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Original 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 (MSC) therapy is now an effective therapeutic modality for treating various diseases. In its application, stem cells require signaling molecules, including growth factors, cytokines, and chemokines. Signaling molecules function in an orderly manner and are greatly influenced by the physiological environment. Stem cell culture techniques with hypoxic conditions can produce growth factors similar to those found in fracture conditions. This study aimed to evaluate the differential expression of growth factors in cultured normoxic and hypoxic bone marrow stem cell (BMSCs).
Methods: This in vitro laboratory experimental study examined normoxic and hypoxic BMSC cultures. BMSCs were harvested from rabbits, propagated in vitro, and cultured under normoxic and hypoxic conditions. Vascular endothelial growth factor (VEGF), Transforming growth factor-β (TGF-β), and Bone morphogenetic protein-2(BMP-2) levels were measured using ELISA.
Results: VEGF, TGF-β, and BMP-2 expression showed significant differences between the normoxia and hypoxia groups. The VEGF, TGF-β, and BMP-2 expressions were higher in the hypoxia group compared with the normoxia group ($p < 0.05$).
Conclusion: The expression of TGF-β1, VEGF, and BMP-2 growth factors in cultured BMSCs was significantly different between normoxic and hypoxic conditions. TGF-β1, VEGF, and BMP-2 expression increased under hypoxic conditions.

Keywords: Bone morphogenetic protein-2; Human and medicine; Hypoxic secretome; Vascular endothelial growth factor; Transforming growth factor-β

INTRODUCTION

Stem cell therapy/mesenchymal stem cell (MSC) therapy is an effective therapeutic modality for treating various diseases due to the protective and reparative mechanisms of tissues.[1,2] 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 in the fracture healing process.

Bone healing is divided into two types: primary healing and secondary healing. Primary healing develops with minimal callus formation when rigid fixation and good contact between fracture fragments are both achieved. This process occurs due to low strain in the fracture area, leading to the formation of new blood vessels through the Haversian system. Damaged bone is resorbed by osteoclasts and filled by osteoblasts, similar to the homeostatic process that occurs in bone infection, bone tumors, and avascular necrosis.[3-5] In fixation techniques that are relatively less stable, secondary healing occurs through the recruitment of stem cells that receive signals from growth fac-

tors (transforming growth factor-β (TGF-β), growth differentiation factor-5 (GDF-5), bone morphogenetic proteins (BMPs)) to create cartilage, which later develops into an adequate bone structure and undergoes remodeling. Most fractures heal by primary and secondary healing. These signaling molecules work in an orderly and gradual manner, 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 stages and molecular processes of stimulation in bone healing; thus, the process can be accelerated. Research conducted by Friedenstein and later Owen found that the iliac crest contains cells that can differentiate into osteogenic cells (osteoblasts).[8] These cells have since become known as bone marrow stem cells (BMSCs). The therapeutic effect of stem cells was initially attributed to the migration of stem cells to injured tissues and the replacement of dead cells. This mechanism was later revised by Gnecchi et al., who proposed that mesenchymal stem cells (MSCs) mediate their therapeutic effects by releasing paracrine factors known as secretomes.[9] The MSC secretome is comprised of bioactive molecules secreted as free soluble factors, including cytokines, chemokines, growth factors, and insoluble nano/microstructured vesicles known as extracellular vesicles.[10]

The physiological environment and tissue conditions strongly influence the signaling molecules in the fracture area. Vascular damage in the surrounding microenvironment causes relatively low oxygen levels (hypoxia). Therefore, hypoxic stem cell culture techniques are believed to produce growth factors similar to those found in the physiological state of fractures. This study is a preliminary study on the production of freeze-dried secretomes, aiming to evaluate the differential expression of growth factors in cultured normoxic and hypoxic BMSCs. Stem cell therapy requires storage media that is not available in every health center in Indonesia. A comprehensive understanding of further research on freeze-drying techniques for secretomes will facilitate transportation and storage, benefiting medical colleagues in remote areas.

MATERIAL AND METHODS

Study Design

This in vitro experimental laboratory study utilized a randomized controlled post-test-only group design to compare normoxic and hypoxic bone marrow stem cell cultures. The study was conducted over three months at the Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya, Indonesia and the Cell and Tissue Bank-Regenerative Medicine, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia. This study was approved by the ethical committee at our institution (certificate number 2.KE.129.07.2019).

Equipment and Materials

The study utilized a hypoxic chamber, a Class III biological safety cabinet (BSC), a centrifuge equipped with a brake button, an incubator with 5% CO_2 humidity and a temperature of 37°C, and an inverted phase-contrast microscope with a super long working distance condenser (SLWDC). The following materials were used for the isolation: α-MEM with L-glutamine, fetal bovine serum (FBS) (Biowest, Cat. S1650), L-glutamine, and 200 mM (nitrogen) solution. The complete culture medium (CCM) consisted of: 500 ml α-MEM, 100 ml FBS (final concentration ~16.5%), and 6 ml L-glutamine (final concentration 2 mM). The following reagents were used for the culture: low glucose α-MEM (Sigma, Cat. M0894) and 50 ml fetal calf serum (FCS) selected for MSCs (Gibco/Invitrogen).

Cell Source and Grouping

BMSCs were harvested from one healthy male New Zealand White rabbit and propagated in vitro. The cells were divided

into two groups: treatment group 1 (P1) - BMSC normoxic culture, and treatment group 2 (P2) - BMSC hypoxic culture.

