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Hypoxia enhances self-renewal properties and markers of mesenchymal stem cells

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ABSTRACT

BACKGROUND
Mesenchymal stem cells (MSCs) are multipotent stromal cells that express CD73, CD90, and CD105 surface markers, but not CD14, CD45, CD34, CD11b, and HLA-DR. MSCs under hypoxic conditions have the essential role of maintaining the stemness capacity by releasing several growth factors into their medium, known as hypoxia conditioned medium (HCM). This study was performed to compare the effect of percentage of HCM to normoxic medium (NM) in increasing MSC proliferation marked by proliferation rate and surface marker expression.

METHODS
This study was of post-test only control group design using human umbilical cord-MSCs (hUC-MSCs) as subjects. The HCM treatment group was obtained by culturing MSCs under 5% O_2, whereas the NM control group was grown under 20% O_2. The hUC-MSCs were divided into 4 groups with different dose ratios of HCM to NM (25%:75%; 50%:50%; 75%:25% for P1, P2 and P3, respectively and 100% of NM for the controls). All of these groups were maintained at 37°C and the data was collected after 72 hours incubation. MSC marker expression of CD73, CD90 and CD105 was analyzed using flow cytometry and MSC proliferation by trypan blue assay.

RESULT
There were significant differences in MSC marker expression of CD73, CD90 and CD105 and proliferation at all dose ratios of HCM to NM ($p<0.05$).

CONCLUSION
Low oxygen concentration promotes MSC proliferation and stemness thus it might be beneficial for maintaining the MSC physiologic niche in-vitro.

Keywords: MSC, MSC-HCM, proliferation, stemness

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stromal cells with the capacity for self-renewal and differentiation into mesodermal lineages such as chondrocytes, osteoblasts, adipocytes, and neurons.[1] According to the International Society for Cellular Therapy (ISCT) MSCs' main characteristics are adherence to plastic, expression of CD73, CD90, and CD105 and lack of expression of CD14, CD45, CD34, CD11b, and CD31 and HLA-DR.[2] Mesenchymal stem cells have been widely used for tissue engineering and treatment of degenerative diseases and immune disorders, due to their trophic activity, immunomodulatory and angiogenic properties and multipotent differentiation capacity.[3]

Mesenchymal stem cells can be isolated from a wide range of source tissues such as the umbilical cord, bone marrow, adipose tissue and dental pulp.[4-6] Moreover, an essential goal for expansion of MSCs *in vitro* is to obtain sufficient cell numbers for research and clinical application. This depends mainly on tissue source of MSCs and processing method. In umbilical cord-derived MSCs (UC-MSCs) the stemness is highest in comparison with bone marrow and adipose-derived MSCs, due in part to their higher telomerase activity, and pluripotent properties under certain conditions.[7–9] A previous study has shown that the major concerns of in vitro MSC propagation are poor growth kinetics, early senescence and DNA damage during expansion.[6] Therefore, isolation techniques, culture medium, oxygen tension, supplements, cell seeding density and their modification such as under hypoxia and TNF-α are essential for MSC propagation.[10,11]

One previous study reported that oxygen concentration is a critical environmental factor for maintaining in vitro stem cell plasticity and proliferation.[12] Other studies also demonstrated that hypoxic conditions (O_2 pressure under 5% have positive effects on MSCs in terms of *in vitro* survival and self-renewal, particularly in preserving the stemness and enhancing proliferation.[13–15] There was an enhancement of MSC proliferation in hypoxic conditions compared to normoxia even following 2 weeks of incubation of the cultures.[16] However, this study did not report the use of the hypoxia chamber as a culture device. In contrast, another study reported that continued hypoxia reduced the clonogenic ability and differentiation potential of MSCs.[7] Another study described that 24-h hypoxia is beneficial for the functional properties of MSCs.[17]

Since hypoxia is the most similar to the physiological conditions occurring in living organisms, we decided to examine MSC culture both in normoxic and hypoxic conditions. In this study, we analyzed the comparative effect of hypoxia-conditioned medium (HCM) obtained after 24-h hypoxia incubation to NM (with HCM:NM ratios of about 25%:75%; 50%:50% and 75%:25%, respectively) in enhancing MSC stemness through analyzing MSC proliferation and CD73, CD90 and CD105 expression for 72 hours incubation.

