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Identification of factors that may Affect Endothelial Progenitor Cells (EPC) Homing with Treatment of High Glucose

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ABSTRACT

Endothelial dysfunction can be caused by various conditions, among others, insulin resistance and hyperglycemia. Endothelial dysfunction may occur due to the formation of reactive oxygen compounds (ROS), thereby increasing the production of free radicals, which is an initial occurrence of oxidative stress and may bring about some complications of diabetes mellitus. Factors that play a role in migration, mobilization and homing of EPC to the damaged vascular area is *Stromal cell-derived factor-1* (SDF-1α / CXCR4), eNOS and NO. SDF-1α is a chemokine that plays a role in enhancing the EPC chemotaxis in the area of vascular injury. SDF-1α activation may mediate EPC homing through the increased PI3 Kinase / Akt / eNOS pathway, so that EPC can form neovascularization. The increased number of EPC, EPC migration, mobilization and EPC homing can repair the damaged vascular tissue so as to prevent further vascular complications. In the chronic diabetes mellitus condition, the SDF-1α expression decreased thus influencing the decreased mobilization of EPC from the bone marrow and EPC homing. The results showed that some factors affecting the EPC Homing are including SDF-1α , CXCR4 receptor and Nitric Oxide (NO) with treatment D-(+) - glucose 5 mM and D-(+)-glucose 22 mM decrease significant compared to control group ($P < 0.05$).

KEY WORDS: *Stromal cell-derived factor-1* (SDF-1α) / CXCR4, Nitric Oxide (NO), Endothelial Progenitor Cells (EPC)

INTRODUCTION

Endothelial dysfunction may occur due to the formation of reactive oxygen compounds (ROS), thereby increasing the production of free radicals, which is an initial occurrence of oxidative stress and may bring about some complications of diabetes mellitus. Increased EPC migration, mobilization and EPC homing to the damaged area can repair vascular endothelial disfunction [1]. Factors affecting the increase in EPC mobilization, migration and EPC homing are including through the increased chemotaxis of Stromal cell-derived factor-1 (SDF-1α/CXCR4), eNOS and NO. The mechanism of SDF-1α can affect EPC homing during ischemia which will cause the hypoxia and releases several growth factors such as HIF-1α and SDF-1α, which are chemokines that stimulate mobilization of EPC from the bone marrow to the circulation. After being in circulation, the SDF-1α will affect the recruitment of EPC to ischemic areas resulting in the process of homing in injury area. The EPC will differentiate to form new blood vessels and can repair the damaged tissues and vessels thus preventing the further vascular complications [2].

MATERIALS AND METHODS

The research design used in this study is the true experimental design for the *in vitro* culture of peripheral blood EPCs using *post test only control group design*.

Isolation and Identification of EPC

The peripheral blood of healthy humans was sampled and collected in flacon supplemented with EDTA. The EPC isolated from whole blood was subjected to the density gradient centrifugation using lymphocyte separation media (Histopaque 1.077 g/ml, Sigma). Furthermore, the supernatant consisting of the isolated cells

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was grown in culture medium M199 (Sigma) containing 100 IU/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), 20% FBS (Invitrogen Corp.), 10 ng/ml hEGF (Sigma) and 100 ug/ml ECGF (Sigma). Cells were grown in 24-well culture plate that has been coated with gelatin 1%, a density of 1 x 106 cells/cm2. They were then incubated (5% CO2, 37°C). The culture medium was replaced every 3 day [3,4,5]. Cell morphology was observed every day. The identified EPC was subjected to immunofluorescence staining where the cells were incubated (5% CO2, 37°C, 1 hour) at 10 µg/mL Dil-ac-LDL (Biomedical Technologies), fixed with paraformaldehyde 2%, then incubated with 50 µg/mL FITC-labeled lectin UEA (sigma) for 45 minutes. The intensity of vWF (BIOSS) expression was examined on late EPC. Methods identified EPC with Dil-Ac-LDL, Lectin dan vWF by using immunofluorescence / Confocal Laser Scaning Microscope (CLSM). [5,18].

Treatment in the EPC Culture

Culture medium for the cultured EPCs which were already mature and confluent on day 7 was replaced with medium containing 5 mm and 22 mM D - (+) - glucose (Sigma) and cells were incubated (5% CO2, 37°C) for 24 hours [6].

