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Expression of Growth Associated Protein-43 (GAP-43) and Brain-derived Neurotrophic Factor (BDNF) from Adipose Mesenchymal Stem Cells (ASC) with fresh frozen nerve scaffold in 5% hypoxic condition

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ABSTRACT

Background: Peripheral nerve injury is one of the most common musculoskeletal injuries, and the healing is prolonged and affects the patient's quality of life. Secretomes derived from Adipose Stem Cells (ASC) is widely researched in tissue engineering. Various studies currently focus on observing potential scaffolds in nerve regeneration. This study aims to analyze the expression of Growth Associated Protein-43 (GAP-43) and Brain-derived Neurotrophic Factor (BDNF) produced by ASC culture with fresh frozen nerve scaffold under 5% hypoxic conditions in vitro.

Methods: An *in vitro* experimental laboratory research using the randomized control group post-test only design was conducted at the Dr. Soetomo Hospital Tissue Bank, Surabaya, East Java, Indonesia. Isolated mesenchymal stem cells from fat tissue were characterized using CD-105. If more than 90% of the MSCs membrane surface binds to the CD-105 marker is indicated positive. Hypoxic conditions were applied to culture stem cells with oxygen levels of 5% and several days of cultivation time until they reached passage four and passage eight. ELISA-test for GAP-43 and BDNF was carried out using secretomes obtained from ASC culture with fresh frozen nerve scaffold in normoxic (21%) and hypoxic (5%) conditions. The analysis was performed on the 2nd to 4th day and 6th day of culture using IBM® SPSS® program version 23 for Windows.

Results: In this study, there was a significant difference in BDNF expression in the group given fresh frozen nerve scaffold (p=0.040) but no significant difference in the expression of GAP-43 in the group given fresh frozen nerve scaffold (p=0.214) in normoxic conditions.

Conclusion: The 5% hypoxic medium did not provide a significant difference in the expression of GAP-43 and BDNF.

Keywords: Growth Associated Protein-43, Brain-derived Neurotrophic Factor, Fresh Frozen Nerve, Stem Cell, Hypoxia.
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INTRODUCTION

Peripheral nerve injury is a severe health issue that often causes permanent disability in patients.[1] Motor vehicle accidents contribute as the most frequent cause of nerve injury. Various attempts and studies have been carried out to find a therapy that gives optimal results for managing patients with peripheral nerve injuries.[2] Recovery of peripheral nerve injury improves the patient's quality of life.[1-3]

Recently, stem cell therapy for peripheral nerve injury has become a choice to accelerate nerve healing. Peripheral nerves have advantages over the central nerves because they have the ability to regenerate axonally and re-enter the target person.[4] If the nerve is intact, the myelin sheath wraps around the axon. When there is trauma to the peripheral nerve, a rapid process of tissue destruction occurs at the injury site. After several hours, macrophages in the body accumulate in the injured area. About one day after the nerve tissue is damaged, the Wallerian degeneration process begins. The axon tips in the injured area begin to unravel slowly, and Schwann cells start to produce growth factors. The growth factors produced are either nerve growth factor or brain-derived neurotrophic factor (BDNF). This growth factor also runs on the proximal nerve endings. The distal axon's tip slowly disintegrates by the presence of macrophages that eat from the dirt.[5] Growth factors that positively affect the growth of nerves during injury are nerve growth factor and fibroblast growth factor (FGF). These growth factors are significantly produced when new axons are formed in peripheral nerve injury. Apart from the growth factors above, brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF) also help in healing nerve injuries.[6] In addition to body factors that benefit peripheral nerves' healing process when an injury occurs, intracellular factors are equally important in this process. When a nerve injury occurs, intracellular proteins are metabolized, which helps in the healing process of peripheral nerve injury. One of the intracellular proteins

that have neurogenetic effects is Growth-Associated Protein-43 (GAP-43). This protein is produced by neuron cells as well as Schwann cells during peripheral nerve injury.[7]

Brain-derived neurotrophic factor (BDNF) is a growth factor that is crucial in nerve healing, especially in peripheral nerve injuries. The BDNF growth factor has more effect on healing motor nerves. This growth factor begins to play a significant role after the Wallerian degeneration process.[8]

