ABSTRACT

The endothelial progenitor cell (EPCs) proliferation capability is reduced in the patient with stable coronary artery disease (SCAD). Garlic (Allium sativum), purple sweet potato (Ipomoea batatas), and vitamin C are proven antioxidant which potentially improve EPCs proliferation abilities.

Objective: To investigate the effect of garlic (Allium sativum), purple sweet potato (Ipomoea batatas), and vitamin C in EPCs proliferation from CAD patients and identify the involvement of Extracellular-Signal Regulated Kinase (ERK) Signalling Pathway.

Material and Method: Mononuclear cells were isolated from SCAD patients and cultivated with colony-forming unit (CFU)-Hill medium and divided into untreated (control), garlic extract (10 mcg/ml and 100 mcg/ml), purple sweet potato extract (1 mcg/ml and 25 mcg/ml), vitamin C (10 µg/ml and 250 µg/ml). EPCs proliferation was measured using the MTT Assay. Results: This research shows that EPCs proliferation was increased in the treatment with garlic extract at 10 mcg/ml and 100 mcg/ml dose (0.267 ± 0.003 and 0.391 ± 0.008; p < 0.05), purple sweet potato extract at 1 mcg/ml and 25 mcg/ml dose (0.250 ± 0.005 and 0.365 ± 0.023; p < 0.001), and vitamin C at 10 µg/ml and 250 µg/ml dose (0.259 ± 0.016 and 0.306 ± 0.022; p < 0.001). Increased ERK expression was found in the treatment with garlic extract, purple sweet potato extract and vitamin C. Conclusion: Garlic extract, purple sweet potato extract, and vitamin C can increase EPC proliferation through the ERK signaling pathway.

Key words: Antioxidant, ERK, Endothelial Progenitor, Proliferation.

INTRODUCTION

Coronary artery disease is a major health problem that causes mortality and reduction of life quality worldwide.1 Endothelial progenitor cells (EPCs) from the patients with stable coronary artery disease (SCAD) had a progressive reduction of proliferation abilities, which worsen as the disease progressed. Impaired EPCs proliferation will reduce vascular repair.2 Lower EPCs proliferation in the patient with SCAD is also associated with a higher incidence of cardiovascular events, mortality, and morbidity.3

Multiple pathways were suggested to be responsible for EPCs impairment in SCAD patients. It is suggested that oxidative stress play significant roles in EPCs impairment through intracellular damage and balance disruption which will alter the control of apoptosis, proliferation, self-renewal, senescence, and differentiation of EPCs.4,5 Oxidative stress may disrupt the ERK signaling pathway, which is important in EPCs proliferation.6,7 The antioxidant is suggested to have a beneficial effect on impaired EPCs proliferation from patients with cardiovascular disease.8,9

Plants with antioxidant properties such as Chokeberry (Aronia melanocarpa),9 potato shoot (Solanum tuberosum) and Marigold (Calendula officinalis) has been proven to improve impaired EPCs proliferation.10 Similarly, vitamin C also able to prevent lowering EPCs proliferation caused by TNF-α.11 Garlic (Allium sativum), purple sweet potato (Ipomoea batatas) extract and vitamin C have potent antioxidant capabilities.8,11 Previous studies also have shown purple sweet potato extract and vitamin C on the EPCs from SCAD patients and identify its mechanism.4 Hence, this research aims to identify the effect of garlic extract, purple sweet potato extract and vitamin C treatment on the EPCs and identify the involvement of ERK phosphorylation in the SCAD patient.

MATERIAL AND METHODS

Garlic extract, purple sweet potato extract, and vitamin C preparation

Garlic and Purple sweet potato were obtained from UPT Materia Medica Batu, Indonesia and vitamin C powder was obtained from Sigma-Aldrich, USA. Purple sweet potato and garlic extract were produced with aqueous extraction method as described

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previously. The L-ascorbic acid dose referred to previous research that use dose 250 mcg/mL to improve adipocyte stem cell proliferation. Briefly, PSP chunks were mixed in water with a 1:1 ratio and blended. The mixture was filtered then boiled for 30 min and dried up using a rotary evaporator. PSP extract was diluted with the culture medium to achieve a concentration of 1 mcg/mL and 25 mcg/mL. Vitamin C powder was suspended in double-distilled water and diluted with culture medium to obtain a concentration of 10 mcg/mL and 250 mcg/mL.

