

High Dose Allicin with Vitamin C Improves EPCs Migration from the Patient with Coronary Artery Disease

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ABSTRACT

Endothelial Progenitor Cells (EPCs) have an important role in endothelial dysfunction repairment through neovasculogenesis and cardiac myocytes regeneration. However, EPCs migration is greatly reduced in the patient with Coronary Artery Disease (CAD). Allicin and Vitamin C are hypothesized to improve EPCs migration due to its antioxidant properties. **Objective:** To investigate the effect of Allicin and its combination with Vitamin C in EPCs migration of CAD patients. **Material and Method:** Mononuclear cells were isolated from CAD patients and cultured on fibronectin-coated plates with colony-forming unit Hill medium. The cells were divided into untreated (control), Allicin treatment (dose 100 mcg/ml, 200 mcg/ml, 400 mcg/ml), and each dose of Allicin combined with 250 mcg/mL of Vitamin C. EPCs migration was assessed with Transwell Migration Assay Kit and evaluated by using statistical tests. **Results:** This research shows that EPC migration was significantly higher in the treatment. Allicin at all dose (dose 100 mcg/ml, 200 mcg/ml, 400 mcg/ml) and its combination with 250 mcg/mL of vitamin C compared to untreated group ($p < 0.05$). Allicin increase EPCs migration in a dose-dependent manner. However, the only combination of 400 mcg/ml Allicin with 250 mcg/mL of vitamin C which has significantly higher EPCs migration compared to Allicin treatment alone. **Conclusion:** Allicin improves EPCs migration in a dose-dependent manner. Improvement of the migration only observed on the Allicin dose 400 mcg/ml with Vitamin C. **Key words:** Allicin, Antioxidant, Endothelial Progenitor, Migration.

INTRODUCTION

Coronary Artery Disease is the leading cause of cardiovascular death worldwide and responsible for 17.3 million deaths in 2013.^{1,2} In 2030, it is projected that CAD in the United States reached 49,3%.³ In Indonesia, the Ministry of Health confirmed that CAD caused 12,9% of deaths from non-communicable disease.⁴ Early-stage of CAD is marked by endothelial injury which could progress to atherosclerosis.⁵ Endothelial injury repair requires colonization of EPCs derived from blood marrow-derived progenitor cells.⁶ Previous studies have shown the benefit of EPCs to repair damaged endothelial by differentiation into the mature endothelial cell to induce vasculogenesis. However, EPCs migration, proliferation rate, adherence capability and survival rate is greatly decreased in the patient with CAD.^{7,8} Several pathways were involved in the impairment of EPCs, including inflammation and oxidative stress.⁹ Emerging evidence showed that the decrease of EPCs number in patients with CAD is related to the long term exposure to pro-inflammatory cytokines and oxidative stress.¹⁰

Recently, antioxidants receive more attention as conjunctive therapy in cardiovascular diseases. Allicin is a volatile oil that commonly found in garlic, onion, and shallot which able to prevent platelet aggregation, cardiac hypertrophy, hyperlipidemia and hyperglycemia.¹¹ Allicin also acts as an antioxidant which reduces circulating

ROS and scavenging free radicals in cardiac myocyte by 50% compared with control both *in vitro* and *in vivo*.¹² The previous study showed that antioxidant such as vitamin C has a beneficial effect as an antioxidant in CAD patient to prevent endothelial dysfunction and slow down the atherosclerosis progression through increasing glutathione level in the body which reduce free radicals.^{13,14}

An antioxidant may increase EPCs proliferation through multiple pathways. Combination of several antioxidant such as Vitamin C and Vitamin E has been proven to synergically improve EPCs proliferation.¹⁴ Various research has studied natural sources as supplementary therapy for CAD, however, there is no study that evaluates the efficacy of Allicin and its combination vitamin C. Hence, this research evaluates the effect of Allicin and vitamin C in EPCs migration from CAD patients.

