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Research Article

The Effect of Culture Techniques of Hypoxic Stem Cell Secretome on The Number of Growth Factor TGF-ß, BMP-2, VEGF

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ABSTRACT

Background: Mesenchymal stem / stromal cell therapy (MSCs) is now an effective therapeutic modality for treating various diseases. In its application, stem cells require signaling molecules which can be growth factors, cytokines, or chemokines. Signal molecules work orderly and are greatly influenced by the physiological environment. Stem cell culture techniques with hypoxic conditions can produce growth factors close to physiological conditions in fractures. This study aims to perceive the different expressions of some growth factors in cultured normoxic and hypoxic BMSC.
Methods: This study is an in vitro laboratory experimental study of normoxic Bone Marrow Stem Cells (BMSCs) and Hypoxic Bone Marrow Stem Cells (BMSCs) cultures. The BMSCs experimental unit was taken from rabbits and then propagated in vitro and cultured under two conditions, normoxia and hypoxia. Then the number of VEGF, TGF-β, BMP-2 growth fractures was observed using ELISA.
Results: VEGF, TGF-β, and BMP-2 expressions showed significant differences between the normoxia and hypoxia groups. VEGF, TGF-β, and BMP-2 expression were higher in the hypoxia group compared with the normoxia group ($p < 0.05$).
Conclusion: The expression analysis of TGFβ-1, VEGF, and BMP-2 growth factors in cultured BMSC were statistically significant between normoxic and hypoxic conditions. TGFβ-1, VEGF, and BMP-2 expressions increase in hypoxic conditions.

Keywords: Hypoxic secretome; VEGF; TGF-β; BMP-2; Human and medicine

INTRODUCTION

Stem cell therapy/mesenchymal stem cell (MSCs) is an effective therapeutic modality for treating various diseases because of tissue's protective and reparative mechanisms.[1,2] In its development, stem cell therapy has been widely used in various fields of medical science, including orthopedics. A further understanding of bone healing and stem cells provides many opportunities for applying stem cell therapy in orthopedic cases, especially the fracture healing process.

Bone healing is divided into two, primary healing and secondary healing. Primary healing will develop with minimal callus appearance in installing rigid fixation and good contact between fractures. This process occurs because of the fracture area's low strain, thus forming new blood vessels through the Haversian system. Damaged bone will be resorption by osteoclasts and filled by osteoblasts as in a homeostatic process occurs in bone infection, bone tumors, and avascular necrosis.[3-5] In the fixation technique, which is relatively stable, a secondary healing process will occur through the withdrawal of stem cells that will receive the signal from growth factors (TGF-β, GDF-5, BMPs) to create cartilage which will later become an adequate bone structure and undergo remodeling. Most fractures healed by primary and secondary

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healing. These signal molecules work in an orderly and gradual manner and are strongly influenced by the environment and tissue conditions in the fracture area.[5-7]

In stem cell therapy, mesenchymal stem cells are used from various sources in the human body. This therapy follows the conditions of the stages and molecular processes of stimulation in the bone healing process; thus, the process can be accelerated. Research conducted by Friedenstein and then followed by Owen found that the iliac crest contains cells that can differentiate into osteogenic cells (osteoblasts). Since then, they have become known as bone marrow stem cells (BMSC). The therapeutic effect of stem cells was initially recognized by migrating stem cells to injured tissues and replacing dead cells. This mechanism was corrected by Gnecchi and colleagues, that MSCs mediate their therapy by releasing paracrine factors known as secretomes.[8] The MSC-Secretome is composed of bioactive molecules secreted as free soluble factors, including cytokines, chemokines, growth factors, and insoluble nano/microstructured-vesicles, known as extracellular vesicles (EVs).[9]

The physiological environment and tissue conditions strongly influence signaling molecules in the fracture area, where vascular damage occurs in the surrounding microenvironment, which causes relatively low oxygen levels (relatively hypoxic), so the stem cell culture technique is hypoxic—believed to produce growth factors close to the physiological state that occurs in fractures. This study is a preliminary study of producing freeze-dried secretomes which aim to perceive the different expressions of some growth factors in cultured normoxic and hypoxic BMSC. Stem cell therapy requires storage media not owned by every health center in Indonesia, so a comprehensive understanding of further research for the freeze-dried technique from secretome will facilitate the transportation and storage of medical colleagues in the distant area.

