Hypoxic Preconditioning Effects of Bone Marrow-derived Culture Mesenchymal Stem Cells on CD31+ Expression, Vascular Endothelial Growth Factors-a (VEGF-A) and Stromal-deri

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Hypoxic Preconditioning Effects of Bone Marrow-derived Culture Mesenchymal Stem Cells on CD31+ Expression, Vascular Endothelial Growth Factors-a (VEGF-A) and Stromal-derived Sactors-1 Alpha (SDF-1α)

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Abstract. Increasing the ability of survival stem cells can be determined by assessing the state of hypoxic preconditioning (HPC) in stimulated cells. The assessment is carried out through bone marrow-derived stem (BMSCs) cultures obtained from the femoral bone marrow aspiration procedure (Wismar Rat). This study aimed to prove the hypothesis that there is CD31+ expression on HPC cultures; also, the concentration of VEGF-A and SDF-1α were higher in HPC than control. This research is designed as a laboratory experiment for a period of three months. Male Wistar rat (n = 16) was divided into 2 groups: P0 controls (21% [O2]) and P1 treatments (1% [O2]). Each sample was run into eight repetitions and observed for 48 hours. The stages in this study include isolation and culture of BMSCs, identification of phenotypic BMSCs based on the expression of cell-surface markers ($CD105^+$, $CD34^-$) and hypoxic exposure. After that, it was observed that the potential of angiogenesis in samples was based on $CD31^+$ expression, the paracrine activity of vascular endothelial growth factor-A (VEGF-A) and stromal-derived factor-1 Alpha (SDF-1α). The results showed that there is no $CD31^+$ expression on P1. A of VEGF-A and SDF-1 were higher in P1 than P0.

1. Introduction

Until now, the management of myocardial infarction has been still limited to revascularization and medical therapy, which aims to restore coronary blood flow and reduce the burden on the heart muscle. When the progression of the disease develops into terminal heart failure, only a heart transplant can be carried out as an effective therapy. However, its use is very limited due to a lack of organ donors and complications caused by immunosuppressive therapy. Some of the latest research in the tissue engineering field provides new hope in the treatment of myocardial infarction, taking advantage of pluripotent ability from stem cells, which is transplanted to repair and regenerate heart muscle tissue [1].

Stem cells are immature network precursor cells that can renew themselves and differentiate into various types of cells [2]. Mesenchymal stem cells (MSCs), also known as multipotent mesenchymal stromal cells, are cells that can renew themselves and present in several tissues and organs. MSCs have attracted much attention because these cells are easily isolated from bone marrow aspirates in minimal amounts and can be developed for clinical purposes both in vitro and also in vivo [3].

For several years, bone marrow-derived stem cells were one of them the primary sources of stem cells for the application of regenerative medicine. Bone marrow-derived stem cells have been used for myocardial regeneration and neoangiogenesis. However, bone marrow-derived stem cells consist of several heterogeneous cell groups, and there is still much controversy regarding the ideal subtype for cell therapy. Of all the multipotent cells found in the bone marrow, there are bone marrow-derived MSCs (BMSCs) that can differentiate into nerve cells, smooth muscle cells, and vascular endothelial cells. BMSCs are a promising source of stem cells for clinical applications such as tissue engineering and cell-based therapy [4][5].

Hypoxic preconditioning (HPC), a sublethal hypoxic state that can stimulate the endogenous mechanism of MSCs, is responded to by several cellular processes such as protein expression that can protect these cells from lethal hypoxia and other ischemic conditions. Liu et al. (2010)found that MSCs cultured with hypoxic stimulation of 1% or 3% will increase the expression of hypoxia-inducible factor-1 α (HIF-1α) which can increase the potential of MSCs in vitro obtained through increased paracrine effects [6]. In this case, the increasing of paracrine effects will increase the ability of stem cells' survival, and it also plays a role in angiogenesis obtained through the expression of several growth factors, one of them is vascular endothelial growth factor (VEGF), which also is proangiogenic [6]. However, VEGF impact will support differentiation of MSCs into endothelial cells, as evidenced by the expression of $CD31^+$ on the cell surface.[7] The increasing of VEGF expression then activates stromal-derived factor-1 (SDF-1) and its receptor, CXCR4, on the surface of cardiac stem cells (CSC). Activation of the SDF-1/CXCR4 axis may stimulate the ability of either MSCs migration and mobilization of their own cardiac progenitor cells (CPC) endogenous to the area of tissue infarct. The increasing of VEGF and SDF-1/CXCR4 axis stimulation, as well as endothelial differentiation by the effect of hypoxic preconditioning, means that the angiogenesis process will be further improved [7][8]. Angiogenesis potential improvement in MSCs is expected to optimize ischemic and infarct tissues repair and regeneration.