GAP-43 is a specific calmodulin-binding protein known as Neuromodulin, P-57, F-1 or B-50.[7] This protein plays a role in healing injured axons. This protein moves to the ends of the axons that experience healing. The production of this protein is doubled during axonal growth. The GAP-43 protein is present in the cytoskeleton of the axonal growth area and interacts with the cell wall and actin filaments, giving axonal growth. In mice's experimental studies, there was an increase in the GAP-43 protein and the L-1 protein in healing from peripheral nerve injury. Schwann cells produce this protein, which also helps in axonal healing in peripheral nerve injuries.[7,9-11]

Stem cells are immature tissue precursor cells that can self-renew, have the ability to form clonal cell populations, and differentiate into several cell lineages.[12] Adult bone marrow Mesenchymal Stem Cells (MSCs) are a fibroblast-like population that can secrete growth factors and cytokines useful in hematopoiesis and other processes. A new type of biological regulation has emerged, which involves communication between cells through secreted substances, namely secretomes. Secretomes are known as a group of molecules/factors that are secreted into the extracellular region. Some examples of these factors are extracellular vesicles, soluble proteins, lipids and free nucleic acids.[12]

In various tissues, reduced oxygen pressure activates Hypoxia-Inducible Factor-1 (HIF-1), which induces the expression of angiogenic factors such as Vascular Endothelial Growth Factor (VEGF).[13] It has recently been discovered that the hypoxic condition of cell cultures has positive effects on MSCs.[14] It is known that most of the growth factors are regulated in various stem cells under hypoxic conditions.[15]

A previous study revealed that human dental pulp stem cells have a larger nucleus but a smaller cell than cells that grow in a normoxic environment. A study using dental-pulp mesenchymal stem cell culture in 5% O_2 significantly increased their proliferation and migration rate and the expression of stem cell markers and NGF, SOX2, BDNF, VEGF and SOX2.[16] These results show that a sufficient amount of O_2 concentration has the ability to enhance the growth and trait of MSCs, and the trophic effects of their secretions.

Currently, therapy for peripheral nerve healing has not been developed much, so the fresh frozen nerve scaffold is expected to provide neurogenic potential in peripheral nerves' healing process. Based on those mentioned above, this study aims to evaluate the expression of GAP-43 and BDNF from ASC with fresh frozen nerve scaffold in 5% hypoxic condition.

MATERIALS AND METHODS

Study Design

This research is *in vitro* experimental laboratory research using the randomized control group post-test only design conducted at the Dr. Soetomo Hospital Tissue Bank, Surabaya, East Java, Indonesia. This research was carried out by measuring the sample size using the unpaired numerical, analytical research sample formula $n = SD^2 (Z\alpha + Z\beta)^2 / (X1-X2)^2$, the minimum number of samples per group in this study was six samples. Fresh frozen nerves used in this study were pieces of human peripheral nerves obtained from surgery for nerve injury, with a length of ±0.5 cm and preserved in a refrigerator at -80 °C.

Stem Cell Preparation

Mesenchymal Stem Cells (MSC) were taken from the patient's fatty tissue during surgery by asking for the patient's prior consent. The fat tissue taken was 1 cm^3 in size, and it was confirmed that neither connective tissue nor blood contaminated the sample. The sample was then put into the transport medium and directly brought to the stem cell laboratory to be isolated. The fat tissue was then separated from the transport medium to be rinsed with a phosphate-buffered saline solution (PBS) until the fat tissue was clean from any red blood cells. The fat tissue was then finely cut until smooth, mixed with the collagenase enzyme, and poured into a bottle containing a magnetic stirrer. The bottle's tissue was then incubated on a hot plate at 37°C for 30 minutes until the fat tissue completely dissolved. After dissolving, the stopper medium was added and set for another 10 minutes until it became a homogeneous solution. The solution was then poured into a 50 mL conical, filtered using a sterile gauze to separate insoluble fatty tissue. The filter result was then centrifuged at 3000 rpm for 5 minutes until pellets form. The formed pellets were then resuspended using an alpha MEM medium to form a homogeneous solution. Following that, a 10 cm petri dish was used to plant the solution, and a CO_2 incubator was used to incubate the solution for 24 hours so cells would attach to the bottom of the petri dish. The adhered cells were replaced with the medium every two days until they formed colonies and grew to reach 80% confluent.