MATERIAL AND METHODS

This research is an experimental laboratory study in vitro of normoxic Bone Marrow Stem Cells (BMSCs) and hypoxic Bone Marrow Stem Cells (BMSCs) cultures with Randomized Control Post Test-Only Group Design. This research was conducted for three months at the Institute of Tropical Disease (ITD) and the Tissue Bank in our institution. The ethical committee in our institution has approved this study with certificate number 2.KE.129.07.2019. This study used 1 set of hypoxic chambers, a Class III Biological Safety Cabinet (BSC), a centrifuge equipped with a brake button, an incubator with 5% CO_2 humidity and 37°C temperature, and an inverted phase-contrast microscope super long working distance condenser (SLWDC). As for the research materials used for isolation reagents: -MEM with 1-glutamine; Fetal Bovine Serum (FBS) (Biowest, Cat. S1650); 1 Glutamine, 200 mM (nitrogen), solution used for isolation: Complete Culture Medium (CCM): 500 mL -MEM; 100 mL FBS (Final concentration ~16.5%: 6 mL 1-glutamine (final concentration 2 mM) and reagent for culture: low glucose -MEM (Sigma, Cat. M0894); 50 ml Fetal Calf Serum (FCS) selected for MSCs (Gibco/Invitrogen).

The experimental unit BMSCs were taken from 1 healthy male rabbit then propagated in vitro into and divided into two groups: treatment group 1 (P1) Bone Marrow Stem Cells (BMSCs) normoxic culture; and treatment group 2 (P2) Bone Marrow Stem Cells (BMSCs) with hypoxic culture. The conditioning results of each group were observed for the number of growth factors VEGF, TGF-β, BMP-2. Observation of the number of growth factors VEGF, TGF-β, BMP-2. They were carried out using the ELISA test.

The implementation of this research was divided into three research stages: First, the isolation of BMSCs from the bone marrow of healthy male rabbit strains of New Zealand. Second, culture BMSCs on culture plates with two treat-

ments: normal oxygen and hypoxic conditions. We only used one level of hypoxic, which is 5%, because the venous blood contains about 5% oxygen. Third, analyze the number of growth factors after differentiation of stem cells in VEGF, TGF-1, and BMP-2. The data collected will be statistically analyzed using the SPSS 24 program.

RESULTS

Bone marrow samples were taken and then isolated for 10-14 days, and a flow cytometry test was performed for CD 105 and CD 45 to ensure that the preparations were stromal stem cells. Cells from the obtained BMSCs were grown until the fourth passage. Media replacement was done two times per week. BMSCs were separated from the media using 0.05% trypsin/0.53 mM EDTA and replated and rearranged at a density of 10 x 8 cells/cm^2 in the same culture medium as the first section. The culture was separated into two treatments in the next stage: culture under normoxia and hypoxia, and secretomes were taken from each group. The number of growth factors TGFβ-1, VEGF, and BMP-2 was calculated.

The mean value of VEGF expression in the hypoxic group was higher than that in the normoxia group, 2663.89 and 1577.88, respectively. In this study, there was a significant difference between the mean VEGF expression between the normoxia and hypoxia groups (p=0.001). The mean value of TGF-β-1 expression in the hypoxic group was higher than that in the normoxia group, namely 83545.14 and 37960.14, respectively. In this study, there was a significant difference in the mean expression of TGF-β-1 between the normoxia and hypoxia groups (p = 0.000). The mean value of BMP-2 expression in the hypoxic group was higher than that in the normoxia group, 26969.84 and 16637.84, respectively. In this study, there was a significant difference between the mean BMP-2 expression between the normoxia and hypoxia groups (p = 0.003) (Table 1).

DISCUSSION

In the bone healing process, growth factors are produced and stimulated by Mesenchymal Stem Cells (MSCs) that migrate to the fracture area. Microenvironmental conditions in fractures that tend to be hypoxic stimulate the formation of growth factors VEGF, TGF-β1, BMP-2 for the bone healing process through the callus formation phase (secondary bone healing) or the cutting cone mechanism (Primary bone healing) process.

