myricetin at concentration 12.50 µmol/l were not toxic on EPCs as the cells viability were 96.11±4.03%, 95.42±7.75%, and 94.22±9.49%, respectively. Flavonoids decreased intracellular ROS level in EPCs (quercetin: 14.38±1.47%, kaempferol: 20.21±6.25%, and myricetin: 13.88±4.02%) compared to EPCs treated with H_2O_2 (30.70%±1.04). Percetage of EPCs apoptosis was not significantly different among each treatment. Immunophenotyping showed the increasing of CD133 and KDR expression in EPCs treated with flavonoids.

Conclusion: Quercetin, kaempferol, and myricetin were safe for EPCs, decreased ROS levels, and increased CD133 and KDR expression. However, the flavonoids did not significantly affect EPCs apoptosis.

Keywords: Apoptosis, Endothelial progenitor cells, Flavonoids, Reactive oxygen species

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INTRODUCTION

Endothelial progenitor cells (EPCs) are bone marrow-derived cells that can be found in peripheral blood. EPCs promote neovascularization and indirectly regulate local endothelial cells' angiogenic functions [1]. Through this action, EPCs are involved in vascular repair and their impairment leads to an increase risk of various vascular endothelial and cardiovascular dysfunction [2]. Several factors, including oxidative stress, contribute to the development of endothelial dysfunction through EPCs senescence [3]. Oxidative stress impairs EPCs function by activating downstream pathways leading to cellular senescence or apoptosis [4]. Oxidative stress can lead the apoptosis by mitochondrial signaling pathway [5, 6] and cellular senescence by activated DNA damaging response pathway [7].

Flavonoids are phytochemical compounds readily available in daily human diet with a wide range of biological activities. Kaempferol, quercetin, and myricetin are members of flavonoid flavonol class that can be found easily in fruits and vegetables. This type of flavonoids is known for numbers of biological activities, including antioxidant, antidiabetic, antiinflammatory, and many others [8–10]. Population studies found that consumption of food rich in flavonoid was related with decreased risk of cardiovascular health problem [11]. The mechanism of flavonoid in decreasing cardiovascular health problem is likely involving more than one pathway, including antioxidant and anti-inflammatory function and vascular effect [12]. Benefit of flavonoids was likely due to their interaction with endothelial cells and EPCs. For example, quercetin is taken up rapidly in cell and accumulated mostly in mitochondria. Intra-mitochondrial quercetin appears to prevent mitochondrial disfunction as well as for the redistribution to cytosol, if flavonoid fraction retained in cell is consumed progressively either by cell-permanent oxidants such as peroxynitrite or by activation of plasma membrane oxidoreductases [13].

A previous study demonstrated the ability of green tea extract, tea catechins including catechin (C), epigallocatechin (EGG), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) to protect EPCs from cellular injury through reduction of intracellular reactive oxygen species (ROS) [14, 15]. More studies are required to investigate whether other groups of flavonoids may posses similar properties. Another study showed that quercetin treatment was able to ameliorate EPCs number [15]. Despite those facts, the effects of quercetin, kaempferol, and myricetin toward cellular injuries and several important endothelial markers are unkown. Based on the protective properties of flavonoids against free radicals, this research aimed to investigate the effect of quercetin, kaempferol, and myricetin on oxidative stress, apoptosis, and cell markers of peripheralblood-derived-EPCs.

MATERIALS AND METHODS

Materials

Human blood samples (n=3) were provided by healthy human volunteers. The EPCs were characterized based on the binding of lectin *Ulex europaeus* agglutinin-1 (FITC-UEAI) (Sigma-Aldrich, USA) and uptake of acetylated-low density lipoprotein (DiI-Ac-LDL) (Sigma-Aldrich, USA). 2'-7'-dichlorofluorescein diacetate (DCF-DA), 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, USA), Ficoll-

Paque (GE Healthcare, Sweden), human fibronectin (Roche, Switzerland), VascGrow™ medium (Stem Cell and Cancer Institute, Indonesia), Fc Receptor (FcR) blocker (Miltenyi Biotec, Germany), CD133 PE (Miltenyi Biotec, Germany), VEGFR2/KDR/Flk-1 PE (RandD System, USA). quercetin, kaempferol, myricetin used from (Biopurify Phyto-chemical, China).