MSC that successfully grow into colonies can be reproduced to fulfil the dosage required for clinical applications. Cells that have developed a monolayer with up to 80% confluence need to be rejuvenated by doing passage. The passage is a process of removing the medium from the petri dish and then rinsing the monolayer layer with PBS solution. In order to separate the monolayer from the Petri base, the triple express enzyme was used, and the solution was incubated for an additional 5 minutes. After the monolayer was released, adding a medium stopper and conduct resuspension was necessary until it became a single cell. The solution with the single-cell was transferred into a conical tube and then centrifuged until pellets were formed. The pellets were then given Alfa MEM medium and then resuspended until a homogeneous solution was developed. The homogeneous solution was then planted in new Petri dishes.

Hypoxic Condition Medium Preparation

Hypoxic conditions were applied to culture stem cells with oxygen levels of 5% and several days of cultivation time until they reached passage four and passage eight. Cells from MSC obtained from phase 1 research were grown up to passage four and passage eight. Cells were then taken aseptically then placed in disk culture with a density of 2×10^7 cells/cm^2 on a 10^5 cm dish with IMDM medium containing 15% FBS, MSC simulator supplements and antibiotics (100 U penicillin/100µg/mL Streptomycin) at 37 °C, 5% CO_2. Maintenance of quiescence of BMMSC cells was carried out by administering hypoxic conditions at a dose of 5% O_2 concentration, namely by inserting the culture flask into a unique incubator for hypoxic conditions (Modular Incubator Chamber), which was cultured until the early passage (4th) and late passage (8th).

Stem Cell Characterization

Isolated MSC from fat tissue needs to be characterized using CD-105, a specific MSC marker. Flow cytometry and immunocytochemical techniques were used to examine labelled MSC. If more than 90% of the MSCs membrane surface binds to CD-105 markers are indicated positive. Meanwhile, CD 45, a specific marker for Haemopoietic Stem Cells (HSC), needs to be used to ensure that pure mesenchymal stem cells are isolated from the fat tissue. A negative test is indicated by the absence of colour radiance on the surface of MSCs on the reading of flowcytometry.

Sample Collection

The collected conditioning medium was then inserted into the dialysis tubing membrane as much as 50 mL. The dialysis tubing was tightly tied at both ends, then put in a 500 ml beaker filled with PBS solution in cold conditions. Next, the magnetic bar was inserted, and the beaker was placed on the hot plate magnetic stirrer. The stirrer was set at 500 rpm and let sit overnight until the conditioning medium's colour in the dialysis tube faded. After it faded out, it was removed from the PBS solution, and the dialysis tubing was cut using sterile scissors. The solution was poured into a 250 ml beaker, and then

