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The effect of preconditioning hypoxia in schwann-like-cells-derived adipose mesenchymal stem cells and rat sciatic nerve-derived stem cells: experimental research

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Background: The preconditioning hypoxia for stem cells is a strategy to achieve effective conditions for cell therapy, indicate increased expression of regenerative genes in stem cell therapy, and enhance the secretion of bioactive factors and therapeutic potential of their cultured secretome.

Objectives: This study aims to explore the response of Schwann-like cells derived from adipose-derived mesenchymal stem cells (SLCs) and Schwann cells rat sciatic nerve-derived stem cells (SCs) with their secretomes under normoxic and hypoxic conditions *in vitro*.

Material and methods: SLCs and SCs were isolated from the adipose tissue and the sciatic nerve of the adult white male rat strain Wistar. Cells were incubated in 21% O_2 (normoxic group) and 1%, 3%, and 5% O_2 (hypoxic group) conditions. Concentration values of transforming growth factor-β (TGF-β), basic Fibroblast Growth factor (bFGF), brain-derived neurotrophic factor, glial-derived neurotrophic factor, vascular endothelial growth factor, and nerve growth factor were detected and calculated utilizing an enzyme-linked immunosorbent assay, and the growth curve was described.

Results: SLCs and SCs indicated positive expression for mesenchymal markers and negative expression for hematopoietic markers. Normoxic conditions SLCs and SCs showed elongated and flattened morphology. Under hypoxic conditions, SLCs and SCs showed a classic fibroblast-like morphology. Hypoxia 1% gave the highest concentration in TGF-β and bFGF from the SLCs group and TGF-β, bFGF, brain-derived neurotrophic factor, and vascular endothelial growth factor from the SCs group. No significant differences in concentration of growth factors between the SLCs group compared to SCs group in all oxygen groups.

Conclusions: Preconditioning hypoxia has an effect on the composing of SLCs, SCs, and their secretomes *in vitro*; no significant differences in concentration of growth factors between the SLCs group compared with the SCs group in all oxygen groups.

Keywords: Adipose mesenchymal-derived stem cells Schwann-like cells, growth factors, preconditioning hypoxia, rat sciatic nerve-derived stem cells, secretomes

Introduction

The preconditioning hypoxia for stem cells has been proposed as a procedure to achieve effective conditions for cell therapy. This strategy represent comprehensively increased expression of regenerative genes in the therapy of stem cell, enhances the secretion of bioactive factors of the stem cells, and therapeutic potency of their cultured secretome[1–3].

The hypoxic condition is a vital physiological circumstance and pathological symptom that arranges a wide range of processes of cells besides signalling pathway transduction. mesenchymal stem cells (MSCs), including adipose tissues,

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remain in hypoxic microenvironments with low-oxygen tensions[4].

MSCs derived from adipose tissue are highly utilized as an origin of stem cells, in consequence of easily obtainable, abundant, and painless harvesting compared with other origins. These cells can be maintained and cultured for extended periods without losing their differentiation ability[5].

Research utilizing rat adipose tissue-derived mesenchymal stem cells indicates whether these cells have the capacity to differentiate into the glial lineage. Hence, they are an adequate potential source to be utilized as a substitute for Schwann cells in the process of neuronal regeneration. Adipose MSCs (AMSCs) differentiated to a Schwann cell-like phenotype/Schwann-like cells derived from adipose mesenchymal stem cells (SLCs/dAMSCs) *in vitro* were able to encourage neurite outgrowth *in vitro* and increase regeneration *in vivo*. The regenerative possessions of these cells are assigned to the secretion of neurotrophic factors, the competence to enlist Schwann stem cells to facilitate the regenerative activity, the direct subscription to myelin formation, and the competence to increase the viability of sensory and motor neurons[6–9].

Meanwhile, transplantation of autologous Schwann cells-/peripheral nerve-derived stem cells (SCs) results in morbidity, limited cell numbers, and potential for in-vitro expansion. Morbidity in the donor area and sacrifice of one or more nerve functions with loss of sensory function. Schwann cells have a long growth cycle and are difficult to propagate, so they are hard to use for clinical applications[10,11].