MATERIAL AND METHODS

Allicin and Vitamin C preparation

Garlic extract contains Allicin (ChemFaces[®]) 98% was dissolved in dimethyl sulphoxide (DMSO). Vitamin C powder (Sigma Aldrich, USA) was dissolved in double-distilled water and diluted with culture medium to acquire the dose of 250 mcg/mL.

Sample criteria

The blood sample was acquired from eight CAD patients in Dr Soetomo General Hospital who fulfilled the inclusion criteria, which are: male, aged

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40-69, stable angina, and coronary angiography showed 50% stenosis of left main coronary artery or >70% of other coronary arteries. Patients with the history of percutaneous coronary intervention, coronary artery bypass grafting, acute myocardial infarct, diabetes, smoking and anaemia were excluded. This research has been approved by the Health Research Ethics Committee of Dr Soetomo General Hospital, Surabaya with letter number 1117/KEPK/IV/2019. Written consent was obtained from the recruited patients and all personal details were omitted.

EPC isolation and culture

Human EPCs were acquired from Peripheral Blood Mononuclear Cells (PBMCs) isolation by using Ficoll Histopaque 1077. 5×10^5 cells/mL PBMCs were distributed into fibronectin-coated 6-well plate dish 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 incubated at 37 °C and CO₂ level at 5% for 48 hours. Then, the non-adherent cells were removed, and fresh medium was added. In order to confirm the EPCs, the cultured cells were stained with FITC-labeled anti-human CD34 antibody clone 581 (BioLegend, USA) within two weeks and documented with immunofluorescence microscopy.

EPC migration assay

EPC migration was calculated by using the Boyden chamber assay method. Isolated EPCs were detached by using Trypsin EDTA solution (Sigma-Aldrich, USA) and then seed it in the upper chamber with basal media. Meanwhile, the lower chamber was supplemented with basal media and chemoattractant. The culture was incubated at 37 °C for 24 hours. The non-migratory EPCs on the upper chamber were removed by PBS. Meanwhile, the migratory EPCs below the upper chamber were fixed with 3.7% paraformaldehyde and permeabilized with methanol. Then, migrated EPCs were stained with Giemsa and calculated.

Statistical analysis

Statistical analysis on the data was carried out using SPSS Statistic 25.0 program (IBM Corp, USA). Data were considered significantly different if $p < 0.05$. Data were presented as mean \pm SD and evaluated for distribution, and then being compared by using an appropriate test.

RESULTS

Research subjects demography

The blood samples were acquired from eight CAD patients on antihypertensive and statin treatment. Table 1 below shows the demography of the subjects.

Allicin and Vitamin C improved EPCs migration

EPCs were confirmed on the presence of the CD34 on immunofluorescence as seen below:

From Figure 1, it can be seen that EPCs presence was confirmed through the positive CD34 expression. Inverted microscope view showed spindle-shaped cells which also characterize EPCs.

As shown in Figure 2, EPCs migration was significantly higher treated with Allicin dose of 100 mcg/mL, 200 mcg/mL, and 400 mcg/ compared to untreated EPCs ($p < 0.05$, ANOVA). EPCs migration was improved in a dose-dependent manner on the treatment with Allicin.

As shown in Figure 3, EPCs migration was significantly higher treated with Allicin dose of 100 mcg/mL, 200 mcg/mL, and 400 mcg/mL in combination with 250 mcg vitamin C compared to untreated EPCs ($p < 0.05$, ANOVA). It also can be seen that Allicin combined with vitamin C also shown to improve EPCs migration in a dose-dependent manner.

As shown in Figure 4, it can be seen that a significant difference was only observed on Allicin with the dose of 400 mcg/mL combined with vitamin C dose 250 μ g/mL compared to Allicin dose 400 mg alone; ($p < 0.05$). No significant difference was found on the combination of Allicin at a lower dose (100 mcg/mL and 200 mcg/mL) with vitamin C dose 250 mcg/mL.