METHODS

Research design

This post-test only control group design was conducted in the Stem Cell and Cancer Research Laboratory, Faculty of Medicine, Universitas Islam Sultan Agung (Unissula), Semarang from July-September 2018.

Research subjects

Human umbilical cord-MSCs (hUC-MSC) were used as the subjects and divided into 4 groups with different doses of MSC-HCM. The total number of hUC-MSCs was 1.2 x 10^6 cells and was equally divided into 4 groups. The control group was treated with normal medium, whereas the treatment groups were supplemented with 25% HCM (P1), 50% HCM (P2) and 75% HCM (P3), respectively.

Mesenchymal stem cell isolation

Mononuclear cells (MNCs) from an umbilical cord were collected and separated by

Ficoll-Paque solution (density 1.077 g/mL; GE) in centrifuge tubes (15 mL). The mononuclear cells were transferred to a new tube and washed twice through centrifugation at 2000 rpm for 10 minutes. They were then seeded into 25-cm^2 flasks at a density of 1 × 10^7–10^8 cells/cm^2. The flasks contained Dulbecco's modified Eagle's Medium–Low Glucose (DMEM-LG, GIBCO Invitrogen) supplemented with 1% L-Glutamine 200 mM, 1% Antibiotic—Antimycotic comprising 10.000 U/mL sodium penicillin, 1% 10.000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B (GIBCO/Invitrogen Corporation), and 10% Fetal Bovine Serum (GIBCO/Invitrogen Corporation). Umbilical cord blood (UCB)-MSC cultures were maintained at 37 °C in 5% CO_2 and non-adherent cells were removed after 48 h. The medium was changed every other day. The experiments were performed at the fourth passage of the cultures with approximately 80–90% confluence. UCB-MSC lineages were established in culture up to the fourth passage.

Characterization of MSCs

The characteristics of MSCs were analyzed by flow cytometric analysis at the fourth passage. The cells were subsequently incubated at room temperature and in the dark with fluorescein isothiocyanate (FITC)-conjugated, allophycocyanin (APC)-conjugated or phycoerythrin (PE)-conjugated monoclonal antibodies, including CD73, CD90 and CD105. APC-, PE-, and FITC-conjugated isotypes were used as negative controls. The analysis used BD Pharmingen™ (BD Biosciences, Franklin Lakes, NJ, USA) at 4°C for 30 min. The cells were washed twice with 1% BSA/PBS, resuspended in 200 µL 1% BSA/PBS and analyzed using a flow cytometer (BD Biosciences, San Jose, CA, USA).

In-vitro differentiation

For the differentiation test, MSCs were grown on 24-well plates at densities of 1x10^4 cells/well, with DMEM supplemented with osteogenic-induction medium containing 10^{-7} mol/L/0.1 µM dexamethasone, 10 mmol/L β-glycerophosphate, 50µmol/L ascorbate-2-phosphate (Sigma-Aldrich, Louis St, MO) and 10% fetal bovine serum (FBS). The cells were rinsed in PBS and fixed with cold 70% ethanol (v/v) for 1 hour at room temperature, then rinsed three times with twice-distilled water after 21 days of induction. A volume of 1 ml 2% Alizarin Red solution (w/v) (pH 4.1-4.3) was added and the cells incubated for 30 minutes at room temperature, then rinsed four times in twice-distilled water.

