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Research Article

Characteristics of Umbilical Cord Derived Mesenchymal Stem Cell/UCMSC from *Macaca fascicularis* and Its Secretome Under Hypoxic Conditions

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Abstract

Mesenchymal Stem Cell (MSC) secretome has potential as a neuroprotective and neuroregenerative agent. It can have effects due to its paracrine factors, such as Brain Derived Neurotrophic Factor (BDNF) and Stromal-Cell Derived Factor-1 (SDF-1) which can be induced with hypoxia preconditioning. This compound may play a role in the treatment of neurological diseases. Stroke has become a neurological disease that contributes to high rates of mortality and morbidity worldwide. There have been several pre-clinical trials on animal stroke models using MSC secretomes from rats and humans, but no studies have been conducted on Non-Human Primate, such as *Macaca fascicularis*. This species has been widely used in biomedical research and part of it can be utilized for such studies which will reduce the cost of using human MSC. The results of this study, Umbilical Cord (UC)-MSCs of *Macaca fascicularis* have been successfully cultured and characterized in terms of phenotypic and differentiation. Hypoxia precondition was able to induce BDNF secretion up to 264 pg/mL and SDF-1 up to 666 pg/mL in the UCMSC secretome. Hypoxic preconditioning with 3% oxygen can induce the most optimal BDNF and SDF-1 secretion, compared to 1% and 5% hypoxia.

Keywords: *BDNF; Hypoxia; Macaca fascicularis; SDF-1; UCMSC Secretome*

1. Introduction

Mesenchymal Stem Cell (MSC) is a stem cell that is widely used in therapy, which can secrete growth factors (such as Vascular Endothelial Growth Factor (VEGF) including Brain-Derived Neurotrophic Factor (BDNF)), Chemokines (such as Stromal-Derived Factor (SDF-1)/ CXCL12, and CCL2), and Cytokines (such as interleukin-6 (IL-6), IL-8, Tumor Necrosis Factor Alpha (TNF-α)) (Cunningham et al., 2018). When MSCs are exposed to hypoxia condition, they adapt to the microenvironment by reducing the appearance of oxidative stress, changing their metabolism to glycolysis, and increasing their motility to tolerate hypoxia. This situation is also supported by the activation of the Hypoxia-Inducible Factor which can trigger direct and indirect pleiotropic effects (Pulido-Escribano et al., 2022). It makes growth factors, cytokines, and chemokines can be secreted 3-6 times more, thus increasing the function of proliferation, differentiation, migration, and inhibiting cell apoptosis (Cunningham et al., 2018).

The secretion of paracrine factors in the MSC culture medium is called the secretome. One particularly promising growth factor in secretome that functions as a neurotrophin is BDNF. This factor exerts its effects by promoting neuronal cell survival and differentiation through interaction with tyrosine kinase receptors (Liu et al., 2020). Another secreted protein that has potential as an adjuvant therapy is SDF-1. This protein has been shown to promote neuroregeneration after brain injury by stimulating the proliferation, differentiation, and migration of neural precursor cells. (Cheng et al., 2017). Many studies have been conducted to investigate the safety and therapeutic effects of the MSC secretome, particularly in diseases caused by central nervous system disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, Huntington's disease, ischemic stroke, spinal cord injury, and traumatic brain injury (Giovannelli et al., 2023; Pinho et al., 2020). Among the neurological diseases mentioned above, ischemic stroke stands out as it is the second leading killer disease worldwide (Johnson et al., 2019) and has become the number one cause of death in Indonesia (World Health Organization, 2024).

A stroke is a case of brain damage resulting from a non-traumatic circulatory disorder in the brain. This disease occurs suddenly, progressively, and rapidly due to thrombosis or embolism in the brain (Kementrian Kesehatan Republik Indonesia/Kemenkes RI, 2018)(Chance & Hickey, 1982). The treatment mainly aims to unblock the arteries and restore blood flow to the brain, which is usually given fibrinolytic agents to remove the thrombus, such as Tissue Plasminogen Activator (TPA) (Chester et al., 2019). In some patients, thrombolysis therapy is ineffective, necessitating the development of adjuvant therapy to enhance the efficacy of treatment. One such agent is neuroprotective and neuroregenerative, exemplified by MSC secretome (da Silva et al., 2023; Haupt et al., 2023).