The role of MSCs that express VEGF, SDF-1α, and the ability of endothelial differentiation regeneration to repair cells has been widely developed but it still needs to be examined further, especially in terms of basic working mechanisms, synergistic between growth factors and also in the preconditioning technique. In this case, hypoxic preconditioning can increase its potential. This research is needed to find out the effect of hypoxia preconditioning (HPC) with 1% oxygen on the culture of bone marrow-derived mesenchymal stem cells (BMSCs) whether it will increase cell resistance and the potential of these stem cells for angiogenesis so that it will help repair and regenerate tissue if these stem cells are transplanted. This ability will be expressed by $CD31^+$ to show endothelial differentiation, an angiogenic activity played by vascular endothelial growth factor-A (VEGF-A), and the ability of cell migration performed by stromal-derived factor-1 α (SDF-1α). So, this study aims to prove the hypothesis that there is CD31+ expression on HPC culture. Besides, it is suggested that concentration of VEGF-A and SDF-1α were higher in HPC than in the control treatment.

2. Methods

The research was conducted in an experimental laboratory (*in vitro study*) at Stem Cells Laboratory, Institute of Tropical Disease, Airlangga University for three months. These studies include true post-test only experiments with a randomized factorial design and are randomized as the research design. Hypoxia exposure is given to mesenchymal bone marrow-derived stem cells (BMSCs) culture derived from bone marrow tissue of a healthy male rat (Wistar Rat) obtained by the procedure of bone marrow aspiration. The BMSCs were then multiplied in vitro to 16 units, then randomized to be divided into two groups, control group (P0) and treatment group (P1). Each group was performed with eight repetitions.

The next stage, which is the identification of phenotypic (flowcytometry) BMSCs, is carried out by looking at the expression of cell-surface markers ($CD105^+$, $CD34^-$). Then, the P1 group is given a hypoxia exposure with 1% oxygen concentration dose in vitro and normoxia exposure with 21% oxygen concentration to P0 group for 48 hours. Observation of the potential of angiogenesis in BMSCs that

have been given hypoxic exposure in vitro is seen from ;(1) differentiation of BMSCs into vascular endothelial-like cells based on the presence of $CD31^+$ by immunocytochemical methods; (2) angiogenic activity, based on an increase in VEGF-A paracrine activity by immunocytochemical and ELISA methods; and (3) the ability of stem cell migration, based on the paracrine activity of SDF-1α by ELISA methods. To analyze the presence or absence of $CD31^+$ expression (the measurement scale is nominal) in the treatment group, the chi-square test was used. In this study, we use SPSS 20 software to analyze the data. Data analysis on CD31+ was conducted using the chi square test, while VEGF-A and SDF-1α data expressions (measurement scale is numerical) were used for the multivariate test (Hotelling's Test-squared distribution).

3. Results

3.1 BMSCs isolation and culture

BMSCs isolation was taken from the femur bone of a Wistar male strain aged three weeks with a weight of 200 grams. After being isolated, these BMSCs were cultured and expanded to the third phase to maintain properties of BMSCs pluripotency and avoid further cell differentiation. Figure 1 shows a microscopic picture of the cell starting from the time of isolation to the third phase.