DISCUSSION

Patient with unstable coronary artery disease is known to have a 50% lower EPCs number than normal patient, which results in slower endothelial regeneration.^{1,15} Impaired activity of EPCs in the blood is associated with cardiovascular risk factors because It reflects impaired regenerative potential.¹⁶ In this research, Allicin at a dose of 100 mcg/mL, 200 mcg/mL, and 400 mcg/mL was shown to improve EPCs migration in a dose-dependent manner compared to the untreated group. Similarly, Allicin has been proven to enhance the proliferation and migration of cardiac microvascular endothelial cells.¹⁷ While the exact mechanism of the increasing of EPCs migration on Allicin treatment is yet to be investigated, it is suggested that antioxidant pathways may play significant roles. Allicin has been shown to have an antioxidant effect and have scavenging ability.¹⁸ Allicin with its antioxidant capability has been shown able to increase the availability of eNOS, NO, and trigger growth factor such VEGF, SDF-1 and IGF production- which are required for EPCs proliferation,^{11,19} Increasing level of NO also increase the level of Vasodilator-stimulated phosphoprotein (VASP) which plays role in the actin elongation which improves EPCs migration.^{20,21} Allicin also act as H₂S donor which can interact with Cys1045 dan Cys1024

Table 1: Subjects demography.

Variable	Mean \pm SD
Age (year)	54.5 \pm 4.31
Height (cm)	168.0 \pm 1.3
Body Mass Index (kg/m ²)	25.39 \pm 2.13
Systolic Blood Pressure (mmHg)	137.5 \pm 24.35
Diastolic Blood Pressure (mmHg)	80.0 \pm 7.56
Heart rate (beats/min)	86 \pm 8.68
Total Cholesterol (mg/dL)	200.5 \pm 74.75
Triglyceride (mg/dL)	97 \pm 11.64
LDL (mg/dL)	145 \pm 61.11
HDL (mg/dL)	35 \pm 7.64
LVEF (%)	53.5 \pm 4.11

LDL: Low-Density Lipoprotein, HDL : High-Density Lipoprotein, LVEF: Left Ventricle Ejection Fraction

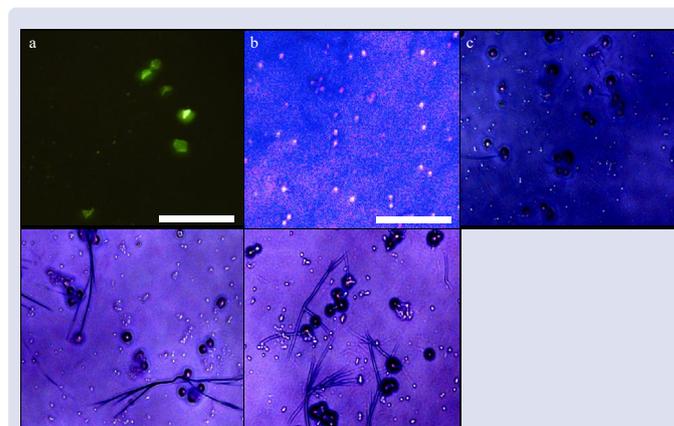


Figure 1: Morphology of EPCs. (a) Immunofluorescence view of isolated EPCs stained with FITC-labelled anti-CD34 antibody; inverted microscope view of EPCs on (b) control group; treatment with Allicin dose (c) 100 mcg/mL; (d) 200 mcg/mL and (e) 400 mcg/mL. White bar represents 100 μ m.