Methods

Isolation and culture

Human blood samples (n=3) were provided by healthy human volunteers. All volunteers signed an informed consent prior to blood collection. Ficoll-Paque density gradient centrifugation was employed to isolate total peripheral blood mono-nuclear cells (PB-MNCs) from peripheral blood samples. PB-MNCs were cultured for 7 d in culture dishes, which were coated with human fibronectin, in VascGrow™ medium (Stem Cell and Cancer Institute, Indonesia) at 37 °C, humidified, and 5% CO_2 to get EPCs that were used in the experiments [14, 15].

EPCs functional characterization

The EPCs was characterized based on the binding of lectin *Ulex europaeus* agglutinin-1 (FITC-UEAI) and uptake of acetylated-low density lipoprotein (DiI-Ac-LDL) [14, 15, 17]. Cultured cells were incubated in DiI-Ac-LDL (1 mg/ml) at 5% CO_2, 37 °C for 4 h. The cells were then fixed for 10 min with paraformaldehyde (3%). Then, the cells were washed and incubated at 37 °C for 1 h using FITC-UEAI (1 mg/ml). The nucleus staining was done using 2'-7'-dichlorofluorescein diacetate (DCF-DA) and 4',6-diamidino-2-phenylindole (DAPI). The cells both positive FITC-UEA-I and Dil-ac LDL were characterized as EPCs [14, 15].

Cytotoxicity assay

Cytotoxicity of quercetin, kaempferol, and myricetin (Biopurify Phyto-chemical, China) toward isolated EPCs were measured using MTS (Promega, USA) assay. EPCs (5×10^3) were inoculated with serum-free medium in 96-well plates then incubated at 37 °C, 5% CO_2 for 24 h. The EPCs were treated using different concentrations (100; 50; 25; 12.5 µmol/l) of quercetin, kaempferol, and myricetin that were diluted in dimethyl sulfoxide (DMSO). MTS was then added and incubated at 37 °C, 5% CO_2 for 4 h [9, 10, 14, 15]. The absorbance were measured using a microplate reader with 490 nm wave length (Biorad, USA).

Intracellular ROS assay

EPCs (5×10^5) were inoculated with serum-free medium in 6-well plates then incubated at 37 °C, humidified, and CO_2 5%. EPCs were treated with quercetin, kaempferol, and myricetin (12.5 µmol/l) diluted in DMSO for 24 h. Oxidative stress in EPCs was induced using H_2O_2 treatment. H_2O_2 treated cells and cells without treatment were used as positive and negative controls, respectively. EPCs were then harvested and stained with DCF-DA (10 µmol/l) at 37 °C, humidified, and CO_2 5% for 30 min. The stained cells were washed with PBS containing KCl solution. FACSCalibur flowcytometry was used to measured the levels of intracellular ROS (Becton Dickinson, USA) [14, 15].

Apoptosis assay

EPCs (5×10^5) were inoculated in 6-wells with serum-free medium and incubated for 24 h at 37 °C, 5% CO_2. EPCs were treated with, kaempferol, quercetin, and also myricetin (12.5 µmol/l) diluted in DMSO and incubated for 24 h. The EPCs were harvested, diluted in PBS, and stained with propidium iodide (PI) for 15 min. FACSs Calibur flowcytometry was used to determine the apoptotic cells as SubG1. The apoptotic cells were determined based on a broad hypodiploid (sub-G1) peak [15, 18].

Immunophenotyping

Flavonoid treated EPCs were harvested and incubated with Fc Receptor (FcR) blocker at room temperature for 15 min. The EPCs then were incubated with each of the following fluorochrome conjugated antibodies: CD133 PE (Miltenyi Biotec, Germany), and VEGFR2/KDR/Flk-1 PE. Expression for each surface marker was analyzed using a FACS Calibur flowcytometry. Calculation of the percentage of markers was done based on percentage reduction of markers with the isotype using BD Cell QuestTM Pro software (Becton Dickinson, USA) [14, 15].

Statistical analysis

Data were depicted as mean±standard deviation. Statistical analysis of the data was evaluated using SPSS software. ANOVA (One-way Analysis of Variance) followed by Duncan post-hoc test was performed to evaluate the statistical significance between the three flavonoid treatments. The result is considered as significant if the P-value was lower than 0.05.