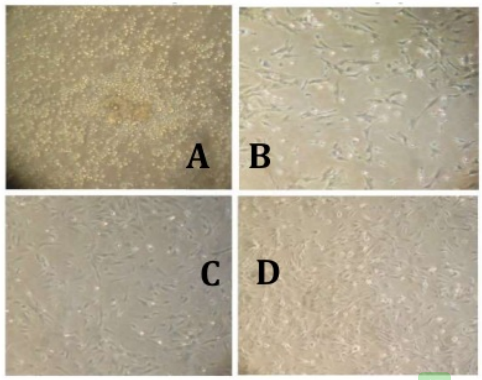

Figure 1: (A) post isolation of BMSCs – Phase 0; (B) Phase 1; (C) Phase 2; (D) Phase 3 BMSCs cultures

3.2 Characterization of BMSCs phenotypes

In this research, we use phase 3 BMSCs cultures taken from the bone femur of a male Wistar rat. Both treatment groups (normoxia and hypoxia) showed expression of $CD105^+$ and $CD34^-$ by flowcytometry examination (see figure 2). Based on the *quadrant statistics*, there is a positive expression of $CD105^+$ in the *upper left* area (UL) both at normoxia and hypoxia cultures, 14.21% and 29.75%, respectively. Therefore, $CD34^-$ shows a negative expression in the *lower left* area (LL) on both cultures.

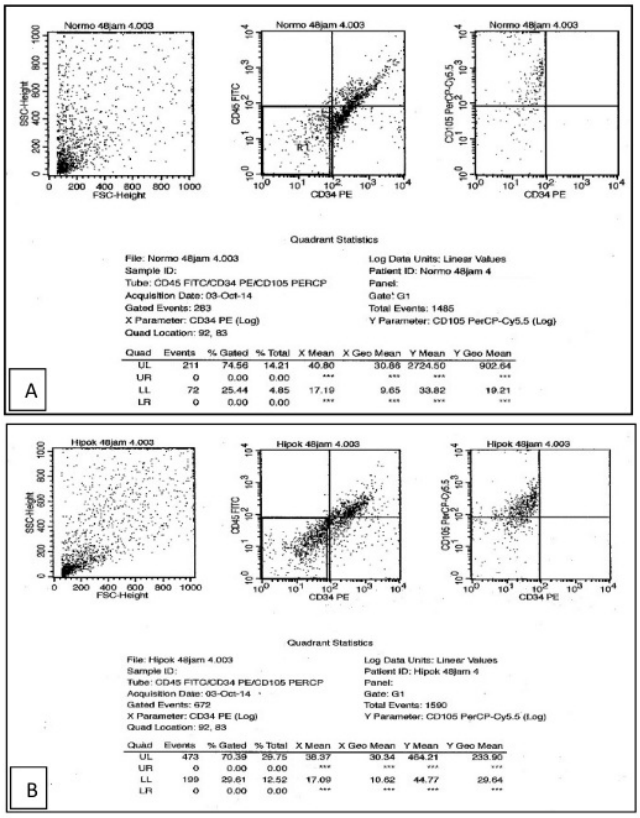

Figure 2: Phenotypes characterization BMSCs cultures; (A) normoxia; (B) hypoxia

3.3 Expression of CD31⁺

An immunocytochemical examination showed that BMSCs given hypoxia or normoxia exposure did not show CD31 expression which was characterized by the absence of green fluorescent BMSCs with immunofluorescence examination (see Figure 3)

3.4 VEGF Paracrine Activity

Stimulation of HPC causes stabilization of HIF-1 α which will form an active HIF complex. Then, along with the hypoxia response element, this will cause upregulation of several growth factors (IGF, bFGF, VEGF). This growth factor has a paracrine activity, which also acts as a survival factor, to act as an angiogenic factor, VEGF. In this study, VEGF expression is stronger at BMSCs with HPC stimulation than at normoxic condition. It was shown on immunofluorescence examination characterized by BMSCs which are glowing green (see Figure 4).