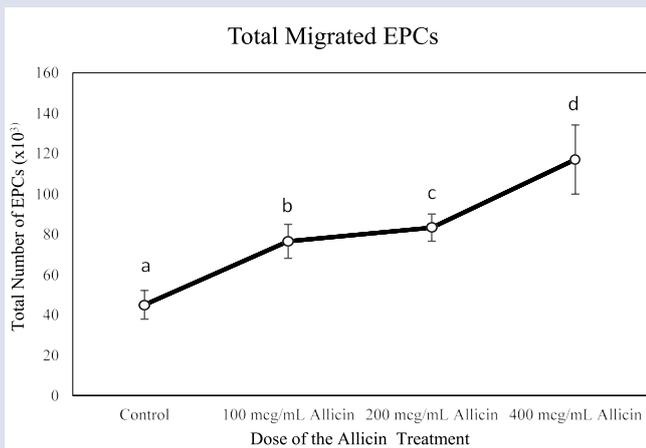


Figure 2: Allicin improves endothelial progenitor cells migration. EPCs were treated with Allicin dose of 100 mcg/mL, 200 mcg/mL, and 400 mcg/mL. No treatment was given in the control group. Viable migrated EPCs were counted by using the Boyden Chamber Assay and evaluated statistically by using SPSS. Different annotation ^{a,b,c,d} showed significant differences ($p < 0.05$).

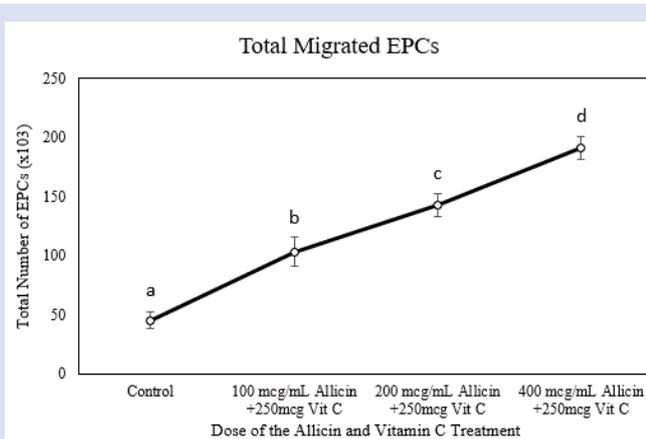


Figure 3: Allicin and Vitamin C improves endothelial progenitor cells migration. No treatment was given in the control group. EPCs were treated with Allicin starting from 100 mcg/mL, 200 mcg/mL, and 400 mcg/mL, each combined with 250 mcg vitamin C. Viable EPCs were counted by using the Boyden Chamber Assay and evaluated statistically by using SPSS. Different annotation ^{a,b,c,d} showed significant differences ($p < 0.05$).

As illustrated in Figure 3, EPCs migration was significantly higher treated with Allicin dose of 100 mcg/mL, 200 mcg/mL, and 400 mcg/mL in combination with 250 mcg vitamin C compared to untreated EPCs ($p < 0.05$, ANOVA). It also can be seen that Allicin combined with vitamin C also shown to improve EPCs migration in a dose-dependent manner.

to activate VEGFR-2.²² Activation of VEGFR-2 will improve EPCs proliferation, migration and survival.²³

The recent research result has shown that ascorbic acid is able to trigger endothelial repair by promoting endothelial proliferation, migration, improving the function, increasing collagen type IV deposition, acting as radicals scavenger, and reducing pro-inflammatory cytokines.²⁴ In this research, we found that EPCs treated with vitamin C dose 250 mcg/mL combined with Allicin dose 400 mcg/mL showed a significant increase of migration compared to the combination of vitamin C dose 250 mcg/mL with Allicin dose 100 and 200 mcg/mL. Previously, it has been shown that Vitamin C treatment alone can improve EPCs migration and proliferation.²⁵ Vitamin C improves EPCs proliferation and migration via suppression of ROS and pro-inflammatory cytokine (TNF- α in particular) which will reduce p-38 phosphorylation and stimulate