Analysis of nitric oxide

Nitric oxide level was detected with colorimetric method using Nitric Oxide Assay Kit D2NO-100 (BioAssay System, USA).

Analysis of SDF-1α and CXCR4

SDF-1α was analyzed using immunofluorescence method using FITC anti-rabbit labeled antibody SDF-1α (BIOSs) (Rockland). CXCR4 were analyzed using immunofluorescence method using the Rhodamine anti rabbit labeled CXCR4 antibody (Sigma) .

Ethics

This study was approved by the Health Research Ethics Committee of the Faculty of Medicine Brawijaya University No. 509A/EC/KEPK-S3-KEDOK/10/2013.

Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using one way ANOVA with SPSS 17.0 statistical package. The post Hoc test was used if the ANOVA was significant. $P < 0.05$ was considered statistically significant.

RESULTS

EPC characterization process in this study consisted of: 1) EPC morphology, 2) the EPC ability to bind lectin and take the LDL, and 3) detection of surface molecular markers of the EPCs with immunofluorescence staining to observe the intensity of vWF expression on the late EPCs. The isolated PBMC from peripheral blood was cultivated in medium Invitrogen Corp. (M199 (Sigma), penicillin (sigma) 100IU / ml, Streptomycin (Sigma) 100 ug / ml, 20% FBS (Invitrogen Corp), hEGF (sigma) 10 ng/ml and ECGF (sigma) 100 ug / ml) for 7 days. Morphologically, MNC was round and not didn't attach at the bottom of a well. On the fourth day of cultivation period, the old medium was replaced with new medium. Non-adherent cells were discarded thus leaving the cells attached to the bottom of a well. Cells attached to the bottom of well were elongated with pointed ends (spindle shape) which were morphological characteristics of the EPC (Figure 1 Spindle shape was seen more clearly at 7 day of incubation period.

a. EPC morphology on day 7 of incubation period

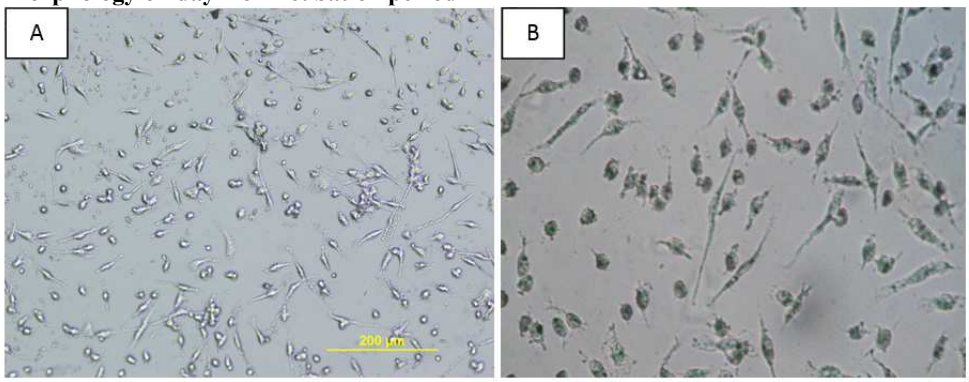


Figure 1. A. EPC Morphology at 200X magnification in the medium, B. EPC Morphology with fixation of Paraformaldehide 2% at 400 X magnification, showing spindle shape at 7 day.

b. Identification of EPC Characteristics using DIL-Ac-LDL , lectin dan vWF

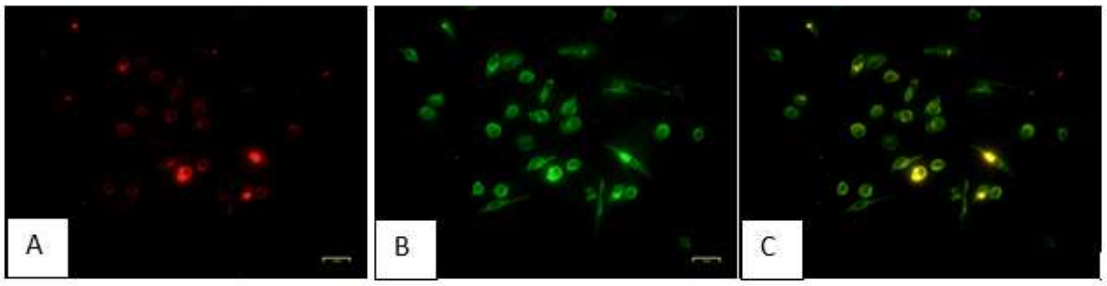


Figure 2. Characterization of the identified EPC with CLSM : A. . Uptake Dil-Ac-LDL (red fluorescence in cells) at magnification, , B. Binding of FITC-UEA-Lectin (Green fluorescence in cells), C. Double Positive Dil-AC-LDL and FITC-UEA-Lectin (orange fluorescence in cells)

