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Ramiprilat Effects on Endothelial Progenitor Cells Migration is Increased by Human Umbilical Cord Blood-Mesenchymal Stem Cells derived Secretome

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ABSTRACT

Endothelial progenitor cells (EPCs) have a critical role in angiogenesis and vasculogenesis of coronary artery disease (CAD) patients. Secretome of human Umbilical Cord Blood-Mesenchymal Stem Cell (hUCB-MSCs) can promote neovascularization. Ramiprilat is an active metabolite of ramipril that has shown benefit in cardiovascular disease. The effect of hUCB-MSCs-derived secretome alone or combination with ramiprilat on EPCs migration is not yet elucidated. This study aimed to identify the effect of hUCB- MSC derived secretome and its combination with ramiprilat on EPCs migration. EPCs were collected from peripheral blood of CAD patient and cultured in the Stemline II medium. Cultured EPCs were then divided into groups of control, ramiprilat 10 μmol, hUCB-MSCs derived secretome (2%, 10%, and 20%), and its combination. The migration of EPCs was assessed using a Boyden chamber assay. Ramiprilat and hUCB-MSCs-derived secretome at all doses increase EPCs migration in dose-dependent manner. Combination of hUCB-MSCs-derived secretome at dose 10% and 20% and ramiprilat significantly increase migrated cells compared to ramiprilat only and secretome only group ($p<0.001$). In conclusion, hUCB-MSCs-derived secretome and ramiprilat enhance EPCs migration and combination of those two substances furtherly increased the migrated cells. hUCB-MSCs-derived secretome has the potential as regenerative treatment for CAD patients.

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CCS CONCEPTS

• **Applied computing**; • **Life and medical sciences**; • **System biology**;

KEYWORDS

endothelial progenitor cells, human umbilical cord blood, mesenchymal stem cells, secretome, migration, ramiprilat

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1 INTRODUCTION

Coronary artery disease (CAD) has a high number of morbidity and mortality around the world. Coronary events are expected to occur in more than one million individuals in United States [1]. While CAD therapies are increasingly progressing in terms of pharmacological and percutaneous intervention techniques, there are some patients that do not take benefits of this advancement therapy including patients with refractory angina [15, 25]. These patients need therapy modalities which can improve their quality of life.

Endothelial progenitor cells (EPCs) are multipotent cell that has an important role in the pathophysiology of coronary artery disease. EPCs are able to differentiate into mature endothelial cell, contributes to reendothelialization after endothelial injury and therefore improve endothelial function. EPCs also play critical role in angiogenesis and vasculogenesis. To do this role, EPCs need to mobilize from bone marrow to circulating blood and migrate to

injured site [20, 31]. Unfortunately, people with low EPCs level, including CAD patients, have an impairment in forming collateral and compensates for the presence of stenosis [5]. Thereby, improving circulating EPCs number and function provide therapeutic option for CAD patients.

Human umbilical cord blood mesenchymal stem cells (hUCB-MSCs) secrete molecules to extracellular space named secretome, that contain soluble proteins, exosomes and micro vesicles [6, 9, 24]. This secretome promotes neovascularization, angiogenesis [6, 32], and improves cardiac systolic function [34]. hUCB-MSCs as source of secretome has advantage in high proliferation capacity and less invasive collecting method [14]. Another researches have shown the migration enhancing capacity of using another source of secretome [3, 12] but the effect of hUCB-MSCs derived secretome on EPCs migration has not yet established. Ramipril, with active metabolite ramiprilat, is an angiotensin converting enzyme inhibitor (ACE-I) that has been shown to increase EPCs proliferation and migration [21, 22]. This study is aimed to identify the effect of hUCB- MSCs derived secretome and its combination with ramiprilat on EPC migration.

2 METHODS

2.1 Sample criteria

This experiment used blood samples that were obtained from chronic coronary syndrome patient at dr. Soetomo General Hospital Surabaya Outpatient Clinic. The inclusion criteria for this study were male, aged 40–59 years old, had conducted coronary angiography that showed >50% stenosis of left main coronary artery or in the other coronary arteries showed 70% stenosis. We exclude patients with acute coronary syndrome, acute limb ischemia, diabetes mellitus and anemia. This study protocol had an ethical clearance from the Health Research Ethics Committee of Dr. Soetomo General Hospital Surabaya. The subject had signed informed consent before recruited. All details which included personal information were omitted.