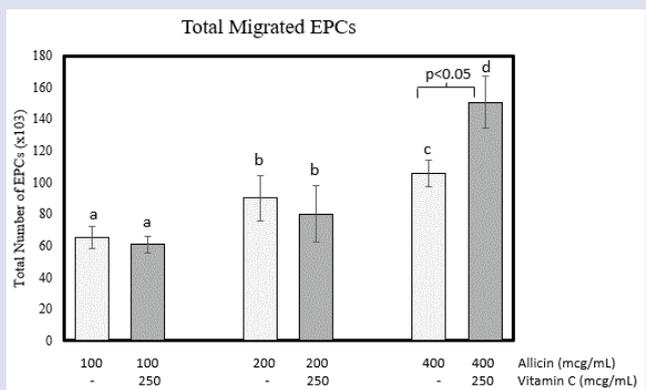


Figure 4: High dose (400 mcg/mL) Allicin combined with 250 mcg vitamin C improve EPCs migration but not for lower dose of Allicin. EPCs were treated with Allicin as a single treatment or in combination with 250 mcg vitamin C, starting from 100 mcg/mL, 200 mcg/mL, and 400 mcg/mL. Viable EPCs were counted by using MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) and evaluated statistically by using SPSS. Different annotation ^{a,b,c,d} showed significant differences ($p < 0.05$).

Mitogen-Activated Protein Kinase (MAPK) activation.¹⁴ Increasing MAPK activation will be followed by improved EPCs proliferation and migration.¹⁰ This suggests that treatment with Allicin combined with vitamin C may have multiple mechanism to improve EPCs migration. However, in this research, the lower dose of Allicin (100 and 200 mcg/mL) was failed to synergically improve EPCs migration while the higher dose of Allicin of 400 mcg/mL shows significant benefit. This finding suggests that the combination of Allicin with vitamin C might not work synergically at the lower dose. It is speculated that low dose Allicin may have a similar mechanism with vitamin C to improve EPCs migration via antioxidant pathway, hence its combination shows no significant benefit. However, at higher dose, Allicin may act as H₂S donor that can activate VEGFR-2 which improve EPCs migration and synergically work with antioxidant capability of vitamin C which stimulate MAPK pathway to increase EPCs migration.^{10,22} It is speculated that EPCs migration can be significantly higher through this multiple mechanism compared to Allicin treatment alone with single mechanism. However, it is suggested to conduct further studies to validate this mechanism in the future.

CONCLUSION

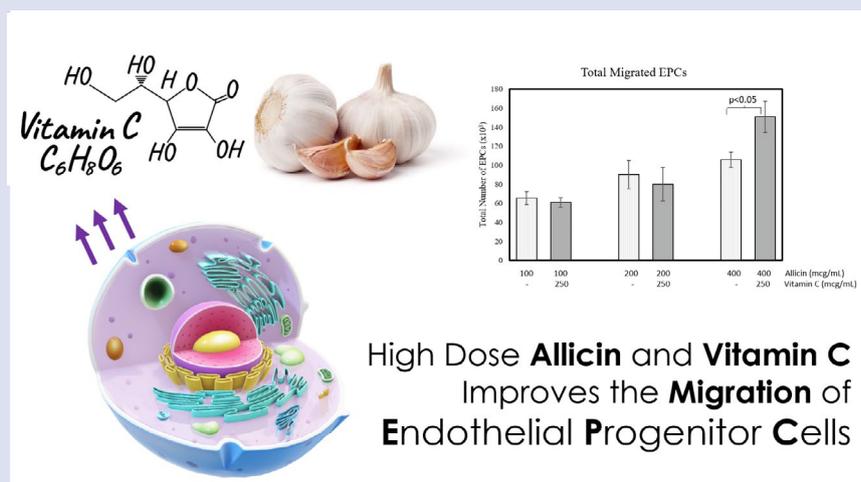
Allicin has a dose-dependent effect to improve the EPCs migration. Combination of Allicin at the dose of 400 μ g/mL and vitamin C dose of 250 μ g/mL showed significantly higher EPCs migration compared to the lower dose of Allicin combined with vitamin C at dose of 250 μ g/mL.

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GRAPHICAL ABSTRACT



High Dose **Allicin** and **Vitamin C** Improves the **Migration** of **Endothelial Progenitor Cells**

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