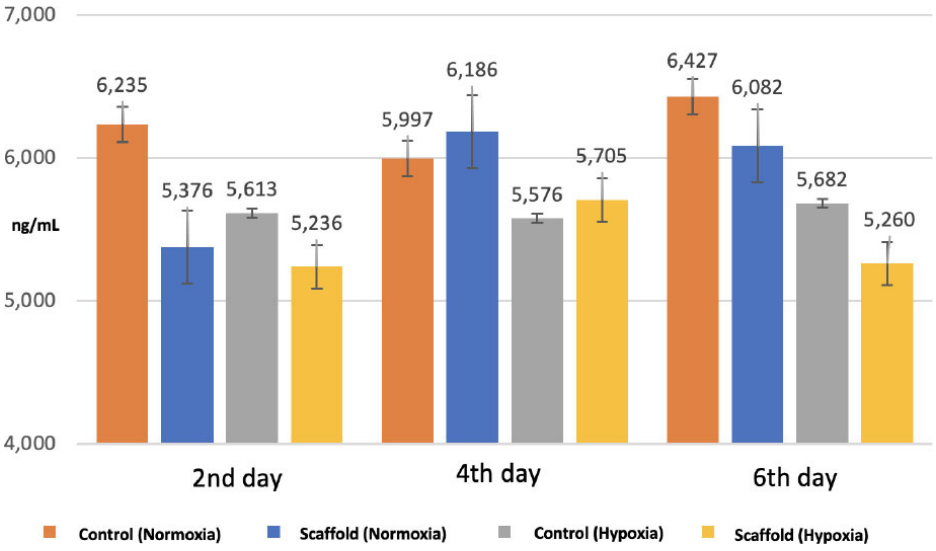

Figure 1. Expression of Brain-Derived Neurotrophic Factor (BDNF) on each group within the same day.

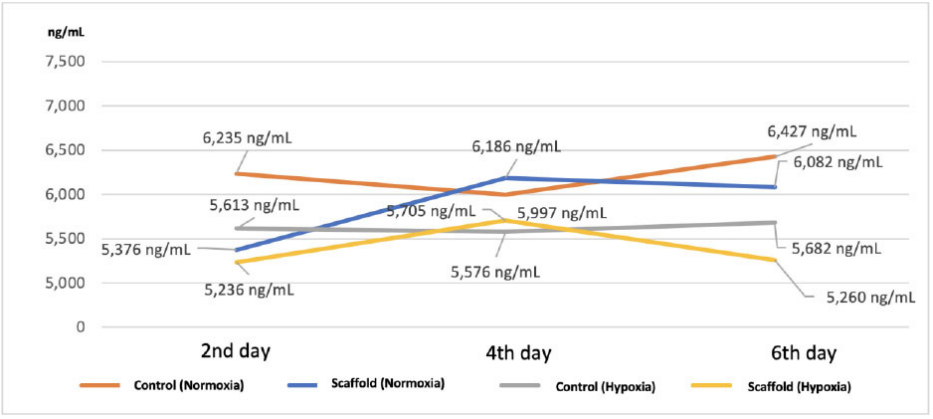

Figure 2. Expression of Brain-Derived Neurotrophic Factor (BDNF) on each day within the same group.

Table 1. Comparison of BDNF means in different days of measurement for each group.

BDNF group analysis	p
Control, Normoxia	0.488
Scaffold, Normoxia	0.040*
Control, Hypoxia	0.504
Scaffold, Hypoxia	0.163

metabolite products were filtered with a 0.22µ pack in 50 mL conical tubes. Lastly, the solution was put in a sterile medipack, sealed and stored at a temperature of -20 °C.

All reagents, samples and equipment were prepared. About 100 µL of initial reagent was added for the Bioassay-Technology Laboratory human BDNF kit to be tested and incubated for 1 hour at 37 °C. Aspiration was performed, and the solution was washed three times. The following 100µL reagent was added, namely the Bioassay-Technology Laboratory human GAP-43 kit. The solution was incubated for 30 minutes at 37°C. Aspiration was performed again, and the solution was washed another five