RESULTS AND DISCUSSION

In this study, the effect of flavonols commonly found in dietary source: quercetin, kaempferol, and myricetin on cellular injury in EPCs were examined. EPCs were isolated from peripheral blood mononuclear cells (PBMNCs) using cultivation under EPCs specific media. PB-MNCs were cultured and isolated; then the result was spindle-shaped endothelial cell-like morphology. The lectin UEA-I binding and Ac-LDL uptake of the cells were confirmed by fluorescent cells [15] and functional characterization of Dil-ac-LDL uptake and lectin binding resulted percentage of the double-stained cells 70.74±16.28 [14].

To determine the cytotoxicity of quercetin, kaempferol, or myricetin, the EPCs were treated using various concentrations of quercetin, kaempferol, or myricetin for 24 h. Cell viability decreased after flavonoid treatment, but there were no significant changes between concentrations 50 to 12.5 µmol/l (table 1). EPCs viability decreased up to 26-30%, at the highest flavonoid concentration relative to the negative control (untreated). Flavonoids with concentrate at 12.5 µmol/l had the lowest point in reducing EPCs viability.

Table 1: Effect quercetin, kaempferol, and myricetin toward cell viability in EPCs

Sample	Cells viability (%)		
	Quercetin	Kaempferol	Myricetin
Untreated	100.00±6.67[a]	100.00±6.67[a]	100.00±6.67[a]
DMSO	94.50±12.91[a]	94.50±12.91[a]	94.50±12.91[a]
12.5 µmol/l	96.11±4.03[a]	95.41±7.75[a]	94.22±9.49[a]
25 µmol/l	93.53±9.69[a]	92.65±16.05[a]	89.86±12.21[ab]
50 µmol/l	90.54±10.20[ab]	86.95±8.95[ab]	84.08±14.83[ab]
100 µmol/l	74.31±13.61[b]	72.46±5.75[b]	70.71±20.07[ab]

*Based on Duncan's post-hoc comparisons (P<0.05), data are presented as mean±standard deviation. Different superscripts (a,ab,b) in the same column show significant differences among concentrations of flavonoids (quercetin, kaempferol, myricetin).

The results of cytotoxicity tests were calculated using probit analysis, which is logistic regression (method of analyzing the correlation between a stimulus (dose) and the quantal response, then the value of median inhibitory concentration (IC_{50}) was determined (table 2). IC_{50} of kaempferol (767.50 µmol/l) was the highest compared to IC_{50} of quercetin (566.81 µmol/l) and myricetin (399.17 µmol/l). Myricetin has the lowest IC_{50} compared to two other compounds; thus myricetin was the most cytotoxic toward EPCs among flavonoids used in the present study. Three flavonoids compounds were safe on EPCs.

Table 2: IC50 value of quercetin, kaempferol, and myricetin in EPCs

Sample	IC_{50} (µmol/l)
Quercetin	566.81
Kaempferol	767.50
Myricetin	399.17

The effect of the three flavonoids compounds on oxidative stress was examined. Percentages of ROS level in EPCs significantly increased after H_2O_2 induction, compared to the untreated control. The EPCs treated with quercetin, kaempferol, or myricetin has lower ROS level compared to control (untreated) (table 3).

Table 3: Effect quercetin, kaempferol, and myricetin toward ROS level in EPCs

Sample	ROS level (%)
Untreated	8.43±1.59 [a]
H_2O_2 200 µM	30.70±1.04 [c]
Quercetin 12.5 µmol/l+H_2O_2 200 µM	14.38±1.47 [ab]
Kaempferol 12.5 µmol/l+H_2O_2 200 µM	20.21±6.25 [b]
Myricetin 12.5 µmol/l+H_2O_2 200 µM	13.88±4.02 [ab]

*Based on Duncan's post-hoc comparisons (P<0.05), data are presented as mean±standard deviation. Data are presented as mean±standard deviation. Different superscripts (a, ab, b, c) in the same column show significant differences among treatment (quercetin, kaempferol, myricetin).