2.2 Secretome preparation

The preparation of hUCB-MSCs derived secretome was done in accordance to previous study [26]. hUCB-MSCs cell line (3H Biomedical AB, Uppsala, Sweden) was cultured in Mesencult media (StemCell Technologies Inc., Vancouver, Canada) which contained penicillin and streptomycin. While the confluency was reaching 80%, the media was replaced with the newer media with no supplementation. hUCB-MSCs with supplement-free media then incubated for 24 hours. After incubation, the media was collected and centrifuged. Supernatant that was resulted from centrifugation was used as a conditioned medium that contained secretome.

2.3 EPCs isolation and culture

EPCs were collected from mononuclear cells (MNCs) of the peripheral blood of CAD patient. Forty milliliters of blood were diluted with phosphate buffer saline with 2% fetal bovine serum then ficoll histopaque was added. Centrifugation of the mixture was done until peripheral blood MNCs (PBMNCs) layer was formed. PBMNCs were cultured with basal Stemline II hematopoietic stem cell expansion medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 15% fetal bovine serum and several growth factors in the fibronectin-coated 6-well plates. The culture was maintained at 37^{o}C with 5% CO_2 in a humidified atmosphere. Non-adherent cells were removed, and fresh medium was added.

2.4 EPCs identification

After three days of isolation and culture, EPCs were confirmed using immunofluorescence microscope examination with FITC-labeled anti-human CD34 antibody (Biolegend, USA) staining.

2.5 EPCs treatment

Cultured EPCs were divided into control group, treatment with 10 μmol Ramiprilat, various concentrations of hUCB-MSCs-derived secretome (2%, 10%, and 20%) and combination of 10 μmol Ramiprilat and each dose of secretome. The control group was not treated with secretome or ramiprilat. For the Ramiprilat group, cell culture was incubated for 48 hours before transferring to the transwell.

2.6 Migration assay

This experiment used Costar®Transwell®Permeable Support (Corning, USA) with a 3.0 μm pore size membrane. EPCs migration was assessed using a Boyden chamber assay method. A total of 5×10^5 EPCs were placed at the upper chamber with basal media and the lower chamber was supplemented with basal media and secretome. The culture was incubated at 37^{o} C for 24 hours. The non-migratory cells were removed manually. The migratory EPCs below the upper chamber were fixed with 3.7% paraformaldehyde and permeabilized with methanol. Migrated EPCs were stained with Giemsa staining and calculated.

2.7 Statistical analysis

Data analyses were done using SPSS Statistics 23.0 from IBM to detect significance level at $p<0.05$. Data distribution was evaluated using Kolmogorov Smirnov test and comparation between groups were calculated using one-way ANOVA test. Correlation between variables was obtained using Spearman correlation followed by a linear regression test.

3 RESULTS

3.1 EPCs identification

Identification of EPCs was done using immunofluorescence and light microscope. Positive CD34 expression was used to mark EPCs. Under a light microscope, EPCs were demonstrated as spindle-shaped cells (Figure 1).

3.2 EPCs migration

This experiment showed that ramiprilat and all doses of hUCB-MSCs derived secretome significantly increased EPCs migration compared to control group ($p<0.001$). hUCB-MSCs derived secretome increase EPCs migration in dose dependent manner (Figure 2). Ramiprilat 10 μmol had significantly higher EPCs migration than secretome 2% (33.80±2.49 vs 17.20±1.92, $p<0.001$) but no statistically significant difference was observed between ramiprilat and secretome 10% (33.80 ± 2.49 vs 27.00 ± 4.00, $p>0.05$). However,