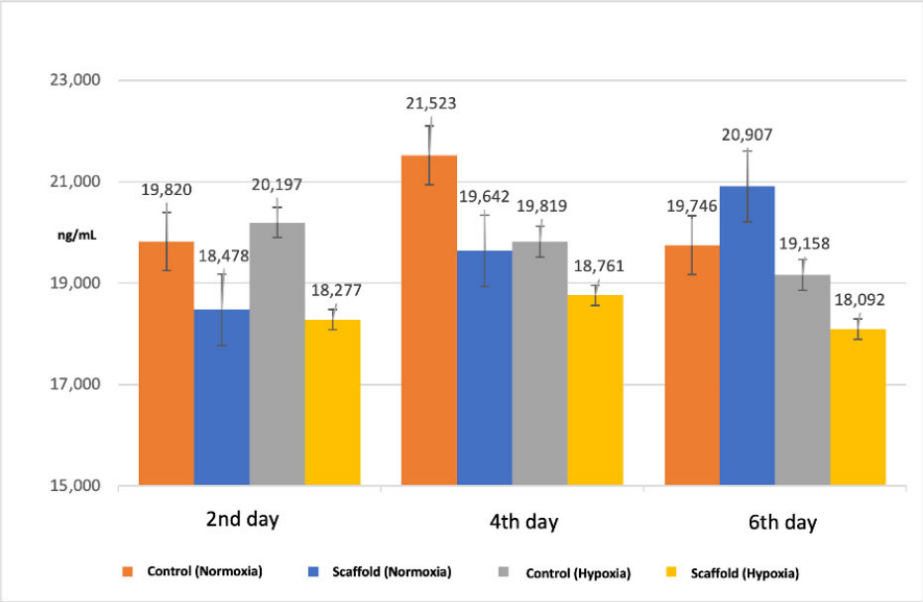

Figure 3. Expression of GAP-43 on each group within the same day.

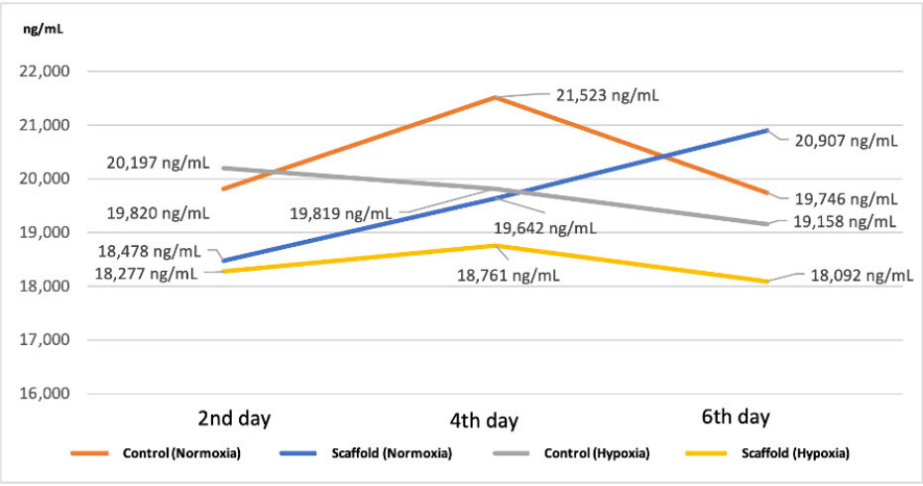

Figure 4. Expression GAP-43 on each day within the same group.

Table 2. Comparison of GAP-43 means in different days of measurement for each group.

GAP-43 group analysis	p
Control, Normoxia	0.366
Scaffold, Normoxia	0.214
Control, Hypoxia	0.661
Scaffold, Hypoxia	0.423

times. 90 µL of substrate solution was added and incubated for 10-20 minutes at 37 °C. Lastly, 50 µL of stop solution was then added to the primary solution. The results were carried out at 450 nm as soon as possible. Samples were collected on the 2nd, 4th, and 6th days. Samples will be analyzed with an ELISA reader Thermo Fisher Multiskan FC™ photometer to measure BDNF and GAP-43 expression concentration. The reader will detect the density of the bond between the protein marker and the protein target and will be converted into a concentration scale (ng/mL).

Data Analysis

Data were analyzed statistically with IBM® SPSS® program version 23. All data were tested for heterogeneity and normality to determine the significance test that will be used. Normally distributed data will use the One-Way ANOVA test and abnormally distributed data will use the Kruskal-Wallis test.

RESULTS

This study was conducted in vitro to analyze BDNF and GAP-43 in 4 sample groups, namely secretions without scaffold in normoxic conditions, secretions in normoxic conditions with fresh frozen nerve scaffolds, secretions without scaffolds in hypoxic conditions and secretions in hypoxic conditions with fresh frozen scaffold nerve. The analysis was performed on each sample on the 2nd, 4th, and 6th day. The results of the normality test on BDNF levels showed that the data were abnormally distributed on the comparison of day 2 and day 4 (p <0.05) and while data was normally distributed on the comparison between day 2 and day 6 (p >0.05) (Figure 1).

