Jan. 2012, Volume 9, No. 1 (Serial No. 86), pp. 22–29
Journal of US-China Medical Science, ISSN 1548-6648, USA

Extract of *Curcuma longa* L. and (-)-Epigallo Catechin-3-Gallate Enhanced Proliferation of Adipose Tissue–derived Mesenchymal Stem Cells (AD-MSCs) and Differentiation of AD-MSCs into Endothelial Progenitor Cells

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Abstract: Objectives: Previous studies reported that proliferation and differentiation of stem cell are influenced by free radicals. Therefore, we conducted an investigation to know whether antioxidants, to our current interest, extract of *Curcuma longa* L. (ECL) and (-)-Epigallo catechin-3-gallate (EGCG), are playing role in differentiation and proliferation of adipose tissue-derived mesenchymal stem cells (AD-MSCs). **Materials and Methods:** ECL and AD-MSCs were prepared. Inhibitory concentration-median (IC_{-50}) of ECL and EGCG were measured based on *1,1-diphenyl-2-picryl-hydrazyl* (DPPH) free radical scavenging activity. To examine the effect of ECL and EGCG on proliferation and differentiation of AD-MSCs, ECL and EGCG in various concentrations were applied in culture of AD-MSCs for different period of time. Cell number was counted by trypan blue exclusion method. Differentiation of AD-MSCs into endothelial progenitor cells (EPCs) was analyzed based on following surface markers: cluster of differentiation 34 (CD34), CD133 and vascular endothelial growth factor receptor 2 (VEGFR-2) with flow cytometer. **Results:** IC_{50} of ECL and EGCG on DPPH scaven ing activity were 7.61 and 0.42 μg/mL, respectively. The highest proliferation rates were achieved by induction of ECL in concentrations of 1 μg/mL, while induction of EGCG in concentration of 0.25 μg/mL. ECL and EGCG enhanced differentiation of AD-MSCs into EPCs, marked by increasing expression of CD34, CD133 and VEGFR-2 at 4 incubation days. **Conclusions:** Our current results suggested that ECL and EGCG as antioxidant could enhance proliferation of AD-MSCs and differentiation of AD-MSCs into EPCs.

Key words: Antioxidant, curcuma longa, EGCG, EPCs, AD-MS, VEGFR-2, CD133, CD34.

1. Introduction

Cardiovascular disease (CVD) is a leading cause of morbidity and mortality in the worldwide, approximately one-third of all deaths in 2002 [1]. The high level of morbidity, mortality of CVD and also significant low quality of life in end-stage of CVD requires an aggressive therapy, such as heart transplantation, although and yet hearts donor transplantation are limited due to the shortage [2, 3]. Therapy with infusion of exogenous cells that will form contractile elements of heart, is one of the potential treatment strategy to increase contractility and cardiac performance [3, 4]. Cells therapy are capable to differentiate into cardiomyocytes, cells are capable to graft within the damaged myocardium may limit the contractile function loss [5, 6]. Stem cells therapy has been reported to have contribution in multiple

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mechanisms for cardiac repair, including: increasing blood flow to the ischemic tissue, reducing cardiac myocyte apoptosis through a paracrine-mediated response, regulating the inflammatory milieu, recruiting endogenous stem cells to assist in regenerating the damaged tissue [7, 8].

Mesenchymal stem cells (MSCs) as multipotent, immune privileged and attractive stem cells therapy [9, 10], had been reported to improve heart function in both myocardial injured animal models and heart failure patients [10, 11]. MSCs can differentiate into smooth muscle cells and endothelial cells, resulting in increased vascularity and improved cardiac function [6]. MSCs can be isolated from human lipoaspirates. AD-MSCs can differentiate to form osteogenic, adipogenic, myogenic, and chondrogenic lineages [12].