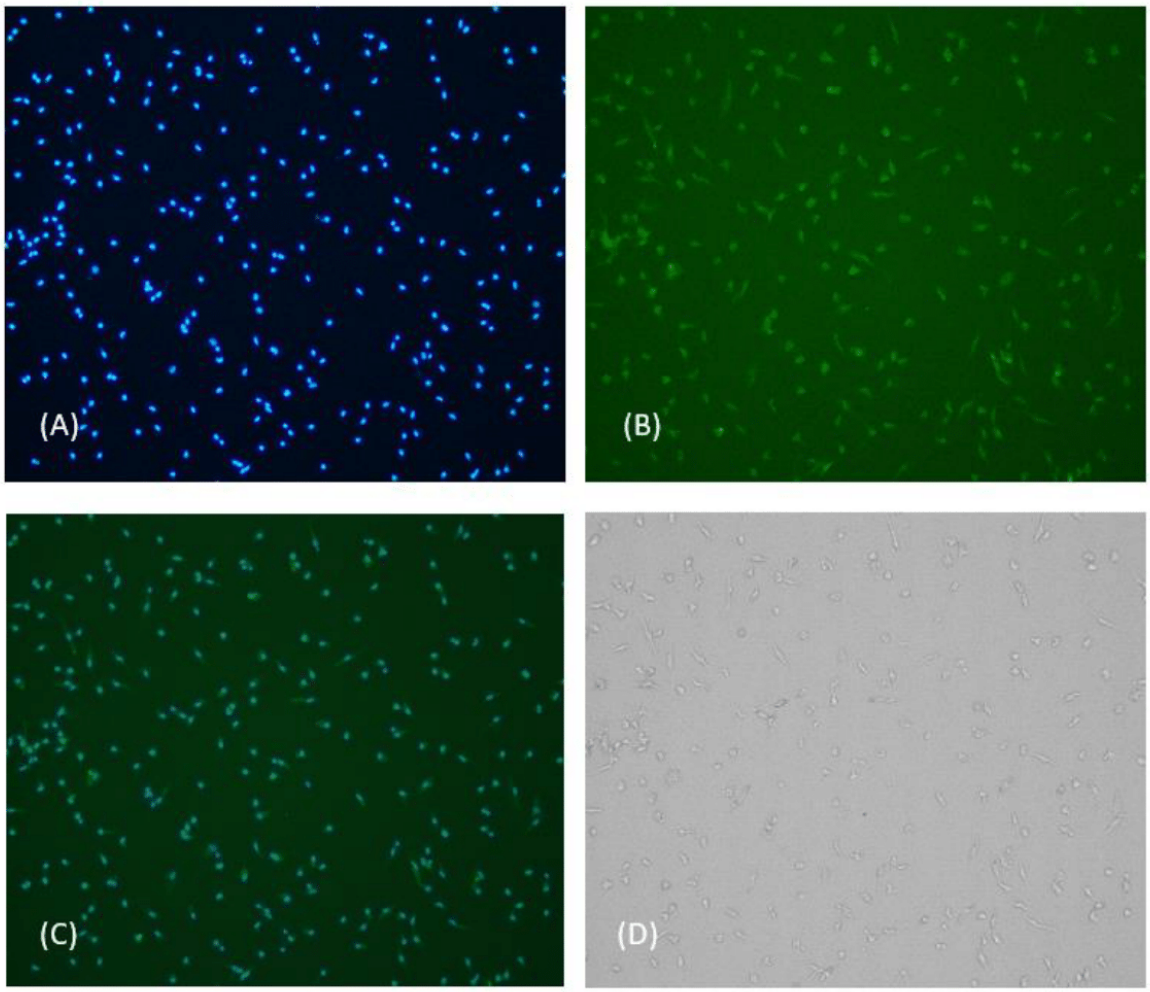

Figure 1: Microscopic picture of endothelial progenitor cells (EPCs) after three days incubation and culture (200x magnification) (A) Staining with diamidino-2-phenylindole (DAPI) showing nuclear image of EPCs with blue fluoresence (B) FITC-labelled anti-CD34 antibody staining of EPCs (C) Merged view of A and B (D) Spindle shape of EPCs were shown in light microscope

secretome 20% showed a significantly higher migration compared to the ramiprilat group (51.00±5.15 vs 33.80 ± 2.49, p<0.001).

