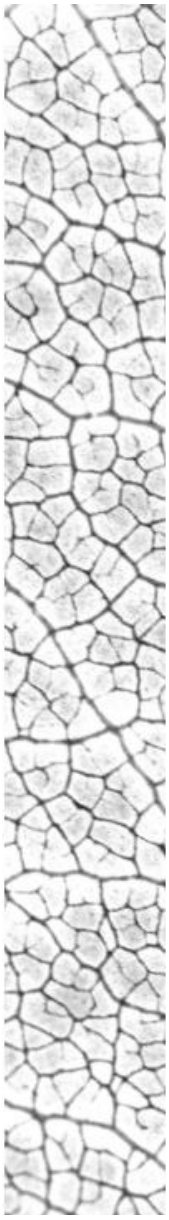

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The effect of low oxygen level on pluripotency and proliferation capability in bone marrow mesenchymal stem cell culture

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Abstract

Background: Physiologically, mesenchymal stem cells (MSCs) require optimal preconditioning in the form of low O_2 tension by 1-3% in bone marrow, 10-15% in adipose tissue, and 2-9% in almost all body tissues. On the other hand, seminiferous tubules in testes require 1-6% of O_2 gradation to perform spermatogenesis. Under these conditions, low O_2 level (hypoxia) is required to reinforce the microenvironment to keep forming quiescent cells. **Purpose:** This study aims to reveal the differences between in-vitro cultured MSCs under normoxic conditions and hypoxic conditions in cell viability, their pluripotency, and proliferative capability. **Method:** This study is an exploratory in-vitro laboratory study on Bone Marrow Mesenchymal Stem Cells (BMSC) cultures using hypoxic conditions. A total of 21 culture plates were divided into 3 groups. The data was analyzed using the ANOVA Multivariate test. **Results:** The highest average of viable cells was found in the P2 group, amounting to 89.29 ± 5.36. On the other hand, the least average of viable cells was found in the P0 group, amounting to 63.67 ± 2.56. **Conclusion:** The cultures with hypoxic conditions and normoxic preconditions are the best culture conditions since they produce cells that were capable of maintaining the pluripotency while still having a better capability of proliferation and viability, compared to direct hypoxic conditions.

Keywords: stem cell, low oxygen level, cell viability, proliferation, pluripotency

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INTRODUCTION

In the past decade, researchers have been using stem cells to find out and study the growth and development processes of human body tissues and the pathogenesis of their illnesses. In addition, there is an increase in the use of stem cells in the treatment of diseases that are no longer possible to be further treated, both conservatively and operatively (Kelly, & Porucznik, (2014). Stem cells have the best potential for many types of cell differentiation and the total potential to regenerate from damaged tissue caused by disease or trauma (Rantam, Ferdiansyah, & Purwati, 2009; Margiana et al., 2019). Although yet to be widely used in the field of orthopedics, stem cells are also expected to be an option, especially in a degenerative joint disease where, currently, the cost of arthroplasty with the prosthetic implant to replace cartilage function in joints gets more expensive 2012; Sun, & Yang, 2014).

In particular, the use of stem cells in the field of orthopedics has attracted the researchers' attention, such as bone grafting (Ferdiansyah 2010), flexor tendon grafting (Suroto, 2010), on the regeneration mechanism of cartilage defects (Utomo, & Abdul Rantam, 2017). which gives highly significant results. The state of low oxygen level, also known as hypoxia, generally considered as a dangerous condition for a human, turns out to be a normal physiological condition and is required by stem cells in the body (niche).

Physiologically, mesenchymal stem cells (MSCs) require optimal preconditioning in the form of low O_2 tension by 1-3% in bone marrow (Chow, et al. 2011)., 10-15% in adipose tissue and 2-9% in almost all body tissues (Gruber, Hoelscher, et al. 2012). On the other hand, seminiferous tubules in testes require 1-6% O_2 gradation to perform spermatogenesis (Wenger, & Katschinski, 2005). Under these conditions, low O_2 level (hypoxia) is required to reinforce the favorable microenvironment on in-vitro culture to keep it viable

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when transplantation is carried out. This condition requires a favorable stem cell during the culturing through hypoxia preconditioning so that quiescent cells are formed.

Until recently, researches are still being conducted to search for absolute factors of in-vitro niches that are capable of controlling stem cell proliferation, keeping it viable and pluripotent, terminating apoptosis, forming senescence cells, or even mutating genes. If accompanied with in-vitro pluripotency, the availability of stem cells can be produced easily by researchers and clinicians and the number of the produced cells can be compressed so that it reinforces the success of cell transplantation therapy.