The effects of MSC secretomes from both rats and humans have been seen through several pre-clinical trials on animal stroke models and prove MSC secretomes can improve nerve function, provide long-term neuroprotection effects, increase neurogenesis and angiogenesis, reduce the infarction zone in the brain, suppress nerve apoptosis, and improve motor recovery in animal models (Giovannelli et al., 2023). However, no studies have identified the types of growth factors, cytokines, and chemokines in MSC secretomes sourced from Non-Human Primates (NHP), such as the long-tailed monkey (*Macaca fascicularis*). These species are known to be organogenetically and genetically similar to humans but can minimize the ethical constraints encountered in using human samples (Mariya et al., 2019)(Krishnan et al., 2022). Moreover, this species has been widely used in biomedical research and part of it can be utilized for such studies which will reduce the cost of using human UCMSC. Based on the above, this study aims to isolate and culture *M. fascicularis* UCMSC and determine the effect of hypoxic preconditioning (1%, 3%, and 5% O2) on cultured UCMSC under optimal conditions on the concentrations of BDNF and SDF-1 in the secretome.

2. Materials and Methods

Macaca fascicularis fetuses were collected from PT Biofarma, Indonesia, which has obtained ethical approval with document number: 224K-Mon-Nef01 by the Laboratory Animal Welfare and Use Commission of PT Biofarma. All UCMSC Isolation And Culture procedures were performed in a Biological Safety Cabinet (BSC)-*Thermo Scientific* aseptically, using pre-sterilized equipment (Rinendyaputri et al., 2023). The Umbilical Cord of *Macaca fascicularis* was taken at 120 days gestation through sectio caesarea. UC was cut with a thickness of about 2 mm and placed on 6-well plates, given a culture medium containing MEM, 10 - 20% FBS, and 1% PenStrep, and then cultured in incubator at 37 ^{o}C and 5% CO_2. The medium was changed every 2 days.

Phenotypic characterization was performed by flow cytometry using a kit from BD Bioscience and Flow cytometer (*BD Accuri™ C6 Plus*) to see positive and negative CD markers (Rinendyaputri et al., 2023). While characterizing the differentiation of UCMSCs using a kit from *StemPro-Gibco* to see the ability to differentiate into Adipocytes, Osteocytes, and Chondrocytes (Widowati et al., 2022). Observation of differentiation into Adipocytes by looking at lipid droplets using *Oil Red O*, Osteocyte differentiation looking at Calcium deposits using *Alizarin Red S* and Chondrocyte differentiation looking at Calcium deposits by *Alcian Blue*.

Hypoxia pre-conditioning was performed in a two-gas incubator-*Thermo Scientific*. UCMSCs that had reached 80% confluence were added to 10 mL culture medium without serum and then placed in the incubator. The preconditioning time was calculated since the oxygen concentration had reached 1%, 3%, or 5%, CO_2 concentration 5%, and the temperature had reached 37 ^{o}C. Hypoxia preconditioning was performed for 48 hours. Secretomes were obtained by centrifuging the culture medium at 1,200 rpm for 10 min, then the supernatant was taken and filtered using a 0.22 μm syringe filter (Rinendyaputri et al., 2023).

BDNF and SDF-1 measurements were performed according to the instructions of the Human BDNF ELISA Kit- Cusabio (Catalogue No.: CSB-E04501h) and Human SDF-1 Kit- ELK Biotechnology (Catalogue No.: ELK1183) (Rinendyaputri et al., 2023). After each step in the ELISA kit was followed, the solution in the well was read using an ELISA reader-*Bio-Rad* at a wavelength of 450 nm, then the concentration of BDNF and SDF-1 was determined. The data obtained were analyzed using *IBM SPSS Version 23.0*. To compare the results between several hypoxia-treated groups and the control, *One-way ANOVA* was used to compare results between multiple hypoxia-treated groups and against controls, followed by a *Post-Hoc Least Significant Difference* test.

3. Results and Discussion
3.1 Results

Macaca fascicularis umbilical cord explants were cut and placed in a 6-well plate in the center so that the MSC growth area when cultured becomes wider. UCMSC growth was observed using an inverted microscope at 100 times magnification. M. fascicularis UCMSC has a fibroblast-like morphology, predominantly characterized by a short spindle shape (Figure 1).