Oxygen tension plays an important role in regulating the expression of various genes. One of them is VEGF mRNA expression induced by low pO_2 exposure in various pathological states.[10] VEGF is the main target of transcription of Hypoxia-inducible Factor (HIF) through the VEGF receptor. This signaling helps heal tissue injury caused by hypoxic and inflammatory conditions. The result of this VEGF signaling is the occurrence of angiogenesis, increased blood flow, tissue perfusion, extravasation of inflammatory cells, remodeling, and tissue repair.[11] In this study, an increase in VEGF levels (2663.89 ng/L) in BM-

Table 1. Independent T-test analysis on the expression of TGFβ-1, VEGF, and BMP-2 between the normoxia and hypoxia treatment groups

	Group	N	Mean	p-Value
VEGF	Normoxia	6	1577.88 ± 433.09	0.001
	Hypoxia	6	2663.89 ± 385.65	
TGF-β-1	Normoxia	6	37960.14 ± 1581.49	0.000
	Hypoxia	6	83545.14 ± 6317.08	
BMP-2	Normoxia		16637.84 ± 711.91	0.003
	Hypoxia	6	26969.84 ± 452.52	

SCs cultured under hypoxia compared to BMSCs cultured under normoxia was 1577.88 ng/L. In this study, an independent t-test was also carried out. A significant relationship was found on the amount of VEGF, namely an increase in hypoxic conditions rather than normoxia conditions. These results align with research conducted by Yin et al., where there was an increase in VEGF levels in the autopsy results of patients with Congenital Heart Disease (CHD). In contrast, there was a decrease in oxygen levels in the blood in patients with CHD. The research conducted by Lin et al. also showed similar results. An increase in VEGF levels was found in human Nasal Polyp Fibroblasts culture under hypoxic conditions induced by Cobalt Chloride ($CoCl_2$).[12]

TGF-β is a multifunctional cytokine required for embryonic tissue development and adult tissue homeostasis.[13] TGF-β stimulates autocrine and paracrine structures important in maintaining and developing BMSCs. Bone and cartilage contain large amounts of TGF-β. TGF-ß stimulates osteoprogenitor proliferation, differentiation, and the formation of osteoblasts.[12] In this study, the results showed a significant relationship to the amount of TGF-β, an increase in hypoxic conditions than normoxic conditions. Hypoxic conditions reported in several studies can stimulate TGF-β in gastric cancer and increase the amount of TGF-β in fibrous tissue.[14] This is in line with a study conducted by Mingyuan et al., which found that the amount of TGF-β, both intracellular and secreted, was significantly higher in Human Foreskin Fibroblast (HFF) and Human Keloid Fibroblast (HKF) hypoxic conditions compared to those under normoxia.[15]

Bone morphogenetic protein (BMPs) is a derivative of growth factor-β and has a role in bone development and formation, tissue homeostasis, and tissue repair. BMPs and their derivatives are chondrogenic factors that stimulate cartilage tissue formation and matrix formation through chondrocyte cells.[16,17] In this study, the concentration of BMP-2 was increased in BMSCs cultured under hypoxic conditions. The average concentration of BMP-2 under normoxia was 16637.84 ng/L, while it was 26969.84 ng/L in hypoxia. In addition, this study also performed an independent t-test and found a significant relationship with the amount of BMP-2. i.e., there is an increase in hypoxic conditions than normoxic. The increase of BMP-2 amount is in line with the research conducted by Tseng et al. that hypoxic conditions increase the expression of BMP-2 in osteoblasts through a HIF-1α-dependent mechanism involving the activation of the ILK/Akt and mTOR pathways. Research conducted by Lafont et al. also showed similar results. There was an increase in the levels of BMP-2 and the products produced by BMP-2 under hypoxic conditions. Increased levels of BMP-2 were obtained through inhibition of the Smad pathway and activation of p38 MAPK.[14]

CONCLUSION

Expression of TGFβ-1, VEGF, and BMP-2 increased under hypoxic conditions. In addition, secretomes can be freeze-dried, so the storage of secretome would be easier and could facilitate doctors in health centers that do not yet have stem cells. In further research, hypoxia culture techniques can be used with the addition of Hydroxyapatite scaffold and Demineralized Bone Matrix to analyze the osteogenic secretome properties produced.

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