Spearman correlation showed a significant and strong correlation between hUCB-MSCs-derived secretome treatment with EPCs migration (r=0.946; p<0.001). The linear regression test showed R-square of 0.877. This indicated that hUCB-MSCs-derived secretome treatment was responsible for 87.7% increase in EPCs migration.

Combination of ramiprilat 10 μmol and hUCB-MSCs derived secretome at all doses showed significant enhancement of EPCs migration compared to secretome only group (30.00±4.06 vs 17.20±1.92, 55.00±4.42 vs 27.00±4.00, 69.00±7.65 vs 51.00±5.15, p<0.001). Ramiprilat only group showed higher number of EPCs migration compared to combination of Ramiprilat and secretome 2% but not statistically significant (33.80±2.49 vs 30.00±4.06, p>0.05). Combination of ramiprilat and secretome at 10% and 20% concentration were significanly superior to ramiprilat only group (55.00±4.42 vs 33.80±2.49 and 69.00±7.65 vs 33.80±2.49, p<0.001). The combination hUCB-MSCs derived secretome 20% and ramiprilat had the highest number of migrated EPCs compared to another groups (Figure 3).

4 DISCUSSION

The result of present study demonstrated that hUCB-MSCs derived secretome, ramiprilat, and combination of both of them enhance EPCs migration. Secretome increased EPCs migration in dose dependent manner. Combination of ramiprilat and high dose secretome has the highest number of EPCs migration compared to ramiprilat alone or secretome alone. This synergistic effect might be beneficial for treatment in chronic coronary syndrome patients.

Mesenchymal stem cells secretome contained pro angiogenic factors including insulin-like growth factor (IGF), interleukin-6 (IL-6), stromal cell-derived factor-1 (SDF-1), prostaglandin E2 (PGE2),

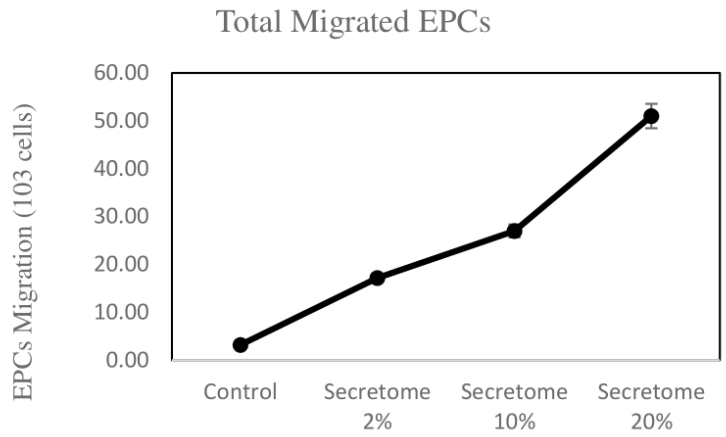

Figure 2: Human umblical cord blood mesenchymal stem cells (hUCB-MSCs) derived secretome improves endothelial progenitor cells (EPCs) migration in dose dependent manner. EPCs were treated with hUCB-MSCs derived secretome at doses 2%, 10% and 20%. EPCs migration was counted using Boyden chamber assay.

vascular endothelial growth factor (VEGF), vascular cell adhesion protein 1 (VCAM-1), microvesicles and exosomes [6, 7, 9, 24]. Secretome played role in cardiac tissue preservation, formation of new vessels in damaged tissue, immunomodulation and cardiac regeneration [6, 32, 33]. The results of this research are in line with previous study using placental-derived MSCs (PL-MSCs). PL-MSCs soluble factors significantly enhance EPC migration. Several secreted proteins identified as candidates for EPC migration enhancing factor [12, 13]. Another study using human amniotic membrane-derived mesenchymal stromal cells (hAMCs) secretome treatment also show increased EPCs migration in a dose-dependent manner [3]. While this research shows that hUCB-MSCs derived secretome does increase EPCs migration, the exact mechanism has not yet been established.