Endothelial dysfunction plays role in the complications of heart failure. EPCs have been reported that it can to provide an endogenous repair mechanism to prevent and replace dysfunctional endothelium. The enhancing number and functional capacity of EPCs may improve the functional capacity of individuals with CVD [13]. EPCs have characteristic in their cell surface's expression including CD34, CD133 and VEGFR-2, and are able to incorporate into sites of neovascularization and EPCs are able to differentiate into endothelial cells *in situ* [14, 15].

Reactive Oxygen Species (ROS) are free radicals, as secondary intracellular messengers and affect cells redox status. The intracellular redox status is critical role in controlling apoptosis, proliferation, self-renewal, senescence, and differentiation [16]. Stem cells have to be equipped with antioxidative defence system to survive and repair cells [17]. Curcumin, is a pigment isolated from turmeric (*Curcuma longa* L.), which has antioxidant and anti-inflammatory properties [18]. Curcumin is able to prevent methylglyoxal-induced oxidative stress and apoptosis in mouse embryonic stem cells and blastocysts. EGCG is one of tea polyphenols that having many biological activities including antioxidative and anti-inflammatory properties [19]. The current knowledge, the effect of antioxidant on AD-MSCs has not been reported yet. Therefore, we conducted our research to evaluate the effect of ECL and EGCG on proliferation and differentiation of AD-MSCs.

2. Materials and Methods

2.1 Extraction Procedure and Sample Preparation.

Turmeric rhizomes (*C. longa* L.), were collected from farmer plantation located in Bogor, West Java, Indonesia (May 2009). The plants were identified by staff of herbarium, department of biology, school of life sciences and technology, Bandung Institute of Technology, Bandung, west Java, Indonesia. Rhizomes were chopped and dried using drying device (40–45°C) until achieve the stable water level (±13%). The dried rhizomes were milled to produce 60 mesh size of flour. Dried, flour materials were extracted with distilled ethanol by maceration extraction, filtered and evaporated using rotatory evaporator at 40–45°C.

For evaluating the DPPH scavenging activity, ECL and EGCG (Sigma-Aldrich) were dissolved and serial diluted with methanol, while in cell culture experiments, ECL and EGCG were reconstituted in DMSO. Various extracts' concentrations, diluted with culture medium, ranged from 0.125 to 4.0 mg/L, were prepared on the day of the experiment.

2.2 DPPH Free Radical Scavenging Activity Assay

Briefly, 50 µL of each extracts' and EGCG's concentrations were introduced in microplate, followed by addition of 200 µL DPPH solution (0.077 mmol/L DPPH in methanol) (Sigma-Aldrich). Mixtures were then mixed gently and kept in the dark for 30 min at room temperature [20–22]. Absorbance of DPPH was determined by microplate reader at 517 nm. DPPH free radical scavenging activity of each sample was measured according to the formula below:

$$\text{scavenging } \% = \frac{A_c - A_s}{A_c} \times 100$$

A_s: absorbance of samples, A_c: negative control absorbance (without sample, only DPPH and methanol).

2.3 Cells Isolation from Lipoaspirates

All protocols were reviewed and approved by the Stem Cell and Cancer Institute Institutional Review Board prior to the study. Lipoaspirates were obtained with informed consent from individuals undergoing tumescent liposuction surgery. Lipoaspirates were stored at 2–8°C for no longer than 24 hours. Isolating methods of the cells from lipoaspirate were adopted from Sardjono, *et al.* [23].

The raw lipoaspirates were diluted with equal volume of phosphate buffer saline (PBS), divided in 50 ml-tubes, and centrifuged at 430×g, 20°C, 10 min, after that the lipid phase containing target cell was transferred into new tubes, washed three times with equal volume of PBS, resuspended with pre-warmed (37°C) 0.075% collagenase type I (Sigma-Aldrich) in PBS, and mixed with an orbital shaker in a humidified, incubated at incubator (37°C, 5% CO_2) for 30 min. Neutralizing the collagenase activity, added and mixed equal volume of DMEM containing 10% fetal bovine serum (FBS). The sample was centrifuged at 600×g for 10 min. The pellet was collected and resuspended in Dulbecco's Modified Eagle Medium (DMEM), 10% FBS, after that filtered using a 100-μm strainer mesh. The cells were collected and counted with trypan blue exclusion method.