This study found the highest BDNF levels on day two in the group without fresh frozen nerve scaffold in normoxic conditions. The lowest BDNF level on day 2 was found in the group with fresh frozen nerve scaffolds in hypoxic conditions. A comparative test was carried out on all groups' levels on day two, and there was a significant difference between the groups (p=0.029). The Post Hoc test results between groups showed a significant difference in the group without scaffold compared to the scaffold group in normoxic conditions (p=0.013) and when compared with the scaffold group in hypoxic conditions (p=0.050).

The analysis results revealed that the highest BDNF level on day four was from the frozen nerve scaffold and normoxic conditions. The group without fresh frozen nerve scaffold and hypoxic conditions had the lowest BDNF levels compared to other groups. The comparative test results showed no significant difference in the mean BDNF levels on day four between each group (p=0.093).

BDNF level on day six was highest in the group without fresh frozen nerve scaffold and normoxia. The group that treated frozen nerve scaffold and hypoxic conditions had the lowest BDNF levels compared to other groups. The comparative test showed a significant difference in the mean BDNF levels on day six between each group (p=0.007). The Post Hoc test results between groups showed a significant difference in the group without scaffold in normoxic conditions compared to the group without scaffold in a hypoxic condition (p=0.025) and when compared with the scaffold group under hypoxic conditions (p=0.001). This analysis also showed a significant difference in the scaffold group in normoxic conditions compared to the scaffold group in a hypoxic condition (p=0.015) (Figure 1 and 2).

The analysis results suggest that BDNF levels in the group without scaffold fresh frozen nerves in normoxia condition were highest on day 4, as shown in Figure 2. The group without fresh frozen nerve scaffold and normoxia had the lowest BDNF levels on day four compared to the other groups. The comparative test showed no significant difference in mean BDNF levels in the group without fresh frozen nerve scaffold and normoxia between each day (p=0.488) (Table 1).

The analysis results reveal that BDNF levels in the scaffold fresh frozen nerve in the normoxia condition group were highest on day two. The scaffold fresh frozen nerves and normoxia group had the lowest BDNF levels on day 2 compared to other groups. The comparative test showed a significant difference in the mean BDNF levels in the scaffold fresh frozen nerve group and normoxia between each day (p=0.040) (Table 1). The Post Hoc test results on the scaffold group in normoxic conditions showed a significant difference on the fourth day compared to the group on the second day (p=0.020). In this analysis, there was also a significant difference in the group on the sixth day compared to the group on the second day (p= 0.038).

The analysis results in this study showed that BDNF levels in the group without scaffold fresh frozen nerves and hypoxia were the highest on day 4. The comparative test showed no significant difference in the mean BDNF levels in the group without fresh frozen nerve scaffold and hypoxia between each day (p=0.504) (Table 1). The group without fresh frozen nerve scaffold and hypoxia had the lowest BDNF levels on day two compared to the other groups.

The analysis results suggest that BDNF levels in the scaffold group of fresh frozen nerves and hypoxia were the highest on day 4. The comparative test showed no significant difference in the mean BDNF levels in the scaffold fresh frozen nerve and hypoxia group between each day (p=0.163) (Table 1). The scaffold fresh frozen nerves and hypoxia group had the lowest BDNF levels on day two compared to the other groups.

The GAP-43 analysis results showed that the highest levels on day 2 were found in the group without fresh frozen nerve scaffold and hypoxic conditions (Figure 3). The comparative test showed no significant difference in the mean of GAP-43 levels on day two between each group (p=0.051). The group given fresh frozen nerve scaffold and conditioned hypoxia obtained the lowest GAP-43 levels compared to the other groups (Figure 3).

The analysis results revealed that the highest levels of GAP-43 on the 4th day was found in the group without fresh frozen nerve scaffold and in normoxic conditions. The comparative test showed no significant difference in the mean of GAP-43 levels on day four between each group (p=0.315). The group given fresh frozen nerve scaffold and conditioned hypoxia obtained the lowest GAP-43 levels compared to the other groups.

The results of this study's analysis showed that the highest levels of GAP-43 on the 6th day was found in the group given scaffold fresh frozen nerves and normoxic conditions. The comparative test showed no significant difference in the mean of GAP-43 levels on the 6th day between each group (p=0.263). The group given fresh frozen nerve scaffold and conditioned hypoxia obtained the lowest GAP-43 levels compared to the other groups.