Hypoxia conditioned medium (HCM)

A previous protocol [18] was adapted for use in this study. Briefly, hMSCs were incubated until confluent, after which they were seeded in a T25 flask (2 × 10^6 cells), washed twice with endothelial basal medium (EBM-2; Lonza), and then placed in a hypoxic chamber (Anaerobic Environment; ThermoForma, Waltham, MA, USA) containing 5 ml EBM-2 for 12 hours. The airtight humidified hypoxic chamber was maintained at 37°C and continuously supplied with 5% CO_2, 10% H_2, and 85% N_2. The oxygen level in the chamber was ~0.5%. After incubation, the medium was collected and centrifuged at 1000 rpm for 10 minutes at 4°C. Then the HCM was added to the treatment groups at concentrations of 25, 50% and 75%, respectively.

Mesenchymal stem cell proliferation essay and stemness

The cells were counted in a hemocytometer for analyzing cell proliferation. In brief, following treatment, the cells are harvested and an aliquot of cells is combined with trypan blue dye solution and loaded into the hemocytometer chamber. The cells are then viewed under the microscope and the number of live cells present within a specific area are counted. The number of cells counted per area multiplied by the dilution factor will determine the number of live cells per milliliter. The stemness of MSCs was analyzed after 72-h induction using a BD Pharmingen™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

All values are expressed as the mean ± SD. Comparisons between the four groups were analyzed using ANOVA and then followed by posthoc Fisher's LSD. A p value of <0.05 was considered significant. All analyses were performed with SPSS 16.0.

Ethical clearance

All research activities were approved by the Commission on Test Animal Ethics (*Komisi Etik Hewan Uji*), Faculty of Medicine, Universitas Islam Sultan Agung, Semarang, under No. 233/ VII/2018/Komisi Bioetik.

RESULTS

Mesenchymal stem cell characterization

The MSCs were successfully isolated from the umbilical cord, based on their adherence to plastic. After reaching 80% confluence, the cells were harvested for 4 passages. The MSCs showed a fusiform appearance, became confluent after 5-7 days in culture and were regularly passaged. Flow cytometry analysis showed that the MSCs were positively expressing 75.8% of CD73, 84.1% of CD90 and 55.2% of CD105 (Figure 1).