The goal of this study is to explore the response of SLCs and SCs with their secretomes under hypoxic and normoxic conditions *in vitro* by comparing the surface marker and the phenotype of the SLCs–SCs; and growth factor levels from their secretomes between normoxic and hypoxic conditions with 1%, 3%, 5% oxygen levels, respectively.

HIGHLIGHTS

- Preconditioning hypoxia for stem cells is an approach to create optimal conditions for cell therapy.
- Adipose mesenchymal stem cells persist in low-oxygen conditions known as hypoxic microenvironments.
- Schwann-like cells derived from adipose mesenchymal stem cells (SLCs) *in vitro* were able to encourage neurite outgrowth *in vitro* and increase regeneration *in vivo*.
- SLCs, Schwann cells rat sciatic nerve-derived stem cells, and their secretome responses in normoxic and hypoxic environment *in vitro*.
- Preconditioning hypoxia has an effect on the composing of SLCs, Schwann cells rat sciatic nerve-derived stem cells, and their secretomes *in vitro*.

Materials and methods

The experimental procedures, including animal laboratory, were conducted according to the ethics and research recommendations for animal care and use and were accepted by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine.

Isolation and culture of AMSCs

Isolation of the AMSCs from the abdominal fat region of the 3-month-old white male rats (*Rattus norvegicus* strain Wistar) and the culture process using the method according to Zuk and colleagues and Gimble and colleagues[12,13]. The adipose tissue acquired from the abdomen was cleansed with phosphate-buffered saline (PBS) to erase blood. Adipose tissue was chopped into 1×1 mm sizes and digested with collagenase I. Adipose tissue was separated mechanically for 80 min at 37°C using 0.1% collagenase I (Gibco). Then centrifuged, the suspension acted to discrete the floating adipocytes from the stromal vascular fraction. Cells in the normal vascular fraction were then preserved at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco) complemented with 10% foetal bovine serum (Gibco)/1% penicillin-streptomycin/2 mM L-glutamine, incubated in a humid atmosphere and 5% CO_2 at 37°C for 48 h. The medium consisting of the non-adherent cells was then erased and replaced with a fresh culture medium. AMSCs cells were passaged and used after passage 3–6 times. Immunofluorescence staining was performed for CD 14, CD 45, CD 90, and CD 105 on AMSCs from passage 3 to ensure the cellular specification of cultured AdMSCs cells[14,15].

Differentiation into SLCs

AMSCs were differentiated into SLCs using an established protocol by Kingham[16,17]. The Schwann-like cells were derived from white male rat AMSCs. In the third passage, the AMSCs cells were differentiated into a Schwann-like cell-differentiated AMSCs (SLCs/dAMSCs) phenotype in the first two steps. First, the growth medium was replaced with a medium complemented with 1 mM-mercaptoethanol (Scharlau Chemicals) for 24 h, followed by administration of 35 ng/ml all-trans-retinoic acid (Sigma-Aldrich) for 72 h. After that, the cells were managed with a differentiation medium containing a growth medium complemented with 5 ng/ml platelet-derived growth factor (PeproTech), 10 ng/ml basic fibroblast growth factor (bFGF) (PeproTech), forskolin 14 M (Sigma -Aldrich)) and 252 ng/ml neuregulin-1 (R&D System) for at least 14 days prior to characterization. The selection of added growth factors is based on their role in modulating the development and survival of Schwann cells[18]. The Schwann-like cell-differentiated AMSCs was confirmed by Glial Fibrilar Acid Protein (GFAP) and S100 protein expression immunocytochemical examination, and used in this study were cells at passages 3–6.

Isolation and culture of SCs

SCs were harvested from the sciatic nerve of the three-month-old white male rats. The sciatic nerve was cut ~1 cm, and the epineurium was detached. The sciatic nerve was chopped into small pieces about 1 mm long and separated by enzyme collagenase NB4 (2 g/ml; 17,454; SERVA) dispersed in DMEM/F-12 for 15 min at 37°C, centrifuged, and resuspended in DMEM/F-12 consisting 10% foetal bovine serum. The cells gained by this procedure were cultured in an incubator at 37°C under a 5% CO_2 atmosphere. Following 48 h, the supernatant was separated, and the cells were resuspended in 1 mg/mL collagenase NB4 to refine these cells from fibroblasts. Refined SC was utilized for all following experiments.