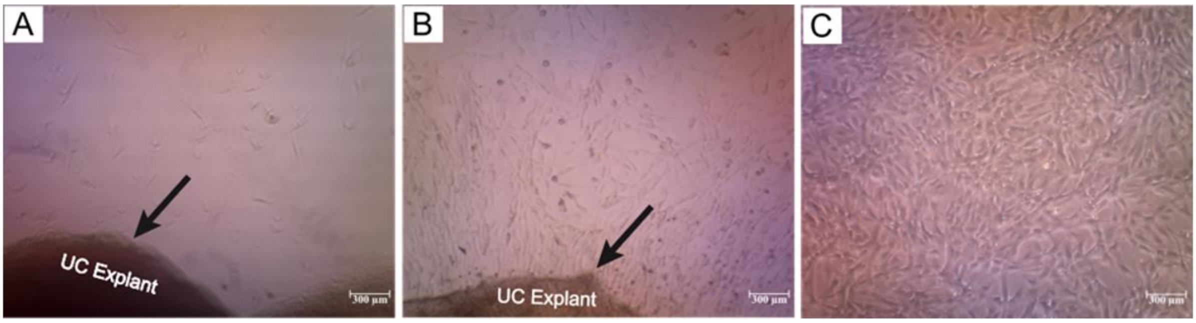

Figure 1. M. fascicularis Umbilical Cord (UC) Explant Day 9 (A). M. fascicularis UC Explant Day 14 (B). UCMSC after fifth passage (C)UC Explants are shown by arrows. After day 14, the growth of MSCs increased until MSCs filled the space of the well plate.

The characterization procedure for UCMSC was performed on cells from the 5th passage. Positive results were seen for CD73, CD90, CD105, and CD44 as indicated by the presence of light scattering readings in the right-upper quadrant of the 5th passage (Figure 2B). The negative CD marker cocktail reading confirmed the lack of CD34, CD11B, CD19, and CD45, as well as HLA-DR markers to differentiate MSCs from hematopoietic cell markers (Figure 2C). The characterized UCMSC can differentiate into the three cells indicated by being colored red for Adipocyte and Osteocyte Cells, while Chondrocyte Cells are colored blue (Figure 3).

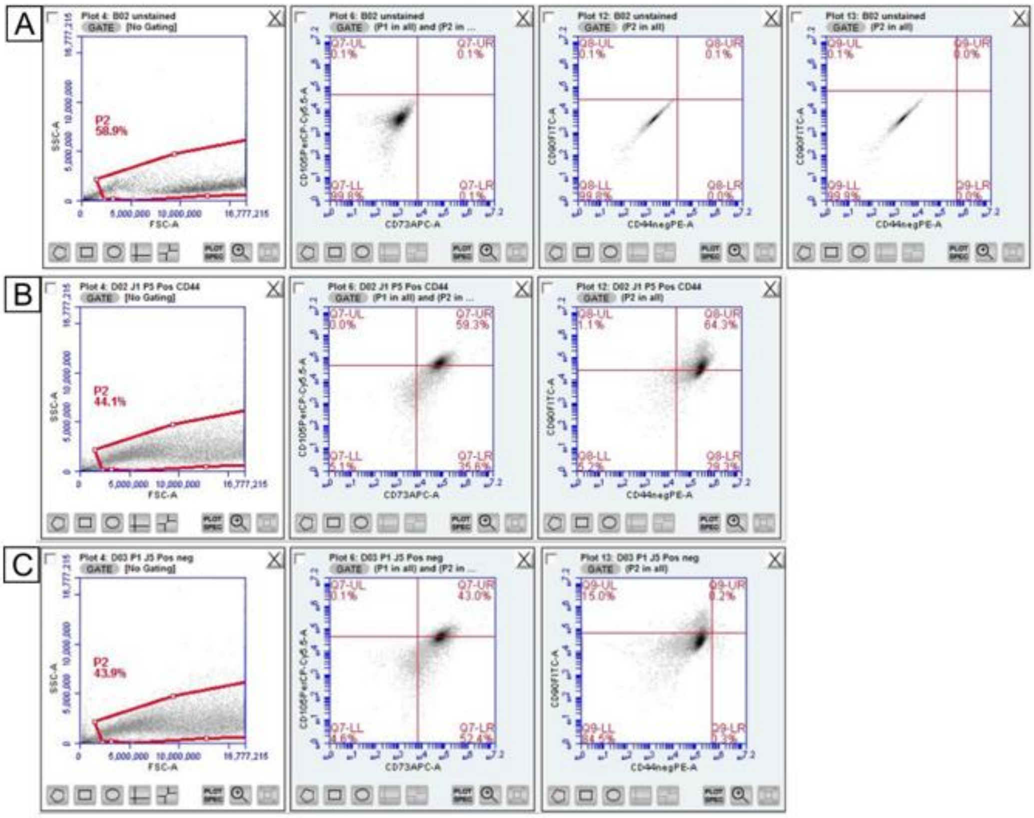

Figure 2. Reading of 5th passage unstained positive-negative marker cocktail (A). Reading of CD44 MSC Positive Marker 5th Passage (B). Reading of MSC Positive-Negative Marker 5th Passage (C).