2.4 AD-MSCs Culture

Purifying AD-MSCs from other contaminating cells, the puryfied cells were seeded on plastic-surfaced culture disks with MesenCult® MSC Basal Medium (StemCell Technologies, Vancouver, Canada), Mesenchymal Stem Cell Stimulatory Supplement (StemCell Technologies), 100 unit/ml penicillin and 0.1 mg/ml streptomycin antibiotics, in a humidified, 37°C, 5% CO_2 incubator. After 4 days, non-AD-MSCs were removed by two washes with medium. In another 6–7 days, when the 80% confluence was reached, AD-MSCs were detached using 0.25% trypsin EDTA solution. Detached cells with fibroblast-like morphology were cultured in another flask for 1 week or until confluence was achieved.

2.5 Cell Viability Analysis

AD-MSCs were seeded on a 24-well plate with the same medium and supplements as described for AD-MSCs culture. Treatments of ECL and EGCG in various concentrations for 3, 4 and 7 days, were conducted. Trypan blue exclusion method was performed to analyze cell viability. Briefly, AD-MSCs were dissociated using trypsin EDTA solution, incubated for 3 minutes in 37°C incubator, harvested, washed, followed by centrifugation at 300×g for 10 minutes. Cell pellet was resuspended with trypan blue solution (0.4% in PBS, 1:1 dilution with culture medium) for 3 minutes. The number of dead cells (retaining the dye) was counted with a hemocytometer and expressed as a percentage of the total viable cell number.

2.6 Differentiation study and detection of EPC Markers using Flow Cytometer

AD-MSCs were seeded on a fibronectin-precoated 6-well plate at a density of 10^4 cells/well. Cells were cultured in EGM Bullet Kit medium (Lonza, Cologne, Germany) 10% FBS, 100 unit/ml penicillin and 0.1 mg/ml streptomycin antibiotics in a humidified, 37°C, 5% CO_2 incubator. AD-MSCs were treated with ECL or EGCG. Then, cells were dissociated and centrifuged. Resulted pellet was collected and incubated in PBS containing 2% FBS + FcR Blocking Reagent (Miltenyi Biotech, Bergisch Gladbach, Germany), at room temperature, dark condition, for 15 minutes. Afterwards, antibody and its isotype were added and placed in dark condition, for 30 minutes. Following are the antibodies and isotypes being used PE-conjugated anti-CD34/FITC-conjugated anti-CD45 (Becton Dickinson, Franklin Lakes, NJ), PE-conjugated

anti-CD34 isotype (Becton Dickinson), PE-conjugated anti-CD133 (Miltenyi Biotech), PE-conjugated anti-CD133 isotype (Becton Dickinson), PE-conjugated anti-VEGFR-2 (R&D System, Minneapolis, MN), PE-conjugated anti-VEGFR-2 isotype (Becton Dickinson), Expressed AD-MSCs were analyzed using flowcytometer, FACSCalibur (Becton Dickinson).

3. Results

3.1 DPPH Free Radical Scavenging Activity of ECL and EGCG

ECL and EGCG showed marked DPPH free radical scavenging activity. Based on DPPH free radical scavenging activity, IC_{50} of ECL showed higher number of concentration as compared to IC_{50} of EGCG. This suggested that ECL has lower antioxidant activity as compared to EGCG (Table 1 and Fig. 1).

3.2 AD-MSCs Proliferation Was Enhanced by ECL and EGCG

Number of viable AD-MSCs was increased along with the treatment of incremental ECL or EGCG concentration. The highest number of viable AD-MSCs was reached by treatment of 1 μg/mL ECL or 0.25 μg/mL EGCG. Declined number of viable AD-MSCs was observed with higher ECL and EGCG concentration.