Preconditioning hypoxia

Preconditioning treatment of 1%, 3%, and 5% hypoxia (conditioned hypoxia) on Schwann cell-like cells derived from AMSCs, SCs, and their secretome preparation was gained with a well-characterized and controlled ProOx-C-chamber system (Biospherix, Redfield, NY) for 24 h with 5% CO_2, and N_2 at 37 °C[19]. After 24 h, the medium was collected.

Assessment of SLCs–SCs surface marker and SLCs–SCs phenotype

Each sample pellet was retained in differing tubes by PBS resuspended, subjected to the anti-rat CD 14, 45, 90, 105, and incubated at a temperature of 4°C for half an hour. Cells were cleansed with PBS. From each sample pellet, the PBS resuspended cell was retained in varying tubes and subjected to anti-rat CD 14-APC, anti-rat CD 45-APC, anti-rat CD 90-APC, and anti-rat CD 105-APC and antibodies incubated at a temperature of 4°C for a half hour. Cells were cleansed with PBS and were pelleted, labelled, and suspended again in the sheath fluid and subjected to flow cytometric analysis (Attune NxT Flow cytometer, Thermi Fischer Scientific). The difference between the isotype (control) and the sample positive staining degree was assessed in %.

Growth factor measurements

Growth factors [transforming growth factor-β (TGF-β), bFGF, brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF)] concentration measurement in each group secretome were done using an enzyme-linked immunosorbent assay according to the laboratory protocol (Bioassay Technology Laboratory), E1688Ra (TGF-β); E0341Ra (bFGF); E0476Ra (BDNF); E0351Ra (GDNF); E0659Ra (VEGF); and E0539Ra (NGF). The collection of the secretome followed the instructions provided by the manufacturer. Within 10 min after adding the stop solution to each well (Bioassay Technology Laboratory), the concentration optical density value was directly measured utilizing a microplate reader set to 450 nm and gain the concentration value for these growth factors.

Statistical Analysis

Data were served as mean and SD. Statistic analysis tests were utilized to measure differences between these groups. All analyses were done with SPSS 26.0 statistical package (IBM Corp.), and Tukey's post hoc tests were used to compare means with P less than 0.05 appraised as statistically significant and 95% CI. This work has been reported in line with the ARRIVE Criteria[20].

Results

SLCs–SCs surface marker expression and SLCs–SCs phenotype

Significant alterations were noticed in the cell-surface markers of CD 90 and CD 73 expression following 7 days of culture between the two groups. Characterization of SLCs and SCs for the expression of the cell-surface markers was carried out by flow cytometry. Cells indicated positive expression for principal mesenchymal markers CD 90 and CD 105 and negative expression for hematopoietic markers CD 14 and CD 45.