Procedures

The study was conducted in three stages. First, BMSCs were isolated from the bone marrow of the rabbit. Second, BMSCs were cultured on culture plates under two conditions, normoxia and hypoxia (5% O_2, consistent with venous blood oxygen levels). Third, growth factor levels (vascular endothelial growth factor (VEGF), TGF-β1, and BMP-2) were analyzed using enzyme-linked immunosorbent assay (ELISA) after stem cell differentiation.

Statistical Analysis

The data was analyzed using SPSS version 24.0 (IBM Corporation, Armonk, NY, USA).

RESULTS

Bone marrow samples were collected and isolated over 10–14 days. Flow cytometry was performed for CD105 and CD45 to confirm the presence of stromal stem cells. BMSCs were grown until the fourth passage, with media replacement performed twice per week. BMSCs were detached using 0.05% trypsin/0.53 mM Ethylenediaminetetra-acetic acid (EDTA), replated, and cultured at a density of 10 x 8 cells/cm^2 in the same culture medium. The culture was then divided into two treatment groups: normoxia and hypoxia. Secretomes were collected from each group, and the levels of TGF-β1, VEGF, and BMP-2 were measured.

The mean VEGF expression in the hypoxic group (2663.89 ± 385.65) was higher than that in the normoxia group (1577.88 ± 433.09). A significant difference in mean VEGF expression was observed between the normoxia and hypoxia groups ($p = 0.001$). The mean TGF-β1 expression in the hypoxic group (83545.14 ± 6317.08) was higher than that in the normoxia group (37960.14 ± 1581.49). A significant difference in mean TGF-β1 expression was observed between the normoxia and hypoxia groups ($p = 0.000$). The mean BMP-2 expression in the hypoxic group (26969.84 ± 452.52) was higher than that in the normoxia group (16637.84 ± 711.91). A significant difference in mean BMP-2 expression was observed between the normoxia and hypoxia groups ($p = 0.003$) (Table 1).

DISCUSSION

In the bone healing process, growth factors are produced and stimulated by MSCs that migrate to the fracture site. The hypoxic microenvironment in fractures stimulates the production of VEGF, TGF-β1, and BMP-2, which are essential for bone healing through callus formation (secondary bone healing) or the cutting cone mechanism (primary bone healing).

Oxygen tension plays an important role in regulating gene expression. VEGF mRNA expression is induced by low pO_2 exposure in various pathological states.[11] VEGF is the main target of hypoxia-inducible factor (HIF) transcription, mediated through the VEGF receptor. This signaling pathway contributes to tissue repair in hypoxic and inflammatory conditions. VEGF

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 ± SD	*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	

signaling leads to angiogenesis, increased blood flow and tissue perfusion, the extravasation of inflammatory cells, remodeling, and tissue repair.[12] In this study, VEGF levels were higher in BMSCs cultured under hypoxia (2663.89 ng/L) compared to normoxia (1577.88 ng/L). An independent t-test revealed a significant increase in VEGF levels under hypoxic conditions compared to normoxic conditions. These results are consistent with the research by Yin et al., who observed increased VEGF levels in autopsy specimens from patients with congenital heart disease (CHD), a condition associated with decreased blood oxygen levels.[13] Similar findings were reported by Lin et al., who demonstrated increased VEGF expression in human nasal polyp fibroblasts cultured under hypoxic conditions induced by cobalt chloride ($CoCl_2$).[14]

TGF-β is a multifunctional cytokine essential for embryonic tissue development and adult tissue homeostasis.[15] TGF-β stimulates the autocrine and paracrine signaling pathways important for the maintenance and development of BMSCs. Bone and cartilage contain large amounts of TGF-β. TGF-β stimulates osteoprogenitor proliferation, differentiation, and osteoblast formation.[16] In this study, a significant increase in TGF-β levels was observed under hypoxic conditions compared to normoxic conditions. Previous studies have reported that hypoxia can stimulate TGF-β production in gastric cancer and increase TGF-β levels in fibrous tissue.[17] These findings align with a study by Mingyuan et al., which demonstrated that intracellular and secreted TGF-β levels were significantly higher in human foreskin fibroblasts (HFF) and human keloid fibroblasts (HKF) under hypoxic conditions compared to normoxia.[18]

Bone morphogenetic proteins are members of the transforming growth factor-β superfamily and play a crucial role in bone development, tissue homeostasis, and repair. BMPs and their derivatives are chondrogenic factors that stimulate cartilage tissue and matrix formation through chondrocyte activity.[19,20] In this study, BMP-2 levels were increased in BMSCs cultured under hypoxic conditions. The average BMP-2 concentration was 16637.84 ng/L under normoxia and 26969.84 ng/L under hypoxia. An independent t-test confirmed a significant increase in BMP-2 levels under hypoxic conditions. This increase in BMP-2 is consistent with research by Tseng et al., who demonstrated that hypoxia increases BMP-2 expression in osteoblasts through a HIF-1α-dependent mechanism involving the activation of the ILK/Akt and mTOR pathways.[21] Lafont et al. reported similar findings, showing increased levels of BMP-2 and its downstream products under hypoxic conditions, mediated by the inhibition of the Smad pathway and activation of p38 MAPK.[22]

CONCLUSION

The expression of TGF-β1, VEGF, and BMP-2 increased under hypoxic conditions. Secretomes can be freeze-dried for easier storage and transport, potentially benefiting healthcare providers in centers lacking access to stem cell therapies. Future research could explore hypoxic culture techniques with the addition of hydroxyapatite scaffolds and demineralized bone matrix to analyze the osteogenic properties of the resulting secretomes.

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None

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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