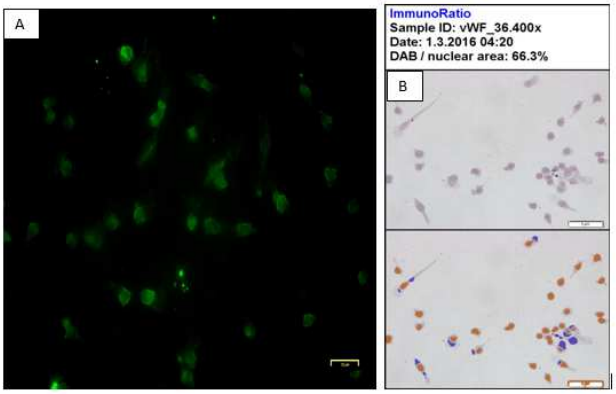


Figure 3. A. Characterization of the identified EPC with CLSM for vWF expression (Green fluorescence in cells), B. Characterization of the identified EPC with immunohistochemistry for vWF expression use ImmunoRatio analysis.

c. Testing of Surface Molecular Markers

EPC surface molecular markers are tested using two markers, namely CD 133, CD 34 and VEGFR-2 in which fluorescence wavelength intensity was measured using flowcytometry. Data were presented in the form of means ± standard deviation. Testing was done by 4 replicates with an average value for the percentages of CD 133 = 77.43% of total 2974 cells, CD 34 = 77.48% of the total 2976 cells, and VEGFR-2 68.08% of the total 2615 cells.

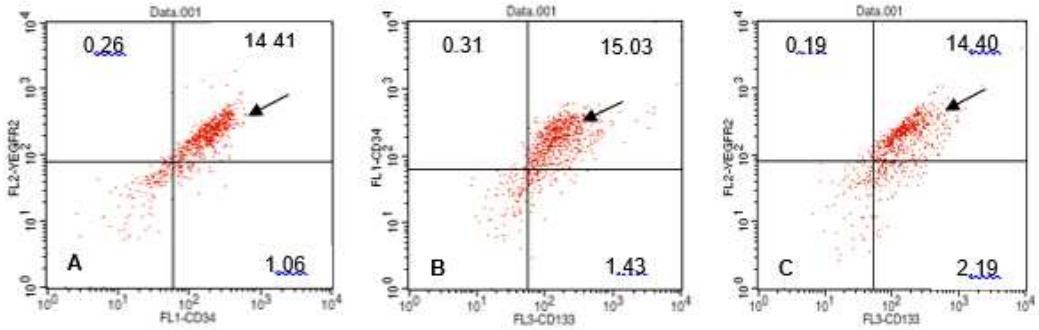


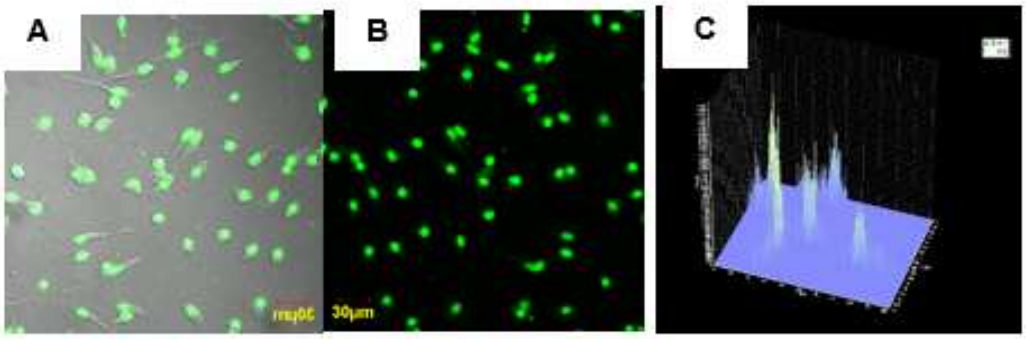
Figure 4. Characteristics of the EPC at day 7 with molecular markers of CD 34, CD 133 and VEGFR-2