**Subject recruitment and sample collection**

The blood sample was obtained from eight patients with SCAD in Dr. Soetomo General Hospital with inclusion criteria as follows: male, aged 40-59, stable angina, and coronary angiography showed >50% stenosis of left main coronary artery or >70% of other coronary arteries. Subjects with a history of percutaneous coronary intervention, coronary artery bypass grafting, acute myocardial infarct, diabetes, smoking, and anemia were excluded. The study protocol was approved by the Health Research Ethics Committee of Dr. Soetomo General Hospital, Surabaya (No.292/Panke.KKE/IV/2016). Each subject has signed informed consent before subject recruitment.

**EPCs isolation and culture**

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood sample by Ficoll Histopaque 1077 (Sigma-Aldrich, USA). To isolate EPCs from PBMCs, a standard protocol was conducted as described previously. Briefly, 5x10⁵ cells/mL PBMCs were cultured in the fibronectin-coated 6-well plate with basal stemline II hematopoietic stem cell expansion medium (Sigma-Aldrich, USA) supplemented with 15% fetal bovine serum and 40 ng/mL vascular endothelial growth factor. The culture was maintained at 37°C with 5% CO₂ in a humidified atmosphere. Two days after, non-adherent cells were discarded, and fresh medium was added. Two weeks after, cultured cells were stained FITC-labeled anti-human CD34 antibody clone 581 (Biolegend, USA) and documented with an inverted immunofluorescence microscope.

**EPCs proliferation assay**

MTT cell proliferation assay kit (Sigma-Aldrich, USA) was used to measure EPCs proliferation as described previously. Treated EPCs were added with MTT reagent and incubated in a 37°C incubator with 5% CO₂ for 4 hours. Proliferation was determined from the reduction of tetrazolium (MTT) into insoluble formazan product by viable EPCs mitochondria. Absorbance was measured with a microplate reader at 595 nm wavelength.

**Western blot analysis**

The protein samples from EPCs were loaded into sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for electrophoresis. The electroblotting apparatus was used to transfer the protein onto the nitrocellulose membrane. The membranes were incubated with primary antibodies rabbit anti-human ERK (Sigma-Aldrich, USA) for overnight. The membrane then washed and incubated with conjugated secondary anti-rabbit IgG antibody. The result was visualized with an image analyzer.

**Statistical analysis**

Statistical analyses were carried out using IBM SPSS Statistics 25.0 (IBM Corp, USA). Data were compared using the ANOVA test and considered to be significantly different if p < 0.05 or p < 0.001.

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**RESULTS**

**Demography of the subjects**

The blood samples were obtained from eight patients with SCAD and history of antihypertension and statin treatment. Demography of the subjects is shown in Table 1.

**EPCs confirmation**

EPCs were confirmed by CD34 expression, the most commonly-used surface marker to identify EPCs (Benjamin et al. 2013). EPCs colony and CD34 expression can be seen in Figure 1.

**The effect of garlic (Allium sativum) extract, purple sweet potato (Ipomoea batatas L.), and vitamin C on the EPC proliferation**

As shown in Figure 2, under the treatment with garlic extract, purple sweet potato extract and vitamin C at all doses, the proliferation capability of EPCs was significantly higher compared to the negative control (p < 0.05, ANOVA). EPCs proliferation capability was increased in a dose-dependent manner with the treatment using garlic extract, purple sweet potato extract and vitamin C. Highest EPCs proliferation capability were observed on the EPCs treated with 100 mg/ml garlic extract.

**ERK expression on the EPCs treated with garlic extract, purple sweet potato extract, and vitamin C**

Western blot examination showed positive expression of ERK phosphorylation at the higher dose of garlic extract, purple sweet potato extract and vitamin C (Figure 3). This suggests that a higher dose of garlic extract, purple sweet potato extract, and vitamin C will increase the EPCs proliferation capability followed by increased ERK phosphorylation.