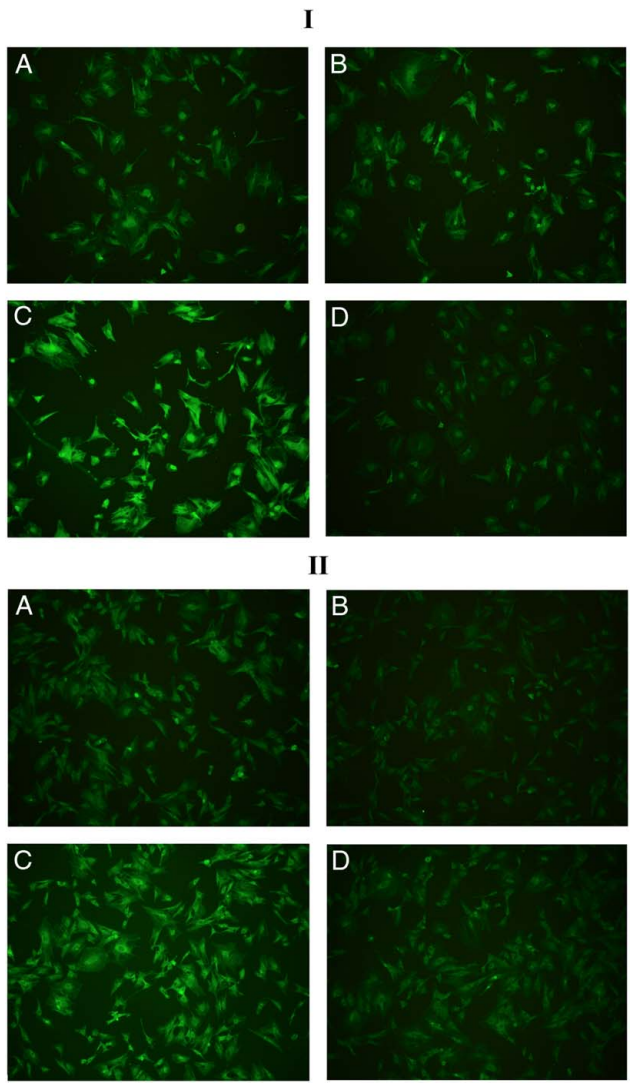

Figure 1. Immunofluorescence staining of SLCs (I) and SCs (II) for CD 14 (A), CD 45 (B), CD 90 (C), and CD 105 (D). SC, Schwann cells rat sciatic nerve-derived stem cell; SLC, Schwann-like cells derived from AMSC.

Characterization of SLCs for the cell-surface markers expression was performed with flow cytometry (Fig. 1). The cell exhibited positive expression (>90%) for principal mesenchymal markers CD 105 and CD 90 and negative expression (<5%) for hematopoietic markers CD 34 and CD 45.

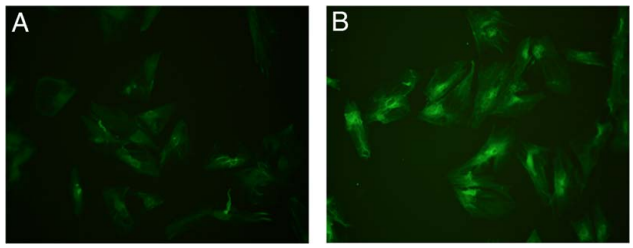

Figure 2. Immunocytochemistry examination of GFAP and S100 for SLCs. SLC, Schwann-like cells derived from AMSC. GFAP, Glial Fibrilar Acid Protein.