Table 1 IC_{50} DPPH scavenging activity of ECL and EGCG. DPPH scavenging activity test was performed three times for each ECL and EGCG. Linear equation, coefficient of regression (R^2), IC_{50} and average of IC_{50} were then calculated.

Agent	Linear Equation	R^2	IC_{50} (μg/mL)	Average of IC_{50} (μg/mL)
ECL	Y=2.552X+29.46	0.91	8.05	7.61±0.46
	Y=2.384X+32.94	0.90	7.16	
	Y=2.252X+32.80	0.91	7.64	
EGCG	Y=16.89X+42.66	0.90	0.43	0.42±0.02
	Y=16.69X+43.14	0.86	0.41	
	Y=16.95X+43.14	0.85	0.40	

3.3 ECL and EGCG Enhanced Differentiation of AD-MSCs into EPCs

Based on the proliferation result, ECL with concentration of 0.25, 0.5 and 1 μg/mL and EGCG with concentration of 0.25 mg/L were selected for

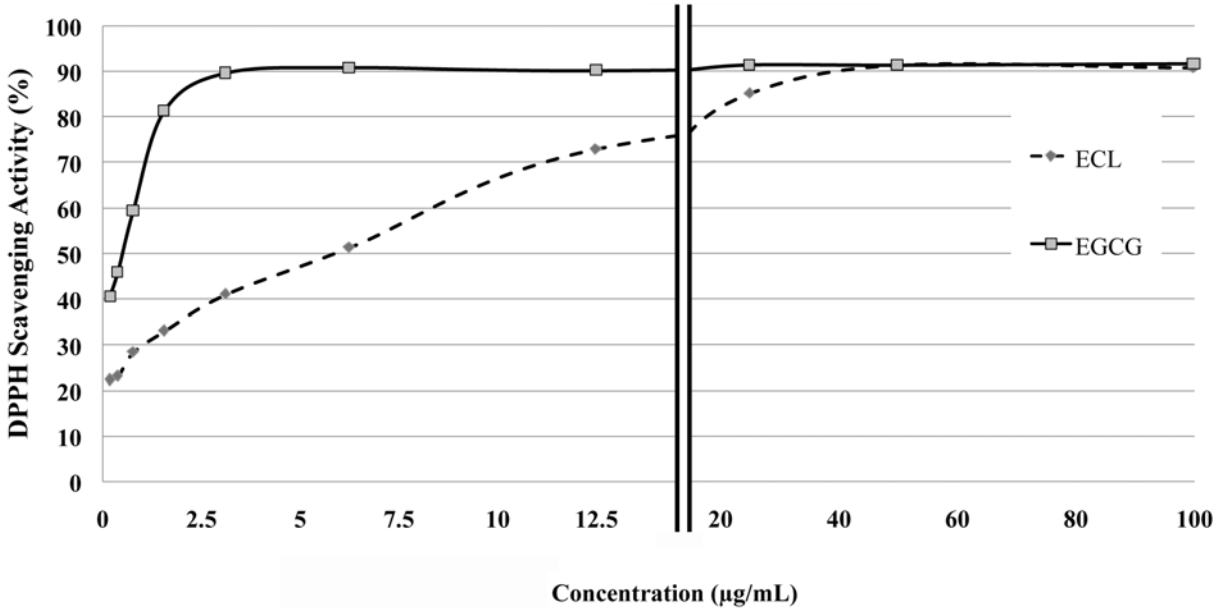

Fig. 1 DPPH Free Radical Scavenging Activity of ECL and EGCG. Dissolved ECL and EGCG were serial diluted with methanol to reach concentration of 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391 and 0.195 μg/mL. Then, DPPH Free Radical Scavenging Activity Assay was performed according to Materials and Methods.