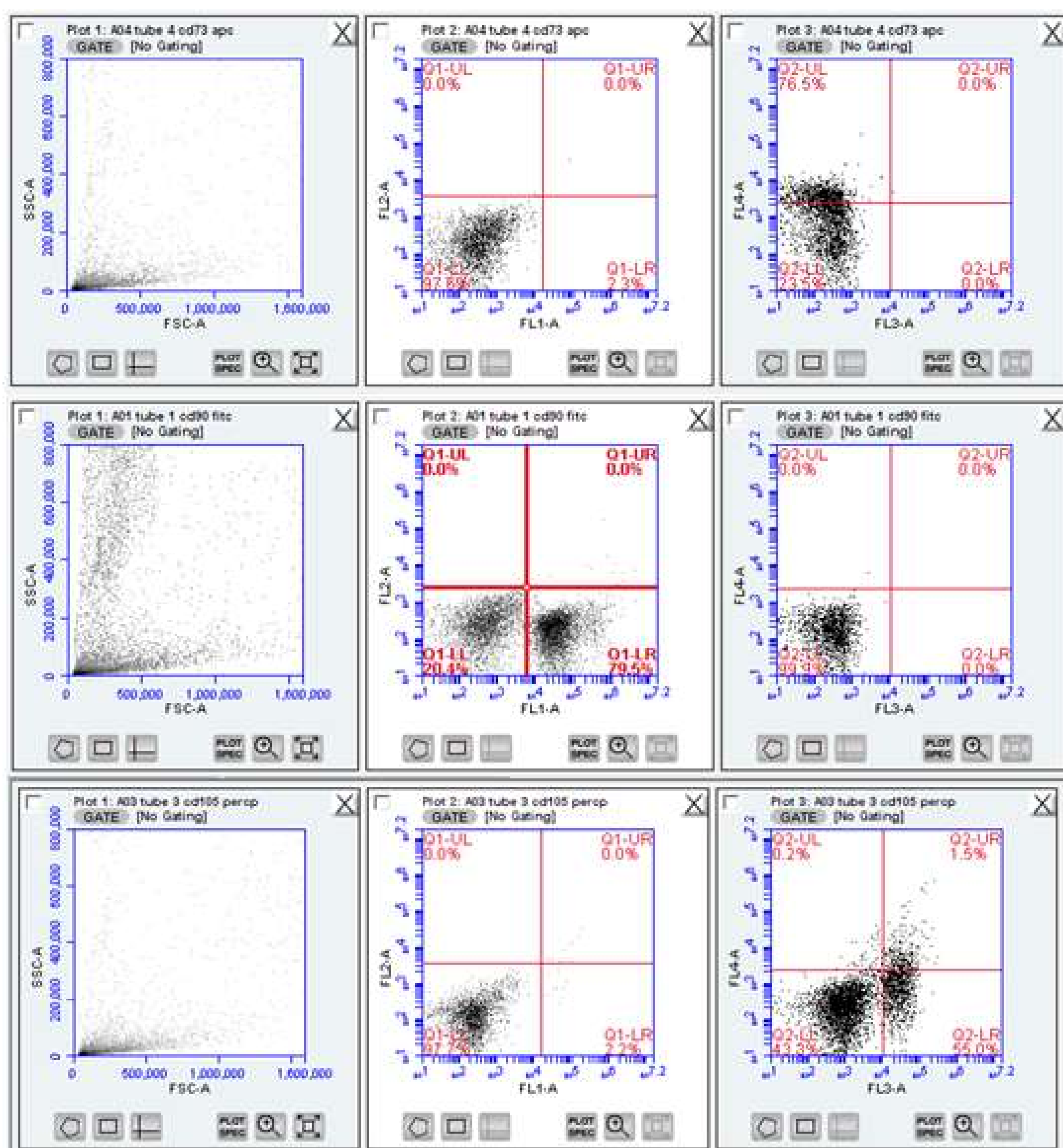

Figure 1. Characteristics of UC-MSCs expressing CD73, CD90 and CD105 by flow cytometry

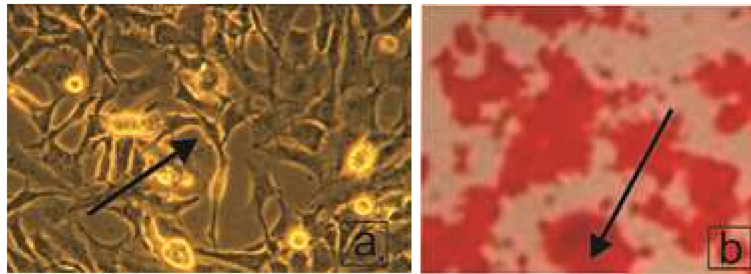

Figure 2. (a) UC-MSC-like from in vitro culture showing fibroblast-like and polygonal cells, at 40x magnification, (b) osteogenic differentiation test with Alizarin Red staining appearing in MSC population

In vitro differentiation

The MSC differentiation assay was done by means of osteogenic medium. The results showed that MSCs were capable of differentiating into osteogenic cells as indicated by their red color from Alizarin red staining (Figure 2b).

MSCs self-renewal and stemness

The MSC appearance was analyzed using the light microscope at 24, 48, and 72 hours after MSC-HCM induction (Figure 3). The results showed significant between-group differences in MSC self-renewal appearance (p=0.000). The highest result was shown by P3 with a high concenteration of MSC-HCM (75%) (Table 1). This is in line with the expression of MSC positive markers (CD 73, CD90, CD105).