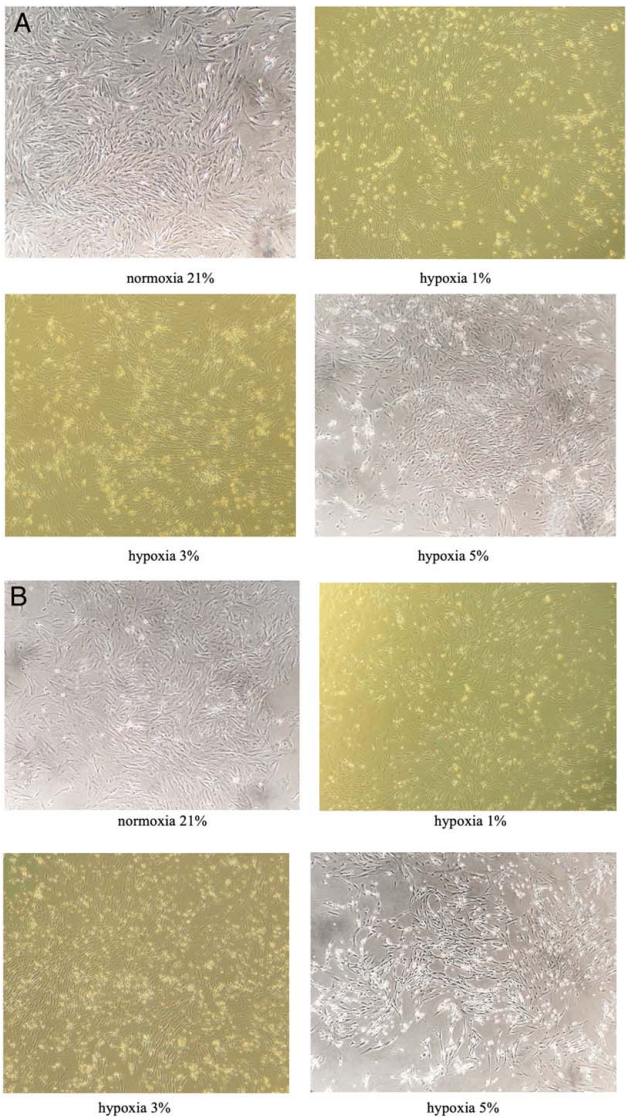

Figure 3. The morphology of SLCs (A) and SCs (B) with dendroid shapes or flat fusiform in a monolayer cell spacing, original magnification × 100. SC, Schwann cells rat sciatic nerve-derived stem cell; SLC, Schwann-like cells derived from AMSC.

The Schwann-like cell-differentiated AMSCs was confirmed by GFAP and S100 protein expression immunocytochemical examination. (Fig. 2).

SLCs and SCs show an MSCs-like phenotype following isolation and culture. Phenotypic characterization of these cells passages 3-6. SLCs and SCs-specific morphological and immunophenotypic changes were characterized in both hypoxic and normoxic populations. Hypoxic SLCs and SCs represent a classic fibroblast-like morphology. Under normoxic conditions, SLCs and SCs represent elongated and flattened morphology (Fig. 3).

Growth factor Measurements

The growth factors (TGF-β, bFGF, BDNF, GDNF, VEGF, and NGF) concentration of each group of secretomes from SLCs and SCs is shown in Table 1, (Fig. 4).

Table 1

The average concentration level of growth factors measurement (ng/ml)

	BDNF	GDNF	TGF-β	bFGF	NGF	VEGF
SLCs						
21%	1.25	1.355	130.05	57.485	196.9	312.41
5%	1.75	1.23	173.005	57.725	244.33	479.055
3%	1.635	1.295	153.105	53.575	270.545	409.495
1%	1.68	1.13	174.13	65.15	208.14	414.425
SCs						
21%	1.695	1.55	140.62	68.57	249.58	382.835
5%	1.715	1.045	161.45	65.105	284.13	443.41
3%	1.725	0.955	127.125	64.57	225.115	396.165
1%	1.91	1.245	188.91	77.93	264.49	483.985

AMSC, adipose-derived mesenchymal stem cell; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; GDNF, glial-derived neurotrophic factor; NGF, nerve growth factor; SCs, Schwann cells rat sciatic nerve-derived stem cells; SLCs, Schwann-like cells derived from AMSCs; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

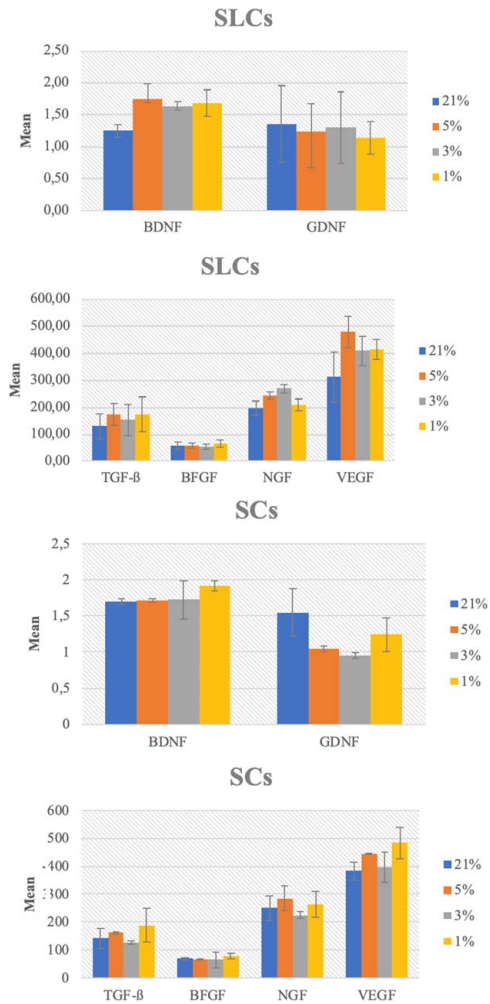

Figure 4. Bar chart of differences in growth factors concentration from SLCs and SCs between groups of 21% normoxia, 5% hypoxic, 3% hypoxic, and 1% hypoxic conditions. SC, Schwann cells rat sciatic nerve-derived stem cell; SLC, Schwann-like cells derived from AMSC.