Remark :

A. UL Value (Upper Leaf)% Total VEGFR-2 (0.15), the value of LR (Lower Right)% of total CD 34 (1.06) and the value of UR (Upper Leaf)% Total Double Positive (14.41)
B. UL Value (Upper Leaf)% Total CD 34 (0.31), the value of LR (Lower Right)% of total CD 133 (1.43) and the value of UR (Upper Leaf)% Total Double Positive (15.03)
C. UL Value (Upper Leaf)% Total VEGFR-2 (0.19), the value of LR (Lower Right)% of total CD 133 (2.19) and the value of UR (Upper Leaf)% Total Double Positive (14,40)

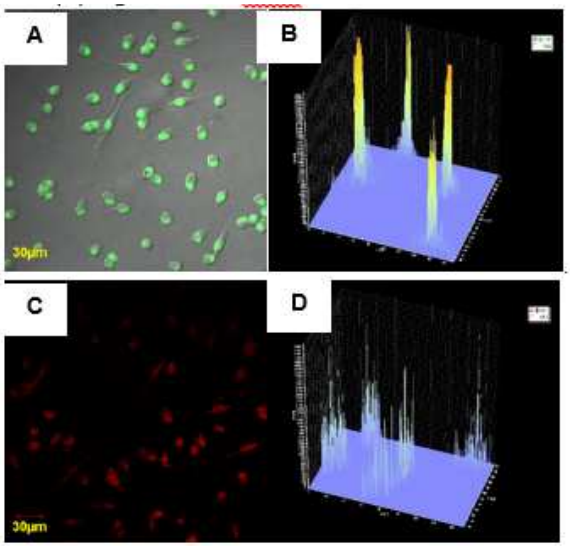
d. Identification of factors influencing EPC homing : SDF-1α, CXCR4, and Nitric Oxide (NO).

1. Intensity of SDF-1α Expression in cultured EPC without administration of D-(+)- glucose



A. Figure 5.A. Immunofluorescence staining of SDF-1α with FITC in cultured EPC without administration of D-(+)- glucose (Sigma) at 400X magnification DIC, B. Immunofluorescence staining of SDF-1α with FITC in cultured EPC without administration of D-(+) -glucose (Sigma) at magnification 400X C. Intensity of SDF-1α expression observed with CLSM 540.94 arbritary units.

2. Intensity of SDF-1α and CXCR4 expressions in cultured EPC by administration of D- (+)- glucose 5 mM.



B. Figure 6.A. Immunofluorescence staining of SDF-1α with FITC in cultured EPC by administering D-(+)-glucose 5 mM; B. Intensity of SDF-1α expression at 364.11 arbritary units; C. Immunofluorescence staining of CXCR4 with Rhodamine in cultured EPC by administering D- (+)- glucose 5 mM; D. Intensity of CXCR4 expression at 165.48 arbritary units.

3. Intensity of SDF-1α and CXCR4 expressions in cultured EPCs by administration of D-(+)-glucose 22 mM.

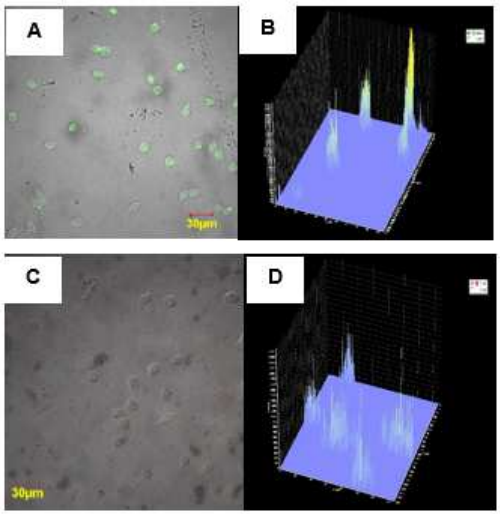


Figure 7.A. Immunofluorescence staining of SDF-1α with FITC in cultured EPC with administration of 22 mM glucose; B. Intensity of SDF-1α expression at 254.05 arbritary units; C. Immunofluorescence staining of CXCR4 with Rhodamine in cultured EPC with administration of 22 mM glucose; D. Intensity of CXCR4 expression at 68.4 arbritary units.