DISCUSSION

Several studies have reported that hypoxic conditions may modulate the paracrine activity of MSCs by up-regulating the various secretable factors into a medium such as HCM. These soluble molecules are associated with the

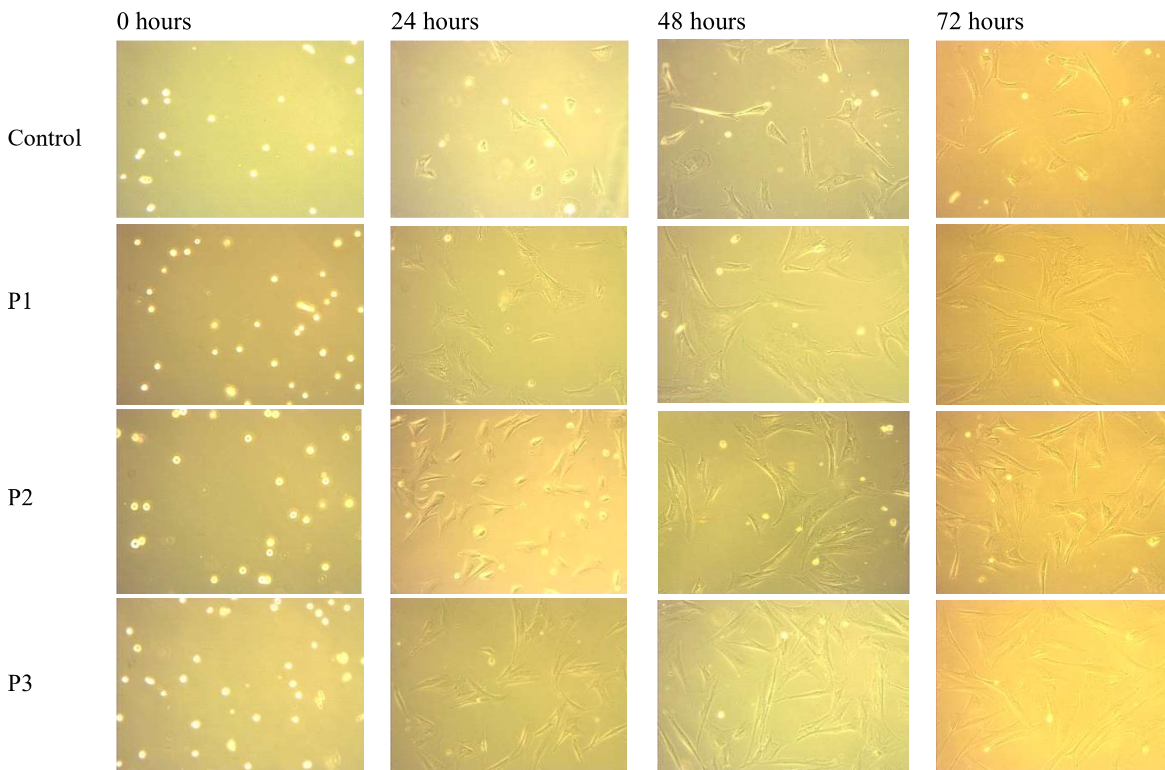

Figure 3. Appearance under light microscope of MSCs after HCM induction

Table 1. Distribution of MSC proliferation and stemness after 72 hour incubation by treatment groups

Variable	Treatment groups				p value
	Control (n=3)	P1 (n=3)	P2 (n=3)	P3 (n=3)	
Proliferation (cell)	411666.67 ± 12583.05	461666.67 ± 10408.33	535000.00 ± 13228.75	571666.67 ± 10606.60	0.000*
CD73 (%)	76.41 ± 0.70	86.5 ± 1.20	90.86 ± 0.40	99.26 ± 0.50	0.000*
CD90 (%)	84.16 ± 0.30	90.83 ± 0.45	93.73 ± 0.45	96.43 ± 0.55	0.000*
CD105 (%)	55.41 ± 0.26	60.81 ± 0.45	63.86 ± 0.40	66.80 ± 0.30	0.000*

Note: P1 medium with 25% HCM, P2 medium with 50% HCM, P3 medium with 75% HCM. *Significant at $p<0.05$

increased proliferation rate and stemness properties of MSCs. Whereas the effects of hypoxia have been investigated previously, the comparison between HCM and NM in enhancing MSC proliferation in vitro by constantly maintaining stemness properties remains unknown. In this study we introduced various HCM to NM dose ratios in MSCs using the hypoxic condition chamber as in a previous study,[17] then analyzed the increase in MSC proliferation rate and their expression of CD73, CD90, and CD105.