During ischemic condition, the affected area releases variety of signal factor including proangiogenic chemoattractant which triggers homing of EPCs to the ischemic area and enhance angiogenesis and vasculogenesis [20, 31]. Elevation of EPCs was seen in myocardial infarction and this increase is in line with increase of VEGF. VEGF increased EPCs migration by binding to VEGFR1 and VEGFR2, shifting G protein signaling toward RAC and RHO which was important for cytoskeletal rearrangement [8, 18, 29].

SDF-1 was the most potent chemoattractant for EPCs. SDF-1 concentration gradient from peripheral blood to ischemic area played a critical role in EPCs migration [19]. SDF-1 bound to the CXCR4 and activated Rac GTPase protein. Its downstream pathway regulates cellular polarity and cytoskeleton changes that accomplish directional migration [27]. The important role of CXCR4/SDF-1 axis was regulated by hypoxia-inducible factors 1α (HIF-1α) [30]. Another research suggested that SDF-1-induced EPCs migration was mediated through the PI3K/Akt/eNOS signal transduction pathway [35]. hUCB-MSCs derived secretome contained SDF-1 which can increase chemoattractant gradient. Giving hUCB-MSCs derived secretome with a higher concentration will increase the gradient so that EPCs will move faster toward the ischemic area. This is indicated in our result which EPCs migration is increase along with the increase in secretome doses.

This study shows that ramiprilat treatment increased EPCs migration better than hUCB-MSC derived secretome at dose 2% and 10%. Ramiprilat is an active metabolite of ramipril, a non-sulfhydryl angiotensin converting enzyme (ACE) inhibitor that blocks the conversion of angiotensin I to angiotensin II, and inhibit degradation of bradykinin [2]. Previous studies had shown that increased in EPCs migration occurred after 7 days of ramipril administration in stable coronary artery disease patients [21]. The underlying mechanism was thought to be related to the bradykinin pathway. ACE inhibitors block the degradation of B2R agonist and enhancing B2R signaling [4]. EPCs stimulation with bradykinin can increase the formation of philopodia and accelerate EPC migration [16]. Bradykinin has an important role in vascular function and involves in eNOS expression through activation of PI3K/Akt-dependent and independent pathway [11, 28].

The combination of ramiprilat with hUCB-MSCs derived secretome shows a synergistic effect where the amount of migrated EPCs increase significantly. The combination of ramiprilat and 20% hUCB-MSC derived secretome shows the highest EPCs migration number, exceeding ramiprilat only group and secretome only group. It is speculated that the combination of these two substances accelerate the migration of EPCs through various mechanism. Apart from the paths mentioned above, hUCB-MSCs also contains a high level of the exosome that has been investigated as proven to reduce myocardial ischemia by inducing neovascularization and increasing vascular tube formation [32]. hUCB-MSCs derived secretome also has anti-inflammatory and antioxidant properties that is expected to improve EPCs migration [10, 17]. Previous research showed that antioxidants can increase EPCs migration [23].

5 STUDY LIMITATIONS

This research has not yet identified which molecules contained in the hUCB-MSCs derived secretome that has major influence