The results showed that flavonols were able to decrease ROS in EPCs. Flavonoid could act as a potent antioxidant because of the combination between chelating activity via ortho-dihydroxy structures, and its ability to scavenge free radicals. There's three stages needed: formation of the hydroxyl radicals (*OH) and anion superoxide (O2*) by Fenton's reaction, mechanisms that decrease lipid peroxidation, and formation of lipid radicals [19]. This finding was in line with a previous study where quercetin could protect EPCs from oxidative stress that was induced by high concentration of glucose [16]. Oxidative stress was known to contribute to the progression of cardiovascular disease through known mechanism involving senescence of EPCs [3].

Oxidative stress is known to affect nitric oxide (NO), a key factor that regulates EPCs function. This fact is due to the regulation of endothelial nitric oxide synthetase (eNOS), which was an enzyme that synthesize NO, by ROS [20]. Many polyphenols, such as EGCG, genestein and anthocyanin was reported to enhance NO [21–23]. Polyphenol acts on NO signaling and metabolism, reducing eNOS uncoupling and improving eNOS expression, activity [24].

Consumption of beverage with high content of polyphenolic compound was found to increase circulating EPCs level through the enhancement of NO bioavailability [25]. A previous study found that quercetin induced rapid eNOS phosphorylation through Akt-independent and PKA-dependent mechanism [26]. Increased eNOS activity was demonstrated to prevent apoptosis in EPCs [27]. Thus the protective effect of flavonoid against cellular injury is likely caused by its antioxidant properties and NO promoting activity [28].

The apoptosis assay was measured to know effect of quercetin, kaempferol, and myricetin in EPCs. The EPCs treated with 12.5 µmol/l of quercetin, kaempferol, or myricetin resulted in similar percentage of apoptotic cells compared to control (table 4.). The lowest percentage of apoptosis was found in EPCs treated with myricetin (12.07±3.61%), but all treatment group was not significantly different among each other.

Table 4: Effect quercetin, kaempferol, and myricetin toward apoptosis in EPCs

Sample	Apoptosis (%)
Untreated	13.5±1.1
DMSO	14.4±1.6
Quercetin 12.5 µmol/l	12.4±1.1
Kaempferol 12.5 µmol/l	14.1±2.2
Myricetin 12.5 µmol/l	12.1±3.6

*Data are presented as mean±standard Deviation. Data was not significant differences among treatment

H_2O_2 is one example of ROS that mean when H_2O_2 increase the ROS level must be increase too [28-39]. EPCs are characterized based on their cell surface expressions, including CD133, and KDR [1, 14, 15]. This study finding showed that quercetin, kaempferol, and myricetin significantly increased the percentages of KDR expressions but not for CD133 (table 5). EPCs that were treated with quercetin had the highest percentage of KDR (2.15±0.54%) cell population. Whereas myricetin had the highest percentage of CD133 (0.73±0.06%) cell population.

Table 5: Effect quercetin, kaempferol, and myricetin toward CD133, KDR expression in EPCs

Sample	CD133 (%)	KDR (%)
Untreated	0.14±0.03 [a]	0.28±0.02 [a]
Quercetin 12.5 µmol/l	0.19±0.01 [ab]	2.15±0.14 [c]
Kaempferol 12.5 µmol/l	0.16±0.02 [ab]	0.77±0.13 [b]
Myricetin 12.5 µmol/l	0.73±0.06 [b]	1.13±0.16 [b]

*Based on Duncan's post-hoc comparisons (P<0.05), data are presented as mean±standard deviation. Data are presented as mean±standard seviation. Different superscripts (a, ab, b) in CD133 and (a, b, c) in KDR show significant differences among treatment (quercetin, kaempferol, myricetin)

A previous study showed that EPCs with CD133 expression promoted neovascularization of tissue-engineered constructs *in vivo* [30]. Moreover, FACS analysis revealed that KDRof EPCs decreased with a progression to stroke in an animal study, suggesting their

roles in vascular health for ischemic-hemorrhagic stroke [31]. This study showed that flavonols like quercetin, kaempferol, and myricetin were able to increase the population of cells with important phenotypes in cardiovascular health. Thus, this fact might show the benefit of these common flavonols toward lowering the risk of cardiovascular diseases.

CONCLUSION

Quercetin, kaempferol, and myricetin were safe for EPCs, decreased ROS level, and increased CD133 and KDR expression. However, the flavonoids did not significantly affect EPCs apoptosis.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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