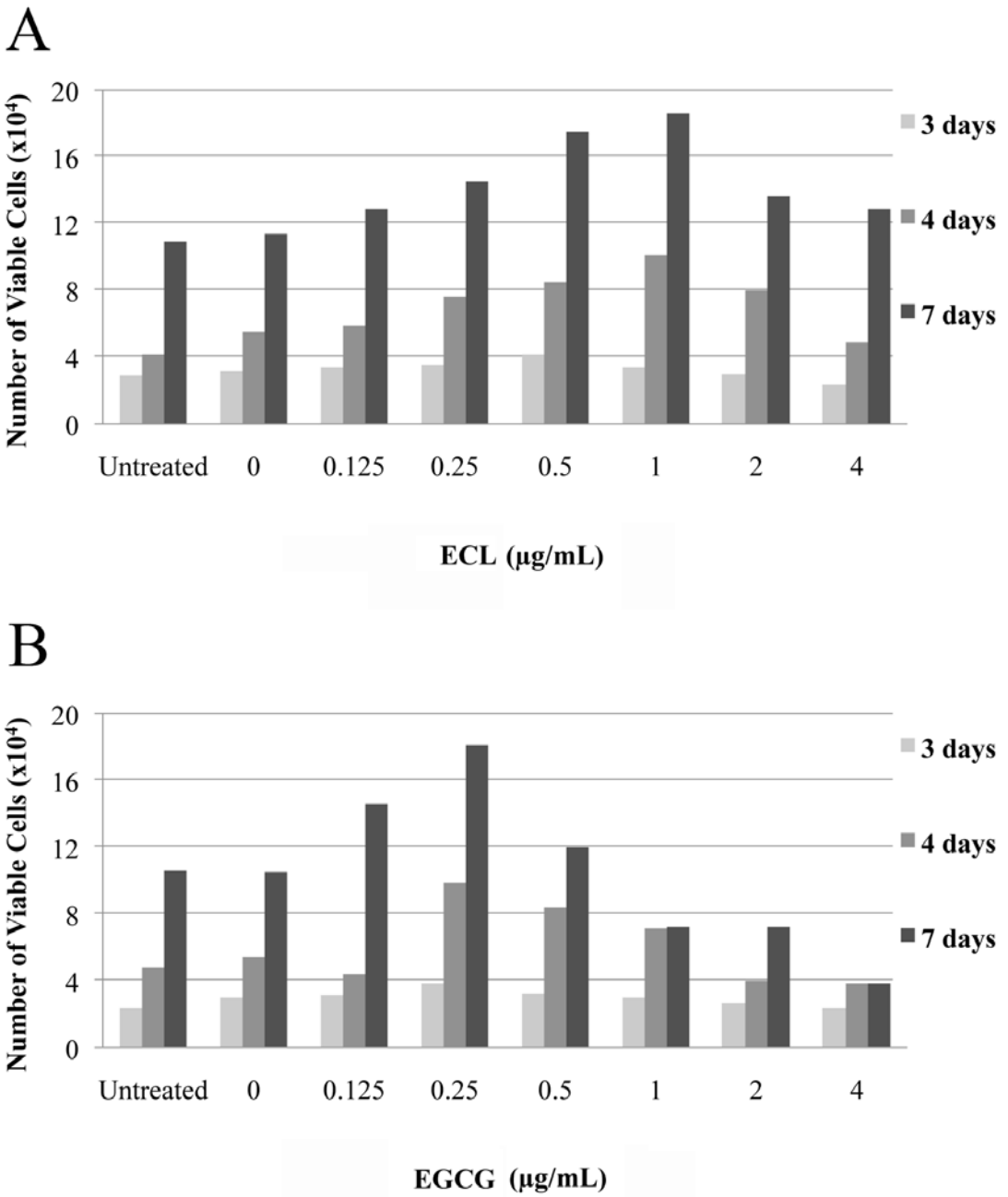

Fig. 2 ECL or EGCG enhanced proliferation of AD-MSCs. AD-MSCs at a density of 10^4 cells/well, were cultured with/without treatment of ECL (A) or EGCG (B), in the time period as indicated in the panel. Then, viable cells were counted with trypan blue exclusion method as described in Materials and Methods.