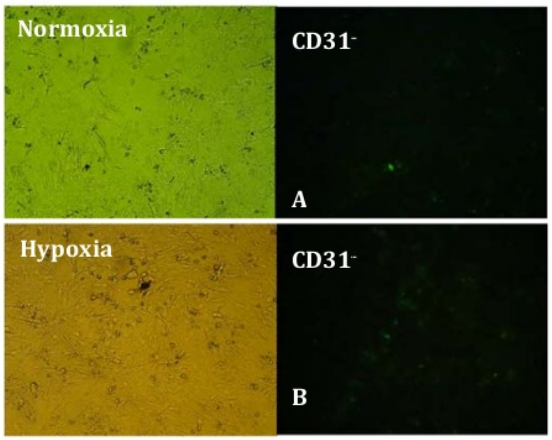

Figure 3: Immunocytochemical examination of CD31 expression with FITC staining.

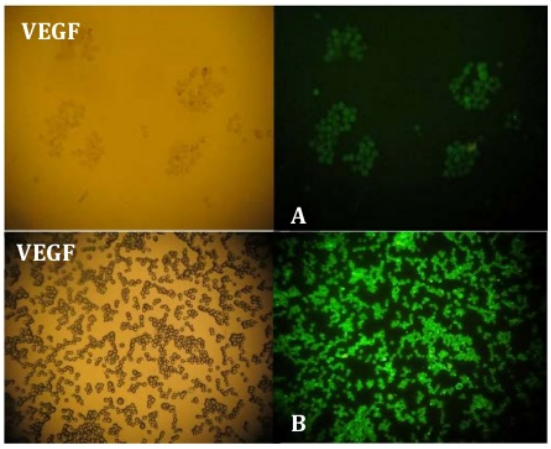

Figure 4: Immunocytochemical examination of VEGF expression in phase three BMSCs cultures

3.5 SDF-1α Paracrine Activity

Hypoxia condition is one of the main factors that stimulates neoangiogenesis and neovascularization, which are the primary molecules which play an important role is HIF-1α. In addition to affecting several growth factors, HIF-1α can also cause upregulation of several chemokines such as SDF-1, IL-8, MCP-1, and MCP-3 which enhance the migration and homing processes of PGCs towards hypoxic/ischemic areas.

The concentration data of both variables were tested for data normality, and it showed a normal distribution. It also showed very significant results ($p = 0.0001$; $r = 0.878$). However, the results of the data analysis showed that the mean levels of VEGF-A and SDF-1α in BMSCs cultures with HPC are significantly higher than normoxic condition cultures with $p < 0.0001$ (Table 1).

Table 1. Multivariate Analysis of Research Variables

Variable	Group		P value
	Normoxia O_2 21%	Hypoxia O_2 1%	
VEGF-A[a]	145.87 ± 7.15 pg/ml	182.69 ± 11.09 pg/ml	<0.0001
SDF-1α[b]	4.529 ± 0.313 pg/ml	9.694 ± 1.638 pg/ml	<0.0001

[a] vascular endothelial growth factors-a
[b] stromal derived factors-1 alpha

4. Discussion

The surface antigen in the cell marker indicates the nature or character of the cell. MSCs have many surface antigens so that the International Society of Cellular Therapy (ISCT) determines the minimum criteria for characterizing MSCs, most of which show positive expressions of CD73, CD90, and CD105, and negative expressions of CD11b or CD14, CD19, CD34, CD45, and HLA- DR.[9] Expression of $CD105^+$ markers, along with $CD73^+$ and $CD90^+$, is still the primary molecule to be used in identifying MSCs. In MSCs, this molecule is also strongly positively expressed and is not affected by the duration of culture or culture passages. [10] According to ISCT, negative expressions of CD34 and CD45 are also one of the minimum criteria in characterizing MSCs. However, CD34 expression would be negative if the MSCs were developed in plastic surfaces but would be positive if CD34 was identified in MSCs directly from the bone marrow [11]. This indicates that CD34 negative expression is based on plastic-adherence MSCs, which is also one of the minimum criteria for MSCs based on ICT. [11] In this study, $CD105^+$ expression was used as a marker for characterization of the MSCs phenotype because the expression is the primary molecule for MSCs identification. CD34- expression was used to indicate the characterization of the MSCs phenotype, and it also shows one of the criteria of MSCs plastic-adherence.