**DISCUSSION**

Patient with SCAD has impaired EPCs proliferation and migration capabilities. This research showed that garlic extract, purple sweet potato extract, and vitamin C had beneficial effects to improve the proliferation of the EPCs derived from peripheral blood of SCAD patients in a concentration-dependent manner. This finding is consistent with previous researches, which showed the ability of garlic extract, purple sweet extract benefit to improve the proliferation of several stem cell lines. Garlic extract was proven to improve neovascularogenesis in a dose-dependent manner for an animal model of neovascuclogenesis. Garlic extract contains a high level of allicin, which has been proven to increase the EPCs proliferation capability followed by increased ERK phosphorylation.

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**Table 1: Subjects demography.**

| Variable             | Mean ± SD   |
|----------------------|-------------|
| Age (year)           | 54.5 ± 4.31 |
| Height (cm)          | 168.0 ± 1.3 |
| Body Mass Index (kg/m²) | 25.39 ± 2.13 |
| Systolic Blood Pressure (mmHg) | 137.5 ± 24.35 |
| Diastolic Blood Pressure (mmHg) | 80.0 ± 7.56 |
| Heart rate (beats/min) | 86 ± 8.68  |
| Total Cholesterol (mg/dL) | 200.5 ± 74.75 |
| Triglyceride (mg/dL)  | 97 ± 11.64  |
| LDL (mg/dL)          | 145 ± 61.11 |
| HDL (mg/dL)          | 35 ± 7.64   |
| LVEF (%)             | 53.5 ± 4.11 |

LDL: Low-Density Lipoprotein, HDL: High-Density Lipoprotein, LVEF: Left Ventricle Ejection Fraction.
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improve EPCs proliferation and migration.\textsuperscript{15} It is also suggested that garlic extract improves EPCs' neovasculogenesis capability through modulation of the PI3/Akt pathway.\textsuperscript{14}

Purple sweet potato contains a high amount of anthocyanins, which was proven to improve impaired EPCs proliferation and migration \textit{in vivo}.\textsuperscript{16} High-level of anthocyanin also has been proven to increase EPCs proliferation capability in a dose-dependent manner through the reduction of intracellular ROS.\textsuperscript{9} This suggested that the benefit of purple sweet potato extract on the EPCs proliferation might involve the ROS pathway. Interestingly, purple sweet potato showed an inhibitory effect on breast cancer, gastric cancer, bladder cancer cell, and colon adenocarcinoma proliferation.\textsuperscript{17-19} This suggests that purple sweet potato might both stimulate or inhibit cell proliferation depending on the type of cells.

Vitamin C treatment has been proven to improve the proliferation of adipocyte stem cells, cardiac progenitor cells, and intestinal stem cells.\textsuperscript{13,20,21} Similar to this research, vitamin C at the dose of 10 mcg/mL was shown to prevent the impairment of EPCs proliferation
caused by TNF-α through P38 inhibition. Interestingly, vitamin C at the dose of 100 mg/dl can reduce the proliferation of the EPCs and vasculogenesis. This suggests that dose-dependent effect of vitamin C to the EPCs proliferation.

In this research, the higher dose of garlic extract (100 mcg/mL), purple sweet potato extract (25 mcg/mL), and vitamin C (100 mcg/mL) were proven to increase phosphorylated ERK of the EPCs compared to sweet potato extract (25 mcg/mL), and vitamin C (100 mcg/mL) were proven to increase phosphorylated ERK of the EPCs compared to the lower dose. While the exact mechanism of impaired proliferation in the EPCs in CHD patients remains unclear, it is suggested that oxidative stress may be involved in the EPCs impairment. Several antioxidants such as vitamin E, resveratrol, and L-arginine have proven to improve EPCs proliferation in-vivo and in-vitro. Oxidative stress downregulates ERK signaling pathway in the embryonic stem cell. While ERK signal transduction pathway is responsible for promoting cell proliferation, nutrient uptake, and cell survival. Reduced ERK signaling pathway also has proven to reduce EPCs proliferation in patients with stable angina. Hence, It is speculated that the beneficial effect of the garlic extract, purple sweet extract, and vitamin C extract to improve EPCs proliferation may involve its antioxidant capability which increases ERK phosphorylation.

CONCLUSION

Treatment with garlic extract, purple sweet potato extract and vitamin C increase EPC proliferation which might involve ERK signaling pathway.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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### GRAPHICAL ABSTRACT

[Diagram showing ERK pathway and increased proliferation and migration of EPCs treated with garlic extract, purple sweet potato extract, and vitamin C.]

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