Table 2

Comparison of growth factors levels (ng/mL) between SLCs and SCs

O_2%	SLCs (AMSCs)	SCs (sciatic nerve)	*P*
BDNF			
21%	130.05 ± 45.79	140.62 ± 35.89	0.655
5%	173.01 ± 41.19	161.4 ± 53.07	0.655
3%	153.11 ± 58.44	127.13 ± 5.37	0.655
1%	174.13 ± 63.19	188.91 ± 58.86	0.655
GDNF			
21%	1.36 ± 0.60	1.55 ± 0.33	0.317
5%	1.23 ± 0.44	1.05 ± 0.04	0.655
3%	1.30 ± 0.56	0.96 ± 0.04	0.655
1%	1.13 ± 0.25	1.25 ± 0.23	0.180
TGF-β			
21%	130.05 ± 45.79	140.62 ± 35.89	0.655
5%	173.01 ± 41.19	161.4 ± 53.07	0.655
3%	153.11 ± 58.44	127.13 ± 6.37	0.655
1%	174.13 ± 63.19	188.91 ± 58.86	0.655
bFGF			
21%	57.49 ± 12.89	68.57 ± 3.07	0.180
5%	57.73 ± 9.41	65.11 ± 1.70	0.180
3%	54.58 ± 8.73	64.57 ± 27.14	0.655
1%	65.15 ± 13.92	78.17 ± 11.72	0.180
NGF			
21%	196.90 ± 25.68	249.58 ± 44.19	0.180
5%	244.33 ± 13.97	284.13 ± 43.93	0.180
3%	270.55 ± 15.63	225.12 ± 12.68	0.180
1%	208.14 ± 22.15	264.49 ± 46.13	0.180
VEGF			
21%	312.41 ± 92.63	382.84 ± 32.38	0.655
5%	479.06 ± 58.61	443.41 ± 1.64	0.655
3%	409.50 ± 54.51	396.17 ± 53.69	0.180
1%	414.43 ± 36.07	483.99 ± 57.38	0.180

AMSC, adipose-derived mesenchymal stem cell; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; GDNF, glial-derived neurotrophic factor; NGF, nerve growth factor; SCs, Schwann cells rat sciatic nerve-derived stem cells; SLCs, Schwann-like cells derived from AMSCs; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor.

Subsequently, the growth factor concentration was compared between the SLCs and SCs groups. There was no statistically significant difference between these two groups for the six growth factors for all oxygen concentrations (Table 2).

Discussion

The existence of viable cell-based therapies to treat peripheral nerve injury provides promising hope for improving microsurgical outcomes, which are currently less than optimal for peripheral nerve restoration[21]. Cell transplantation is one of the cell therapies, and tissue engineering procedures are purposed at creating an approving microenvironment for tissue regeneration[22].

The restriction of stem cell curative efficiency is bad viability of transplanted cells in recipient tissue which have a nutrient-deficient and oxygen-deficient condition. Almost all transplanted cells will die within a few days following transplantation, resulting in very low cell viability. This is due to the extreme changing in the microenvironment. Therefore, it is critical to ameliorate cell engraftment efficiency prior to transplantation to increase stem cell therapeutic efficiency[23–25].

Some procedures were used to enhance the curative potency of AMSCs by conditioning the cells prior to transplantation. Preconditioning hypoxia represents comprehensively increased expression of regenerative genes in stem cell therapy[19]. Preconditioning performed by culturing mesenchymal stem cells in a hypoxic milieu, which looks like the native oxygen milieu of the tissues (1–7%) and not under basic culture circumstances (21%), will increase the viability of these cells[5]. Regarding the production of growth factors, preconditioning hypoxia increases the secretion of bioactive factors from the AMSCs and the curative potency of the cultured AMSCs secretome. It is a hopeful procedure to enhance the resistance to ischaemia and refine their curative potency[23,26].