One of the most exciting debates about MSCs capacity is regarding the angiogenic potential. Although MSCs support this process through the release of angiogenic factors and proteases, the relevance of the ability of MSCs in endothelial differentiation is still being debated. The ability of MSCs to differentiate into endothelial lineages is shown by upregulation molecule surface cell endothelium, which are CD31, CD34, VEGFR1, VEGFR2, and vWF [12].

The strategy of using HPC in stem cell therapy has many advantages, for instance, increasing cell survival, increasing paracrine activity to create a supportive environment rich in tropic and angiogenic factors, increasing motility/migration and proliferative ability cells, and also improving cell differentiation according to functional integration. Hypoxia is an inductor of potential differentiation of stem cells and accelerates differentiation of MSCs when cultured under hypoxic conditions (5-8% O_2) compared to normoxia [13][14]. The ability of BMSCs to differentiate into cells from ectodermal and endodermal layers is inseparable from transcription factors HIF-1α.

The ability of BMSCs given hypoxic stimulation to differentiate into cells in the endothelial lineage has been demonstrated by Chacko et al. [15]. They used rat MSCs third phase in EGM-2 medium and stimulated 5% hypoxia O_2 for 24 hours. The results obtained were positive CD31 expression on immunocytochemical examination. This result is different from our study, which shows negative CD31 expression. Even though BMSCs cultures, both normoxia, and hypoxia, are in the same cell phase [15]. However, markers such as CD31 and CD34 will be expressed along with the increase of cultures time or endothelial maturation [16]. Thus, the longer the MSCs cell phase culture, the higher the tendency for MSCs to differentiate. This finding may explain the CD31 negative expression in our study, which was examined in the early phase.

Besides hypoxic stimulation, the ability of differentiated BMSCs to become endothelial-vascular like cells is also inseparable from the medium used and stimulant materials such as growth factors or special supplements [16]. It was seen in Siddiqui's study, who used the α-MEM medium with an

additional 50 ng/ml VEGF and 2% FCS in BMSCs culture without hypoxic stimulation where the results showed higher BMSCs ability for endothelial differentiation compared to BMSCs without stimulants [16]. In this study, the ability of BMSCs to differentiate between being endothelial-vascular like cells was only stimulated by using hypoxic exposure by expecting the paracrine effect of HIF-1α in the process of endothelial differentiation. However, this is not proven to be characterized by negative CD31 expression in hypoxic BMSCs cultures (see Figure 3). One possibility that can explain these results is because we use medium -MEM on second groups where this is different from Chacko et al. [15]. Besides that, in this research, we do not use any stimulants, for instance, VEGF and FCS, which can increase the ability of endothelial differentiation as in Siddiqui's research. However, the addition of VEGF in hBMSCs would significantly increase mRNA expression from CD34, VEGFR1, and VEGFR2 but not Tie -2 and vWF or CD31, which even decreased [17]. Similarly, in hBMSCs culture, together with VEGF, do not show the increase of CD31, vWF, or VEGFR2 [18].

One very interesting assumption regarding the potential for endothelial differentiation in HPC-BMSCs in our study is that the negative expression of CD31 shows the ability of HPC-BMSCs to maintain its pluripotency and undifferentiated state stabilization of these cells. The fact that supports this statement is from Boyette et al. which reported that HPC is a perfect way to expand MSCs however, can blunt the differentiation potential of MSCs so that it can affect its usefulness in regenerative tissue engineering [19]. A similar thing is stated by Basciano et al. who examined the effect of 5% hypoxia O_2 on BMSCs through gene expression in extracellular matrix preparation and epithelial, muscle, and nerve development [20]. They found that the duration of hypoxic culture was an essential parameter in terms of MSC differentiation capacity. Even in cultures that support growth, hypoxia enhances the genetic program that maintains these cells under undifferentiated conditions and remains multipotent. These controversial facts widen our insight into the potential of MSCs, which still need to be explored further, especially in terms of differentiation capacity.