4. Diagram of SDF-1α and CXCR-4 concentration intensity in 3 treatment groups.

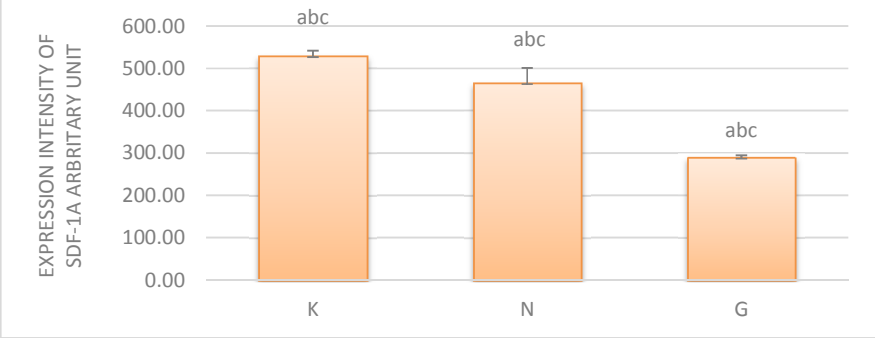


Figure 8. The SDF-1α intensity in each experimental groups. Values are presented as mean ± SD; [a]P < 0.05 in comparison with control (K) group; [b]P < 0.05 in comparison with D-(+)-glucose 5 mM. group; [c]P < 0.05 in comparison with D-(+)-glucose 22 mM; Statistical test results showed significant between the three groups

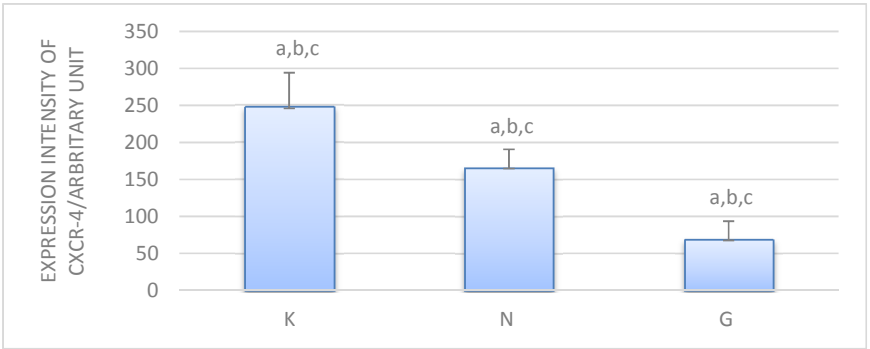


Figure 9. The CXCR-4 intensity in each experimental groups. Values are presented as mean ± SD; [a]P < 0.05 in comparison with control (K) group; [b]P < 0.05 in comparison with D - (+) - glucose 5 mM. group; [c]P < 0.05 in comparison with D - (+) - glucose 22 mM; Statistical test results showed significant between the three groups.

5. Nitric Oxide (NO) concentration of EPC medium (µM)

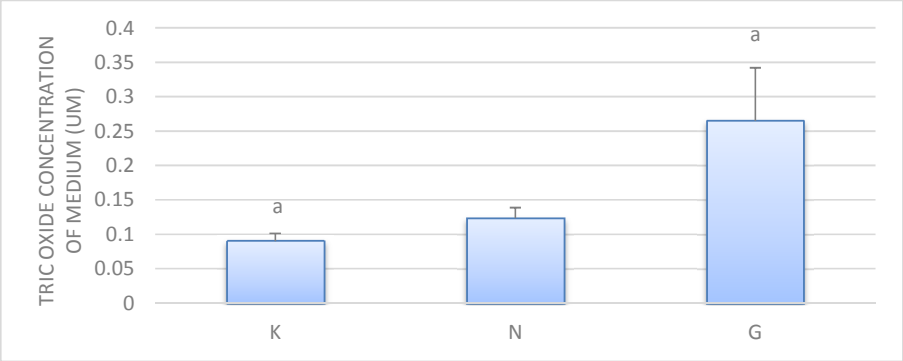


Figure 10. Nitric Oxide (NO) Concentration of EPC medium by the method of Nitric Oxide Assay Kit D2NO-100 (Bioassay System, USA). Cultured EPC with administration of N (D-(+)-Glucose 5 mM) and G (D-(+)-22 mM) glucose compared with the control group p≤0,05.