The presence of hypoxic preconditioning indicates the expression of neuronal markers derived from differentiated neuron-like cells from AMSCs. Conduits installed to connect the gap between the sciatic nerves by administration of hypoxic AMSCs also demonstrated the degree of gastrocnemius muscle recovery in animal models. In addition, it was found that the percentage of myelin fibres that were regenerated from hypoxic AMSCs was higher than normoxic ones. In-vitro study, hypoxic preconditioning increased the potential for AMSCs neuronal differentiation. Whereas studies in experimental animals treated with hypoxic AMSCs showed similar results, which strengthens that hypoxic preconditioning increases the potential for AMSCs neuronal differentiation *in vitro* and increases the therapeutic potential *in vivo*[27].

Some growth factors were believed to have responsibilities for peripheral nerve regeneration, including TGF-β, bFGF, BDNF, GDNF, VEGF, and NGF. These are the key growth factors when the peripheral nerve has damage and followed by regeneration.

BDNF, GDNF, and NGF are affluent just in the nervous system, whereas bFGF, VEGF, and TGF-β serve on a number of cell varieties throughout the body besides the nervous system[28,29]. Research conducted by Su and colleagues in 2009 and 2011 indicated that neural progenitor cells have the ability to compose BDNF, NGF, and GDNF. This ability of these cells can be obtained *in vitro* and *in vivo*[30].

Hypoxic preconditioning has been shown to increase paracrine secretion of neuroprotective, regenerative, and angiogenic factors (such as BDNF, NGF, neurotrophin-3, GDNF, and VEGF) having higher relevance to peripheral nerve regeneration[31].

This study found that oxygen levels in hypoxic conditions (1%, 3%, 5%) gave some of these growth factors the highest concentration. However, most tissue cultures were maintained at an oxygen level of 21% *in vitro*. A comparison SLCs and SCs in terms of growth factors concentration was examined in the normoxic condition (21%) and in the hypoxic condition (1%, 3%, 5%). This study indicated no significant difference in the results. However, it is necessary to examine more growth factors and several cytokines for comparing the two groups of SLC and SCs and their secretomes.

The limitations of this study were the limited growth factors examined to compare the SLCs and SCs groups. However, these six growth factors in this study play an important role in peripheral nerve regeneration.

Conclusion

Preconditioning hypoxia has an effect on the composing of SLCs, SCs, and their secretomes *in vitro*. The hypoxic 1% of oxygen level gave most of the highest growth factor concentrations in this study. No significant differences concentration of growth factors between the SLCs group compared with the SCs group for all oxygen groups.

Ethical approval

The experimental procedures including animal laboratory were conducted according to the ethics and research recommendations for animal care and use, and were accepted by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia, number 2. KE.038.04.2020.

Consent

Animal studies, not human studies.

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Author contribution

T.S., F.M., H.B.N.: Conceptualization, study design, performed experiments, data analysis and interpretation, data curation, formal analysis, funding support, investigation, methodology, project administration, resources, validation, visualization, writing original manuscript draft, critical review, editing and approval of the final version of the manuscript. T.S., H.S., N.A.: performed surgery and experiments, data collection, analysis & interpretation, critical review. editing and approval of final version of the manuscript. data curation, formal analysis, investigation, visualization, writing, review and editing. H.S., C.R.S.P., D.T.: performed experiments, data collection, analysis and interpretation, critical review, editing and approval of final version of the manuscript, data curation, formal analysis, and investigation, writing, review and editing. H.S., F.A.R.: microsurgery advise, critical review, editing and approval of final version of the manuscript. D.N.U., H.S., F.M.: Scientific and microsurgery advise from clinical perspective, and critical review, editing and approval of final version of the manuscript. S.R., F.M.: resources, scientific/protocol advice, critical review, editing and approval of final version of the manuscript.

Conflicts of interest disclosure

No conflict of interest for all of authors regarding this study.

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Guarantor

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Data availability statement

Not applicable.

Provenance and peer review

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