VEGF characteristics are unique compared to other pro-angiogenic factors because of their potential and specificity in the vascular endothelium. This angiogenic growth factor is released in response to several conditions such as hypoxia. Hypoxia can regulate VEGF gene expression, and as previously explained, hypoxia can increase the stability of HIF-1α so that transcription of genes can increase the release of VEGF and VEGFR [21]. In our study, from ELISA examination, VEGF concentration higher in BMSCs given 1% hypoxia O_2 compared to normoxia O_2 21%, i.e. 182.69 ±11.09 pg/ml vs. 145.87 ±7.15 pg/ml with strong significance ($p < 0.0001$). Also, immunocytochemical examination showed VEGF expression in BMSCs with normoxia culture to be stronger after they were given hypoxic exposure, as seen in figure 4. This finding indicates that hypoxia exposure can stimulate VEGF release higher than normoxic conditions. Similar to Razban et al., 2006 who researched hBMSCs culture given 2% hypoxia O_2 exposure showed increased concentration of VEGF by ELISA on the supernatant HIF-1a - overexpressing MSCs. This study shows that genetic modification of MSCs by HIF-1a overexpression has the potential to improve the process of angiogenesis by the influence of hypoxia through paracrine and autocrine mechanisms.

Hoffman et al., who traced the angiogenic potential of the influence of HPC on BMSCs and fibroblast cultures in mice with the hindlimb ischemia model, concluded the same way as the researchers do [20]. In in vitro, BMSCs with hypoxic exposure showed higher VEGF paracrine activity than normoxia (281.1±62.6 pg/ml vs. 154.9±52.3 pg/ml). These BMSCs and fibroblasts were then injected into mice, and after three weeks, it was found that the BMSCs group had significantly higher vascular density than the fibroblast and control groups. This result shows that BMSCs that express more VEGF due to hypoxia exposure will have more potential in the process of angiogenesis.

In cell-based therapy, effective therapy is also influenced by the ability of migration transplanted stem cells to the tissue that is injured/ischemia, which then repairs and regenerates it. Short-term exposure to hypoxia has been shown to increase the ability of stem cell migration in vitro by modulating the expression of several cytokines and chemokine receptors. The bond between SDF-1 and its receptor CXCR4 (SDF-1/CXCR4) has a vital role during the process of neovascularization through regulation trafficking from PGCs and ECs. Research by Hung et al [6] reported that there

was upregulation of expression of CXCR4 and CXC3R1 at protein and mRNA levels when MSCs were cultured with 1% hypoxia O_2 exposure for 48 hours where they showed an increase in cell migration over a period of 14 hours with the Boyden chamber method in response to addition of SDF-1α/CXCL12 and fractalkine/CX3CL1. Although the process for assessing cell migration ability in our study was different and more straightforward, the results showed an increase in SDF-1α paracrine activity that was higher in BMSCs culture with 1% hypoxia O_2 exposure for 48 hours compared to normoxic culture via the ELISA method (9.694 ± 1.638 pg/ml vs 4.529 ±0.313 pg/ml with $p < 0.0001$). It is expected that HPC-BMSCs have more significant potential in the process of angiogenesis, especially in migration and engraftment of cells, shown by the increasing of paracrine expression and activity of SDF-1α. Zemani et al. have proven that in EPCs given SDF-1 exposure at dose 100 ng/ml for 30 minutes in a Matrigel, where precondition using SDF-1α can also increase the ability of cell migration, endothelial adhesion, and differentiation in the form of vascular tubes [23]. A study by Pasha et al. has also shown that preconditions of MSCs with SDF-1 can reduce the incidence of apoptosis, improve survival, homing/engraftment, vascular density, and improve myocardial function through SDF-1 / CXCR4 signaling [24]. All of this proves that SDF-1α has significant importance in the process of angiogenesis, especially in terms of migration and homing of cells.

5. Conclusion

There was no CD31 expression in BMSCs cultured with hypoxic preconditioning (O_2 1%). Paracrine concentration and activity of VEGF-A and SDF-1 were higher in BMSCs culture with HPC compared to normoxic conditions (O_2 21%).

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