DISCUSSION

EPC is defined as clonogenic unipotent cell that can renew itself, proliferate and differentiate into mature endothelial cells, replace necrotic or apoptotic vascular endothelium [7]. Asahara et al., 1997 [8] firstly isolated cells expressing the antigen cluster of differentiation 34+ (CD34+) from peripheral blood in which the CD34+ is known as hematopoietic cell markers [8]. Morphologically, EPC of monocytes is spindle-shaped, resembling fibroblast cells. While EPC derived from bone marrow is cobblestone-shaped, resembling endothelial cells. Based its phenotype, EPC has surface antigens of CD34, CD133, kinase insert domain receptor (KDR) and vascular endothelial growth factor receptor-2 (VEGFR-2) [9]. Based on the functional characteristics, EPC can bind lectin that has the endocytosis of lipoprotein compound [10]. Characterization of EPC can also be known by absorbing ac-LDL in colony formation that has surface marker of VEGFR2, CD 31 [9].

The results of the study show the characteristics of EPC cells from previous studies. EPC has been isolated from peripheral blood as seen in figure 1 or EPC which is still in the medium and EPC which is already fixed with paraformaldehyde and Giemsa staining showed spindle-shaped morphology, which resembles fibroblast cells. The characterization with Dil-Ac-LDL and lectin showed that EPC isolated from peripheral blood absorbed Dil Ac LDL (red fluorescence in cells) as shown in Figure 2.A , Figure 2.B Binding of FITC-UEA-Lectin (Green fluorescence in cells), and Figure 2.C. Double Positive Dil-AC-LDL and FITC-UEA-Lectin (orange fluorescence in cells). Figure 3.A Characterization of the identified EPC with CLSM for vWF expression (Green fluorescence in cells), Figure 3.B. Characterization of the identified EPC with immunohistochemistry for vWF expression use ImmunoRatio analysis

Further detection was done with molecular markers of endothelial cells. In Figure 4.A,B,C the EPC cells isolated from peripheral blood expressed molecular markers of CD 133, CD 34 and VEGFR-2 whose fluorescence wavelength intensity was measured using flowcytometry. The results showed the average value percentages for the CD 133 = 77.43% of total 2974 cells, CD 34 = 77.48% of the total 2976 cells, and VEGFR-2 68.08% of the total 2615 cells.

The results of this study indicated that the APC cells isolated from peripheral blood of healthy humans have morphological characteristics and phenotype similar to EPC of the previous research results. After getting the normal characteristics of the EPC, EPCs were ready to be given a treatment to describe the endothelial dysfunction. The treatment was done by administration of the D-(+)-glucose (Sigma) at a dose of 5 mM and 22 mM in the culture medium of EPC. This treatment had been done by Khotimah., 2004 [6].

In this study, after the EPC cells are treated with D-(+)-glucose, the expression intensity of SDF-1α and CXCR4 are identified in which they are chemoattractants that may affect the the EPC mobilization, migration and homing. The results showed the expression of SDF-1α and CXCR4 in normal EPC cells without treatment of D-(+)- glucose by immunofluorescence examination which is shown Figure 5.A,B Immunofluorescence staining of SDF-1α with FITC in cultured EPC without administration of D-(+)- glucose (Sigma), Figure 5.C. Intensity of SDF-1α expression observed with CLSM 540.94 arbritary units. Figure 6.A Immunofluorescence staining of SDF-1α with FITC in cultured EPC by administering D-(+)-glucose 5 mM; Figure 6.B. Intensity of SDF-1α expression at 364.11 arbritary units; Figure 6.C. Immunofluorescence staining of CXCR4 with Rhodamine in cultured EPC by administering D- (+)- glucose 5 mM; D. Intensity of CXCR4 expression at 165.48 arbritary units. Figure 7.A. Immunofluorescence staining of SDF-1α with FITC in cultured EPC with administration of 22 mM glucose; Figure 7.B. Intensity of SDF-1α expression at 254.05 arbritary units; Figure 7.C. Immunofluorescence staining of

CXCR4 with Rhodamine in cultured EPC with administration of 22 mM glucose; D. Intensity of CXCR4 expression at 68.4 arbritary units. The results of this study indicate that the greater the concentration of glucose in cultured EPC, the lower the expression intensity of the SDF-1α and CXCR4. Statistic analysis with Anova SDF-1α intensity $^{a}P < 0.05$ in comparison with control (K) group; $^{b}P < 0.05$ in comparison with D-(+)-glucose 5 mM. group; $^{c}P < 0.05$ in comparison with D-(+)-glucose 22 mM; Statistical test results showed significant between the three groups. Statistic analysis with Anova CXCR-4 ; $^{a}P < 0.05$ in comparison with control (K) group; $^{b}P < 0.05$ in comparison with D - (+) - glucose 5 mM. group; $^{c}P < 0.05$ in comparison with D - (+) - glucose 22 mM; Statistical test results showed significant between the three groups.