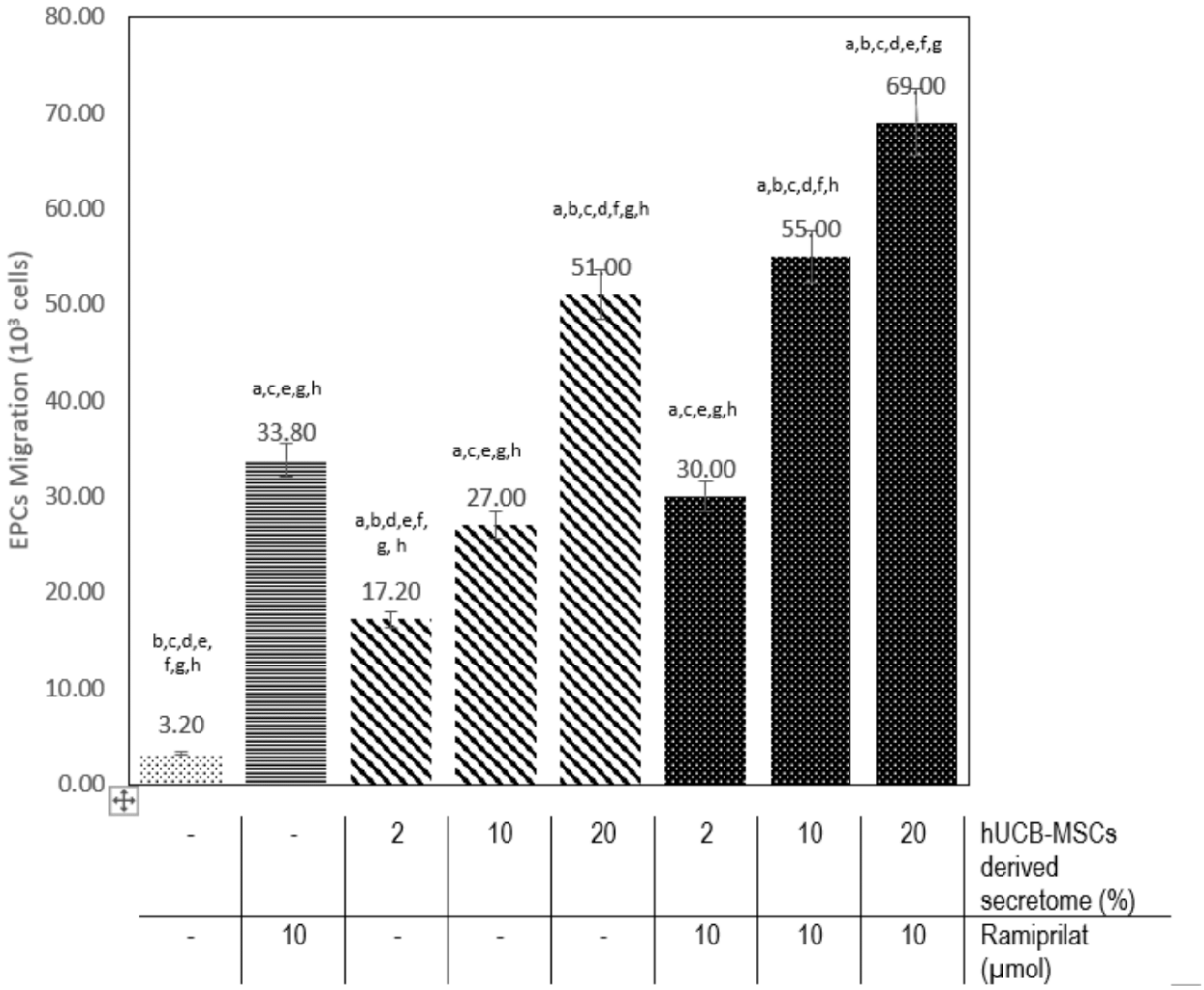

Figure 3: Comparison of EPCs migration between all groups. a: EPCs migration significantly increased compared to control group (p<0.001), b: EPCs migration significantly increased compared to the 10 μmol ramiprilat group (p<0.001), c: EPCs migration significantly increased compared to the 2% hUCB-MSCs derived secretome group, (p<0.001), d: EPCs migration significantly increased compared to the 10% hUCB-MSCs derived secretome group (p<0.001), e: EPCs migration significantly increased compared to the 20% hUCB-MSCs derived secretome group (p<0.001), f: EPCs migration significantly increased compared to the combination of 2% hUCB-MSCs derived secretome and ramiprilat group, (p<0.001), g: EPCs migration significantly increased compared to the combination of 10% hUCB-MSCs derived secretome and ramiprilat group, (p<0.001) and h : EPCs migration significantly increased compared to the combination of 20% hUCB-MSCs derived secretome and ramiprilat group, (p<0.001).

in EPCs migration. Further research is needed to verify various mechanisms speculated to improve EPCs migration in hUCB-MSCs derived secretome group and in combination group.

6 CONCLUSION

hUCB-MSCs-derived secretome and ramiprilat enhance EPCs migration. Combination of those two substances furtherly increased the migrated cells. hUCB-MSCs-derived secretome has the potential as a cardiovascular regenerative treatment for the patient with CAD.

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