differentiation study. AD-MSCs were treated with/without addition of ECL or EGCG to differentiate AD-MSCs into EPCs marked by CD34, CD133, and VEGFR-2 markers. Our results showed that ECL and EGCG induced a marked increase of CD34 (Fig. 3A), CD133 (Fig. 3B), and VEGFR-2 (Fig. 3C) expressions. The increasing of expressions was reached at 4 incubation days for all CD34, CD133 and VEGFR-2, that later tended to increase higher at 7 incubation days for CD34 and CD133.

4. Discussion

ECL contains curcuminoid compounds which have high antioxidant activity. Some of the curcuminoid

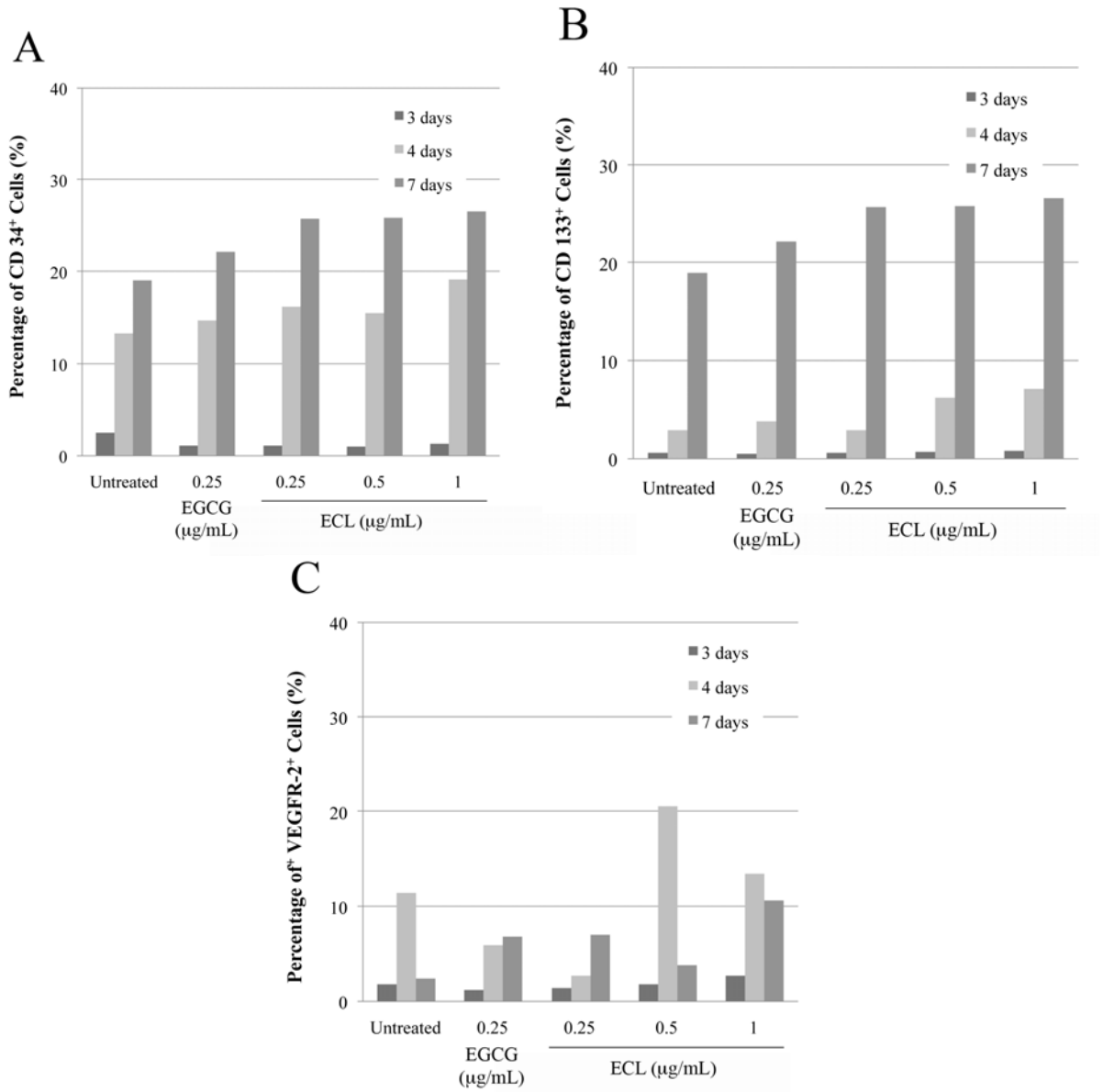

Fig. 3 ECL or EGCG enhanced differentiation of AD-MSCs into EPCs. AD-MSCs were cultured according to Materials and Methods. AD-MSCs were/not treated with 0.25, 0.5 or 1 g/mL ECL or 0.25 g/mL EGCG for 3, 4 or 7 days. Treated-AD-MSCs were further processed according to Materials and Methods, to detect CD34 (A), CD133 (B) and VEGFR-2 (C).

compounds: curcumin, demethoxy curcumin and bisdemethoxy curcumin have 20, 9 and 8 times higher antioxidative activities compared to α-tocopherol, respectively [24, 25]. Antioxidant is capable to neutralize free radicals and stress oxidative during cell metabolism. According to our current results, ECL and EGCG, could increase proliferation.of AD-MSCs. This result is in concordance to other report, showing that low level of ROS and free radicals can enhance cells proliferation [26]. Free radicals are also involved in the production of prostaglandins which modulates cell growth. Free radicals themselves appear to have a down regulatory effect on cell proliferation [26]. Organelles in the mitochondria produce the energy required to drive the endergonic processes of cell life, but now they are considered as the main target for free radical regulatory and as the source of signaling molecules that command cell cycle, proliferation, and