Stromal cell derived factor 1 α (SDF-1α) is chemokine that attracts EPC from the bone marrow to the injury area or injury. SDF-1α induces EPC migration *in vitro* [12] and the bonding between CXCC-chemokine receptor (CXCR4) and SDF-1α is believed to increase the mobilization of EPC. SDF-1α also regulates proliferation and survival of EPC. SDF-1α and CXCR4 are a key regulation of EPC mobilization and recruitment [12]. EPC is believed to improve the integrity of the monolayer endothelium with occupies parts of injured endothelium or dysfunctional endothelium [7].

In the diabetes mellitus condition with hyperglycemia, there is a reduced activation of VEGF, SDF-1 α and bradykinin [2]. In vascular injury as a result of hyperglycemia in acute diabetes mellitus, there is an overexpression of SDF-1 α that may influence the mobilization of EPC through the CXCR4 receptor. It is an important process in vascular repair [17]. The protracted increase in blood glucose can cause chronic phase of diabetes mellitus and lead to complications [2]. The diabetes mellitus condition will add up kinase C protein (PKC), species reactive oxygen (ROS), proinflammatory and anti-angiogenic factor and results in a decreased Nitric Oxide compared with non-diabetic condition. Hyperglycemia may cause vascular injury response and repair in the area of vascular injury. The vascular injury response will secrete growth factors such as hypoxia inducible factor-1 α (HIF-1 α) and stromal cell derived factor 1 α (SDF-1α), which play a role in increasing proliferation, differentiation and migration of EPC from the bone marrow into circulation for repairs of fat tissue, blood vessel walls, especially adventitia of the spleen, liver and intestines. EPC migrates to the area of injury and makes a repair of blood vessels in angiogenesis mechanism [13,20].

In chronic diabetes mellitus condition, there is a decreased chemokine SDF-1 α which mediates phosphorylation of Akt, resulting in decreased mobilization of EPC [14]. EPC is found to play an important role in neovascularization and wound healing in diabetic patients. The protracted hyperglycemia in diabetes can lead to chronic microangiopathy. Hyperglycemia condition will reduce levels of SDF -1α so thereby hinder the process of neovascularization and diabetic wound healing process. EPC mobilization and homing occurs because of the bond between the G protein-coupled receptor CXCR4 and ligand SDF-1α; if this bond does not happen then mobilization of EPC will not occur [15].

The research results also show Nitric Oxide concentration in EPC culture medium. Results of analysis using the colorimetric method, Nitric Oxide Assay Kit D2NO-100 (Bioassay System, USA) indicate concentrations of NO in the medium with 22 mM glucose at the highest compared with EPC medium with 5 mM glucose and control group without glucose treatment (Figure 8). Cultured EPC with administration of N (D-(+)-Glucose 5 mM) and G (D-(+)-22 mM) glucose compared with the control group $p \leq 0,05$.

The results of this study are not consistent with the opinion [16] who state that the hyperglycemia will cause a decrease in phosphorylation of eNOS, which in turn will generate reduction in NO production which can affect the mobilization of EPC and EPC homing to the injury area.

This can occur in the acute diabetes where the early stage of hyperglycemia can increase the concentration of SDF-1α and VEGF and also enhance iNO. The increased iNO can increase some factors that influence the mobilization of EPC. However, in the chronic diabetes mellitus, there is a decrease in production of eNOS and NO in the bone marrow. Hypoxia will stimulate some transcription factors such as *transcription factor like hypoxia inducible factor 1* (HIF-1) whose activity can increase VEGF transcription. Furthermore, VEGF stimulates receptors VEGFR1 and VEGFR2 in endothelium and hematopoietic stem cells as well as increases matrix metalloproteinase-9 (MM) -9) and activates Kit ligand and induces the EPC proliferation and migration [7, 19].

Declaration of interest

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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