Based on the elaboration above, it is necessary to conduct research that reveals the difference between in-vitro MSC culture under normoxic conditions and hypoxic conditions, especially in the conditions of viability to observe their pluripotency, and the ability of MSC proliferation from the produced cultures.

METHOD

This study is an exploratory in-vitro laboratory study on Bone Marrow Mesenchymal Stem Cells (BMSC) cultures using hypoxic conditions. The experimental unit was divided into three different treatment groups, which were then evaluated in the same incubation period. The BMSC experimental unit was taken from three healthy male rabbits which then experienced in-vitro propagation into 21 culture plates (CP). The total of 21 CPs were divided into 3 groups, i.e., the control group (P0) with 7 CPs, treated under normoxic condition with 21% of oxygen concentration; treatment group 1 (P1) with 7 CPs, treated under normoxic condition for 24 hours and then moved to hypoxic condition with certain O2 concentration; and treatment 2 (P2) with 7 CPs under hypoxic precondition with 1% of O2 concentration. The results of conditioning from each group were observed in terms of their viability and proliferation, as well as the pluripotency in passage 4. The observation of slow proliferation was based on the effect of cell mobilization after the scratch test. The viability observation was based on penthanil blue staining, where dead cells absorbed blue color. The pluripotency observations were based on the expression of OCT4 and SOX2 coding genes.

The experimental unit in this study included 21 mesenchymal stem cell cultures taken from the femur bone marrow of male New Zealand white rabbits, weighing at least 3 kg, aged between 6-9 months. In the initial stages, the isolation and MSC culture of the healthy bone marrow of male New Zealand white rabbits were carried out. Next, hypoxia precondition was administered in stem cell culture with two doses of O2 concentration, 21% and 1%, followed by several days of cultivation time until reaching the 4th passage. The analysis of stem cell viability included: slow proliferation, based on the least like CF-Us microscopically; pluripotency, based on the expression of OCT4 and SOX2 proteins by immunofluorescence; and viability, based on uptake of methylene blue on cells that died after hypoxic preconditioning. The expression of OCT4 and SOX2 proteins from BMSCs after hypoxic precondition can be identified by observing the phenotype using indirect immunofluorescence.

The results of expression through the immunofluorescence method in this study were observed descriptively and quantitatively. The obtained data, such as the number of CFU-Fs, viability, and pluripotency. The steps of multivariate comparison of hypothesis testing included testing the data normality with the Kolmogorof Smirnov test, and Factorial Multivariate Analysis of Variants (MANOVA).

Table 1. Number of Least-like CFU-Fs in the 4th Passage

Sample No.	Control (P0)	Treatment 1 (P1)	Treatment 2 (P2)
1	39	17	5
2	35	15	7
3	31	18	5
4	29	19	4
5	34	21	9
6	27	13	8
7	38	15	8

Table 2. Average Number of Least-like CFU-Fs on the 4th Passage

Groups	Number of Samples	Mean+ SD	Minimum	Maximum
Control (P0)	7	33.286 ± 1.70	27.00	39.00
Treatment 1 (P1)	7	16.857 ± 1.03	13.00	21.00
Treatment 2 (P2)	7	6.571 ± 0.72	4.00	9.00

Table 3. Statistical Results of Average Slow Proliferation Comparison Based on Number of Least-like CFU-Fs (One-Way ANOVA with post hoc test)

Groups	Mean + SD	Sig.
Control (P0)	33.286 ± 1.70	P <0.001
Treatment 1 (P1)	16.857 ± 1.03	
Treatment 2 (P2)	6.571 ± 0.72	

RESULTS

The obtained data, the number of CFU-Fs, were interpreted quantitatively and qualitatively. The quantitative interpretation was conducted through direct statistical analysis using Factorial ANOVA with various concentrations of O2 (21%, 21-1%, and 1%). On the other hand, the qualitative interpretation was done by identifying the form of a macroscopic description, presented descriptively. The numbers of least-like CFU-Fs, as seen in **Table 1** and **Table 2**, suggest that the highest average was in the control group, amounting to 33.286 ± 1.70. Conversely, the lowest number of least-like CFU-Fs was in the treatment group 2 (P2), amounting to 6.571 ± 0.72. **Table 3** shows a p-value of less than 0.05, meaning that there is a significant