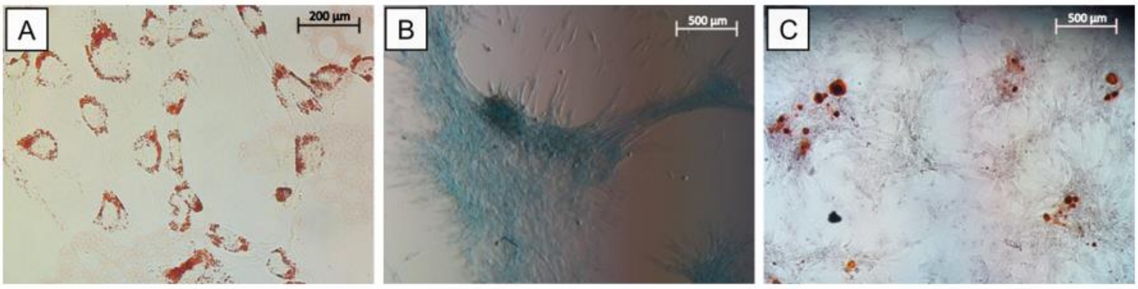

Figure 3. Results of UCMSC Differentiation Characterization. Differentiation of MSC Into Adipocytes (A), Chondrocytes (B), And Osteocytes (C).
Observation of adipocyte differentiation was performed at 100x microscope magnification, while chondrocytes and osteocytes were observed at 40x microscope magnification.

BDNF and SDF-1 profiles of UCMSC secretomes were examined using the ELISA method. Both standard curves used Exponential Association Regression, with the equation y=a(b-exp(-cx)). This equation has a correlation value (r) that is closest to 1.0000, so it is expected to provide a more accurate concentration calculation.

Based on the calculation results, hypoxia treatment was able to increase BDNF secretion up to 9 times and SDF-1 up to more than 100 times. BDNF and SDF-1 concentrations peaked in hypoxia conditions with 3% oxygen concentration. This concentration was higher than when using a lower (1%) or higher (5%) oxygen concentration (Table 1).

Table 1. The concentration of BDNF and SDF-1 in Hypoxia-Preconditioned UCMSC Secretomes

Sample	BDNF		SDF-1	
	Concentration Mean (pg/mL)	*Std. Dev*	Concentration Mean (pg/mL)	*Std. Dev*
Kontrol	28.0	-	6.0	-
Hypoxia 1%	181.5	6.36	517.0	36.77
Hypoxia 3%	264.0	16.97	666.0	11.31
Hypoxia 5%	244.3	12.06	267.5	38.89

The results of statistical analysis showed that there were significant differences between the treatment groups, except for the mean BDNF concentration between the 3% and 5% hypoxia treatments. Likewise, in the mean SDF-1 concentration, non-significant differences were only found between the mean concentration of 1% and 3% hypoxia treatment (Figure 4).