Our results showed that there were significant differences between all HCM to NM dose ratios in cell proliferation rate after 72 hours. This is in accordance with a previous study that found that hypoxic conditions may increase the proliferation and stemness in MSCs.[18] We suggest that the hypoxic state induces hypoxia-inducible factor-1a (HIF-1a) as transcription factor for inducing HIF-1a survival target genes including self-renewal and proliferation.[19] This is in line with one other study reporting that MSCs under hypoxic conditions also have the ability to express the transcription embryonic factors such as Sox2.[18]

In undifferentiated mouse and human embryonic stem cells (ES), Oct3/4 and Sox2 also interact with the Nanog promoter to strengthen the stemness of the cells.[18,20] These factors serve as candidate molecules for mastering the regulation of initiation, maintenance, and differentiation of pluripotent cells. This is also in accordance with our finding that there are significant differences between all HCM to NM dose ratios in MSC positive markers (CD73, CD90, CD105) with the highest result in group P3 (75%:25%). We suggest that the secretome released by MSCs under hypoxic conditions may induce the in vitro MSC progeny cells to grow over with maintaining their stemness.

Table 2. Multiple comparisons of MSC proliferation and stemness

Groups	Mean differences	p value
Proliferation		
Control P1	-50000.00	0.001
P2	-123333.33	0.000
P3	-155833.33	0.000
P1 P2	-73333.33	0.000
P3	-105833.33	0.000
P2 P3	-32500.00	0.020
CD73		
Control P1	-10.033	0.000
P2	-14.43	0.000
P3	-22.83	0.000
P1 P2	-4.40	0.000
P3	-12.80	0.000
P2 P3	-8.40	0.000
CD90		
Control P1	-6.66	0.000
P2	-9.56	0.000
P3	-12.26	0.000
P1 P2	-2.90	0.000
P3	-5.60	0.000
P2 P3	-2.70	0.000
CD105		
Control P1	-5.40	0.000
P2	-8.46	0.000
P3	-11.40	0.000
P1 P2	-3.06	0.000
P3	-6.00	0.000
P2 P3	-2.93	0.000

Note: P1 medium with 25% HCM, P2 medium with 50% HCM, P3 medium with 75% HCM

Our findings indicate that HCM plays an important role in stemness potentials of the adult stem cell (ASC) including UC-MSC under hypoxic conditions. The limitation of this study is that we did not analyze the transcription marker molecules such as Sox2, Oct3/4 and Nanog.

CONCLUSIONS

Low oxygen concentration in MSCs promotes cell proliferation and stemness, thus it might be beneficial for maintaining the MSC physiologic niche in-vitro.

CONFLICT OF INTEREST

Competing interests: No relevant disclosures.

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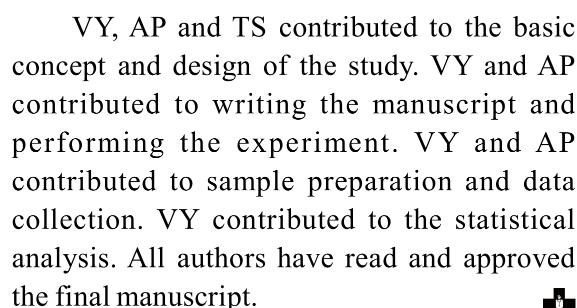
CONTRIBUTORS

VY, AP and TS contributed to the basic concept and design of the study. VY and AP contributed to writing the manuscript and performing the experiment. VY and AP contributed to sample preparation and data collection. VY contributed to the statistical analysis. All authors have read and approved the final manuscript.



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