According to the results obtained, the GAP-43 levels in the group without scaffold fresh frozen nerves and in normoxic conditions were highest on day 2 (Figure 4). The comparative test showed no significant difference in the mean level of GAP-43 in the group without fresh frozen nerve scaffold and in normoxic condition between each day (p=0.366). The group without fresh frozen nerve scaffold and in normoxic condition obtained the lowest GAP-43 levels on day six compared to other groups.

The analysis results showed that the GAP-43 levels in the control group in normoxic conditions were the highest on day 2. The comparative test showed no significant difference in the mean level of GAP-43 in the group without fresh frozen nerve scaffold in normoxic conditions between each day (p=0.366) (Table 2). The group without fresh frozen nerve scaffold and in normoxic condition obtained the lowest GAP-43 levels on day six compared to other groups.

This study's results showed that the GAP-43 levels in the scaffold fresh frozen nerve group in normoxic conditions showed the highest levels on day 6. The comparative test showed no significant difference in the mean levels of GAP-43 in the scaffold fresh frozen nerve group in normoxic conditions between each day (p=0.214) (Table 2). The group without fresh frozen nerve scaffold in normoxic condition obtained the lowest GAP-43 levels on day two compared to the other groups.

The analysis results showed that the highest GAP-43 level in the group without scaffold fresh frozen nerves and hypoxic conditions was the highest on day 2. The comparative test showed no significant difference in the mean of GAP-43 levels in the group without fresh frozen nerve scaffold in normoxic conditions between each day (p=0.661) (Table 2). The group without fresh frozen nerve scaffold and in normoxic condition obtained the lowest GAP-43 levels on day six compared to the other groups.

According to the results, the highest level of GAP-43 in the scaffold fresh frozen nerve with hypoxia group was the highest on day 4. The comparative test showed no significant difference in the mean levels of GAP-43 in the scaffold fresh frozen nerve with hypoxia group between each day (p = 0.423). The scaffold fresh frozen nerves

with the hypoxia group had the lowest GAP-43 levels on day six compared to the other groups.

DISCUSSION

Impaired motor and sensory abilities are alarming conditions in patients with peripheral nerve injuries.[5] Even with the peripheral nerves' axon ability to regenerate, the healing ability of the nerves is generally inferior.[17] This has led to efforts to find alternative treatments for managing peripheral nerve injuries and various innovations related to this matter.[5]

One of the treatment options for peripheral nerve injury is stem cell therapy because of its ability to accelerate nerve healing.[4] Stem cells are precursors of immature tissue that have the ability to differentiate into several cell lineages, self-renew and form clonal cell populations.[12] A previous study found that stem cell transplantation effectively increased neurotrophic factors and restored function in acute brain injury.[18] In another study, it is indicated that stem cells have significant therapeutic potential in treating neurological and brain disorders such as ischemic stroke and bleeding.[19]

One of the neurotrophic that is important in the process of nerve regeneration is the BDNF. Apart from having a neuroprotective function, BDNF has a vital role in developing and connecting neurons and their ability to survive.[20,21] Besides BDNF, nerve cells also secrete a unique protein during injury, namely GAP-43. GAP-43 is an F-actin that acts specifically on the nervous system, which regulates phosphoproteins' expression during development, which causes different neurons to respond to extracellular signals.[22] A study by Aigner L et al., found that BDNF and GAP-43 were expressed simultaneously and could induce spontaneous nerve sprouting and neurite outgrowth.[23] These findings indicate that high levels of GAP-43 can induce the growth of axonal branches in injured nerves.[23] This has led to significant interest in BDNF and GAP-43 as treatment options for peripheral nerve injury.