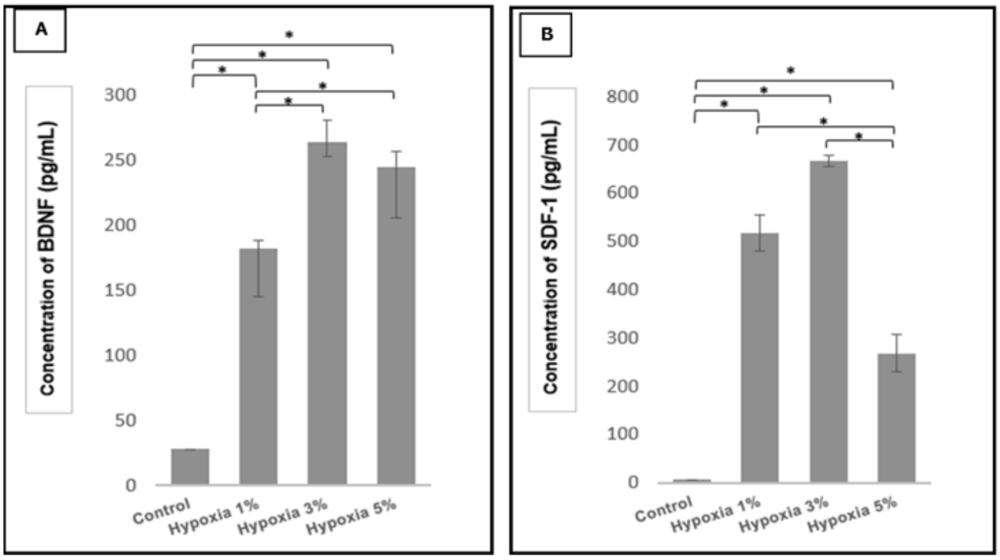

Figure 4. Concentration of BDNF (A) and SDF-1 (B) in *Macaca fascicularis* UCMSC Secretome with 1%, 3%, and 5% Hypoxia Preconditioning
* There is a significant difference between two treatment groups (P value <0.05)

3.2 Discussion

This study successfully obtained BDNF and SDF-1 at several concentrations in *M. fascicularis* UCMSC secretomes that had been cultured under optimum conditions with hypoxia preconditioning treatment (1%, 3%, and 5%). When associated with the cell culture stage, MSC sources from umbilical cord have better proliferation ability if compared to adipose tissue and bone marrow, and also show higher cell yield than sources from bone marrow (Mastrolia et al., 2019). The age of the MSC donor can also affect the protein composition of the secretome (Turlo et al., 2023), thus using sources from fetal tissue can further limit the age variable. In this study, the umbilical cord was taken from *M. fascicularis* at 120 days gestation.

The culture medium used is Minimum Essential Medium (MEM) because it has been widely used to culture mammalian cells. This medium contains a large amount of amino acids so that it can mimic the protein composition in mammalian cells. In addition, this medium also contains glucose, vitamins, and minerals to provide nutrients to cells during culture. However, this medium does not contain protein, fat, and growth factors, so it is often combined with the addition of Fetal Bovine Serum (FBS) (Theodoridis & Kraemer, 2023). MSCs that will be used are verified against the minimum fulfillment of MSC criteria standards set by International Society for Cellular Therapy (ISCT), this is also important in data exchange to compare the results of this study with other similar studies (Dominici et al., 2006). The results of phenotype characterization and multilineage differentiation ability showed that the cultured cells met the minimum requirements (Fig. 2A-2C). Hypoxia conditions were applied to 5th passage UCMSCs in MEM medium without serum (FBS) because serum also contains other protein components that can interfere when analyzing data analysis.

Oxygen is one of the most important factors in metabolic processes and cellular signaling (Nakazawa et al., 2016). During cell culture, controlling oxygen levels is an important thing to consider. In general, cell culture is carried out in a state of normoxia, which is at a concentration of ~21% oxygen, which is higher than in vivo (Kaneko & Takamatsu, 2012). Oxygen levels in the atmosphere drop to around 14.5% - 19.7% after being inhaled through the mammalian respiratory system, then to 13.2% when passing through the arterial blood until it reaches the tissues, where oxygen levels remain around 0.7% - 7% (Carreau et al., 2011). The physiological oxygen concentration of MSC is 2-8% (Mohyeldin et al., 2010) and the oxygen concentration in umbilical cord blood ranges from 1-6% (Sjöstedt et al., 1960). Oxygen levels below atmospheric concentration (21%) during cell culture are often termed hypoxia.

The results of hypoxia preconditioning treatment at 1%, 3%, and 5% oxygen levels in this study showed the highest concentration of BDNF was at 3% hypoxia which was 264 pg/mL. This result is relatively higher when compared to similar studies that show the concentration of BDNF from human UCMSC secretomes in conditions of 10% hypoxia for 48 hours which is 50 pg/mL, and around 120 pg/mL after being treated with hypoxia for up to 96 hours with 5% oxygen concentration (Sidharta et al., 2018). Another similar study using MSC from gingiva, with hypoxia treatment for 7 days at 0.5 - 1% oxygen concentration was able to secrete BDNF in the amount of 150 pg/mL (Patil et al., 2022). MSCs from human umbilical cord Wharton's jelly can secrete BDNF around 4,500 pg/mL with hypoxia at 2% oxygen (Majumdar et al., 2013). This concentration is much more than the BDNF obtained in this study. The difference with this study is in the source of MSCs used, from Wharton's jelly, the use of *Knock out-DMEM* medium, and the method of isolation by digestion instead of explants. When compared to the digestion technique, the explant technique has disadvantage in terms of contamination risk (Segeritz & Vallier, 2017).