apoptosis [27]. Hence, a study to explore the possible potential of antioxidant on mitochondria should be pursued in the future.

ROS and nitric oxide (NO) generation in stem cells could occur in response to transient changes in systemic redox balance and could initiate a feed forward cycle of ROS/NO generation and elaboration of a balanced anti-oxidative response system that might be the basis of stem cell proliferation, migration and differentiation. An increasing number of studies reported on the crucial role of ROS/NO on MSCs differentiation [28]. The neuronal differentiation of MSCs involved upregulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and increased ROS generation [29]. Our results supported that ECL and EGCG could enhance MSCs differentiation into EPCs. Physical shockwave treatment was shown to increase osteogenic activity of human umbilical cord blood (hUCB) mesenchymal progenitor cells through superoxide-mediated transforming growth factor (TGF)-β1 induction [30]. ROS generation through the activity of the NADPH oxidase (Nox)-2 and Nox-4 isoform has been demonstrated in human $CD34^+$ cells which may contribute to the activation of intracellular signaling pathways leading to mitochondriogenesis, cell survival, and differentiation in hematopoietic stem cells [31]. ROS as well as reactive nitrogen species (RNS) are involved in stem cell mobilization, function and differentiation in a very complex way [29].

5. Conclusions

We suggested that ECL and EGCG could enhance proliferation of AD-MSCs and differentiation of AD-MSCs into EPCs. In order to disclose mechanisms of ECL and EGCG in enhancing proliferation and differentiation, further study should be performed.

Acknowledgment

Authors gratefully acknowledge a financial support from Directorate General for Higher Education, Ministry of National Education, Republic of Indonesia for research grant of Hibah Bersaing 2009 (DIPA no 0868.0/023-04.1/-/2009)

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