In this study, an analysis of BDNF and GAP-43 excretion was carried out on secretions from adipose stem cells with fresh frozen nerves scaffold. The role of fresh frozen nerves as a scaffold in adipose mesenchymal stem cells is expected to be a neuroinductive factor that will stimulate the expression of growth factors and cytokines that play a role in the nerve healing process to improve the healing process of peripheral nerve injury.[24] Secretomes are an alternative in tissue engineering, a collection of factors produced by stem cells, which can be cell-free therapy.[24] In this study, secretions were obtained from cultures of human adipose-derived mesenchymal stem cells (ASCs). BDNF and GAP-43 levels were measured using ELISA and observed on day 2, day 4, and day 6.

There were four treatment groups for cell culture, namely the ASC group cultured without scaffold and in normoxic conditions, the ASC group cultured with scaffold in normoxic conditions, the ASC group cultured with scaffold in hypoxic conditions (5%), and the ASC group cultured with scaffold in hypoxic conditions (5%).

In this study, it was found that there was a significant difference in BDNF levels in the group given fresh frozen nerve scaffold in normoxic conditions compared to other groups. This result is similar to a study using bone marrow-derived stem cells. It was also found that the administration of stem cells significantly increased the production of BDNF, which is essential for healing from peripheral nerve injuries.[25] Another study also explained that the BDNF levels produced by stem cells significantly affect nerve healing. Still, this study also showed an increase in another growth factor that helped heal septic nerve injuries, namely BDNF. The combined effect of BDNF and GDNF on healing from peripheral nerve injury provide better healing effects than just one growth factor alone.[26] This is also in accordance with an article that explained that giving stem cells to nerve healing, especially if there is a defect size of more than one cm, provides good benefits for nerve regeneration in vivo. Administration of stem cells can be done in the form of gel or silk fibre.[27]

In this study, there was also no significant difference in the levels of GAP-43 in the group given fresh frozen nerve scaffold compared to those that were not. An experimental study using peripheral nerves from mice not subjected to nerve endings showed no increase in GAP-43 levels than nerves subjected to stress.[28] Another study using the sympathetic nervous system, which had Schwann cells, showed insignificant results. This study estimated that neurons in the central nervous system are still involved in their regulation, although Schwann cells themselves can produce GAP-43.[29] Another experimental study used a direct culture of Schwann cells for four weeks and significantly increased GAP-43 levels in immunohistochemical readings.[30]

This study also showed that there was no significant difference in BDNF levels in the hypoxic-conditioned group compared to those that were not. This study also found that there was no significant difference in GAP-43 levels in the hypoxic-conditioned group compared to controls with normoxic conditions. Previous research revealed that human stem cells using hypoxic conditions (2%) showed an increase in the differentiation rate up to 30 times faster within six weeks. There was an increase in protein Oct-4 and HIF-2a in culture development.[31] Experimental research using brain tissue in mice developed in conditioned media in 2% hypoxia gives a better neurogenetic effect than normoxia.[19] This is also supported by a journal article that stated that a hypoxic condition of 1-5% oxygen would positively affect neural stem cells to express growth factors such as BDNF, FGF, etc.[32] Another experimental study compared human stem cells in normoxic and hypoxic conditions of 5%, which was then viewed with an electron microscope with contrast showed better growth in 5% hypoxic conditions.[33]

CONCLUSIONS

This study shows the expression of GAP-43 in stem cells, given the neuroinductive scaffold is not stimulated, both under normal oxygen and hypoxic conditions. On the contrary, BDNF expression showed an increasing amount in stem cells given fresh frozen nerve scaffold in normoxic conditions but not in hypoxic conditions. In addition, the hypoxia level does not affect the expression of BNDF or GAP-43. Future studies are expected to research lower hypoxia levels to confirm further

the effect of hypoxic conditions on BNDF expression and GAP-43 expression.

CONFLICTS OF INTEREST

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

ETHICS CONSIDERATION

This study was reviewed and approved by the Ethical Committee of Dr. Soetomo General Hospital, Surabaya, with reference number 0059/KEPK/IX/2020.

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AUTHOR CONTRIBUTIONS

IPGPP is responsible for the ethical clearance procedure, improvement and correction of CRFs, research procedure, as well as data collection and analysis in Cell and Tissue Bank of Dr. Soetomo General Hospital, Surabaya, East Java, Indonesia. AAF and HS are responsible for the review and signing of data Sources & CRFs.

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