Observation of SDF-1 concentration in this study also applies the same thing, where the highest concentration was obtained at 3% hypoxia, which amounted to 666 pg/mL. This amount is much more when compared to previous studies using MSCs from human Wharthon's Jelly tissue cultured in normal conditions without hypoxia preconditioning, which amounted to 2.0 pg/mL (Konala et al., 2020). There is also another study that used MSC source from human bone marrow and cultured in a transwell co-culture system with osteosarcoma cells grown in basal dishes, showing results slightly below this study, which was 600 pg/mL (Yu et al., 2015). SDF-1 in the secretome has also been obtained through 1% - 5% hypoxia preconditioning of human bone marrow MSCs that were concentrated 10 times using a protein concentrator and obtained an SDF-1 concentration of 850 ng/mL (Yang et al., 2023). The procedure of concentrating the secretome is a distinguishing factor that is thought to be the key to the high concentration of SDF-1 obtained compared to the current study.

Hypoxia conditions are known to affect the signaling of many cascades triggered by several transcription factors. Of the existing transcription factors, the Hypoxia Inducible Factor (HIF)-1α is a key factor that regulates cellular responses to hypoxia (Stamati et al., 2015). In the state of hypoxia, HIF-1α will form a heterodimer with HIF-1β which then binds to the Hypoxia-Response Element (HRE), which can affect the transcription of about 70 genes involved in various processes in cells, such as angiogenesis, metastasis, migration to cell death (Brahimi-Horn & Pouysségur, 2007). HIF-1α has the same signaling pathway as SDF-1, namely through the Phosphoinositide 3-Kinase (PI3K)/ Protein kinase B (Akt) pathway (Chen et al., 2013). The chemokine SDF-1 has several receptors to bind to, such as CXC Receptor (CXCR)-4 and CXCR-7 which play a role in cell migration. SDF-1 triggers PI3K/Akt signaling after binding to the CXCR-4 receptor (Haque et al., 2013). Activated Akt will trigger cell responses for proliferation, angiogenesis, migration, and survival (Chen et al., 2013). SDF-1 secreted in cerebral ischemic areas is also known to have a role in migration, proliferation, and prevention of cell apoptosis (Janowski, 2009).

The signaling pathway that occurs in BDNF, precisely mature-BDNF, also has a route that has a strong bond with the TrkB receptor. The phosphorylated TrkB will then activate several enzymes including PI3K and mitogen-activated protein kinase (MAPK) (Kowianski et al., 2917). In the PI3K/Akt signaling pathway, BDNF will modulate the synaptic plasticity of the N-methyl-D-aspartate receptor (NMDAR) which increases Ca2+ influx and is followed by the activation of cyclic-AMP response element binding protein (CREB) which ultimately triggers an increase in the expression of protein-coding genes that provide neuroprotective effects. While the MAPK / Extracellular-Signal-Regulated Kinase (ERK) signaling pathway can provide a protective effect on nerve cells from apoptosis (Zhao et al., 2017). BDNF works by minimizing cell death due to stroke, increasing neurite/axon growth and neurogenesis after stroke, and inducing neural plasticity after stroke (Liu et al., 2020). The limitation of this study is that we could not determine the type of BDNF specifically whether it is pro or mature BDNF, we only got the total BDNF value.

Conclusion

M. fascicularis UCMSC has a fibroblast-like morphology, predominantly characterized by a short spindle shape. In addition, these MSCs have a positive phenotype for CD73, CD90, CD105, and CD44 markers and are capable of differentiating into adipocytes, osteocytes, and chondrocytes. Hypoxia preconditioning treatment can induce the secretion of growth factor (BDNF) and chemokine (SDF-1) from *Macaca fascicularis* UCMSCs, where 3% hypoxia can induce the most optimal compared to 1% and 5% hypoxia.

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Authors' Contribution

AD designed and performed testing as well as drafted the manuscript. AD, RR, AM, and SN validate the methodology and tests. AD, RR, and AM conduct the manuscript preparation. RR and AM supervised the entire study. All authors revised and approved the final manuscript.

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