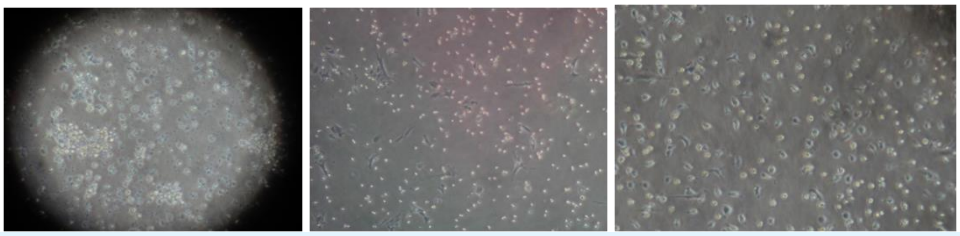

Fig. 1. Like CFU-Fs are formed after cells attach to Petri, using an inverted 2,000x microscope. A. Normoxia, B Normoxia-Hypoxia, C Hypoxia

Table 4. The Number of Cells Expressing OCT4 Coding Genes on the Examination of Immunofluorescence Percentage

Sample No.	Control (P0)			Treatment 1 (P1)			Treatment 2 (P2)		
	OCT4	Cell Total	%	OCT4	Cell Total	%	OCT4	Cell Total	%
1	12	986	1.22	654	879	74.4	245	285	85.96
2	9	787	1.14	623	794	78.46	225	272	82.72
3	7	845	0.83	722	892	80.94	201	232	86.64
4	11	868	1.27	687	897	76.59	175	215	81.40
5	14	683	2.05	769	985	78.07	218	262	83.21
6	5	775	0.65	854	1083	78.86	238	298	79.87
7	17	823	2.07	586	746	78.55	211	263	80.23

Table 5. Average Percentage of Cells Expressing OCT4-Coding Genes from Immunofluorescence Examination in the 4th Passage

Groups	Number of Samples	Mean + SD	Minimum	Maximum
Control (P0)	7	1.32 ± 0.55	0.65	2.07
Treatment 1 (P1)	7	77.98 ± 2.04	74.40	80.94
Treatment 2 (P2)	7	82.86 ± 2.65	79.87	86.64

Table 6. Statistical Results of Average Slow Proliferation Comparison based on OCT4 Expression (One Way ANOVA with post hoc test)

Groups	Mean + SD	Sig.
Control (P0)	1.32 ± 0.55	P<0.001
Treatment 1 (P1)	77.98 ± 2.04	
Treatment 2 (P2)	82.86 ± 2.65	

difference in the average slow proliferation based on the number of Least-like CFU-Fs among the control group, treatment group 1, and treatment group 2. **Fig. 1** shows a picture (form) like CFU-Fs microscopically after the treatment is given a variety of O2 concentrations.

Fig. 1 indicates that, in the normoxia treatment, the number of CFU-Fs was more and the cells were actually already attached to the dish. Besides, the distance between cells that forms a colony is more tenuous. As in both direct and indirect hypoxia treatment, the number of colonies was less and there were still many cells floating, and the cells that made up the colonies piled up densely and had a slightly yellowish color. The pluripotency analysis from MSC can be seen in the expression of the OCT4 phenotype by immunofluorescence methods.

In **Table 4** and **Table 5**, it can be seen that the highest average percentage of the number of cells expressing the OCT4 coding gene on immunofluorescence examination is in treatment group 2 (P2), amounting to 82.86 ± 2.65. On the other side, the least average percentage of the cells expressing the OCT4 coding gene in the immunofluorescence examination is obtained in the control treatment group (P0), amounting to 1.32 ± 0.55. **Table 6** shows a significant difference in the average percentage of the number of cells expressing the OCT4 coding gene on immunofluorescence examination among the control group, treatment group 1, and treatment group 2.

In **Table 7** and **Table 8**, it is clear that the highest average percentage of the number of cells expressing the SOX2 coding gene in the immunofluorescence examination is in treatment group 2 (P2), amounting to 92.92 ± 4.31. Meanwhile, the least average percentage

Table 8. Average Percentage of Cells Expressing SOX2 Coding Genes from Immunofluorescence Examination in the 4th Passage

Groups	Number of Samples	Mean + SD	Minimum	Maximum
Control (P0)	7	2.48 ± 0.74	1.28	3.33
Treatment 1 (P1)	7	90.00 ± 3.79	84.75	95.46
Treatment 2 (P2)	7	92.92 ± 4.31	86.05	97.12

Table 7. The Number of Cells That Express SOX2 Coding Genes on Immunofluorescence Examination

Sample No.	Control (P0)			Treatment 1 (P1)			Treatment 2 (P2)		
	SOX2	Cell Total	%	SOX2	Cell Total	%	SOX2	Cell Total	%
1	24	835	2.87	825	912	90.46	283	305	92.79
2	21	746	2.82	809	865	93.53	258	272	94.85
3	27	812	3.33	731	846	86.41	237	265	89.43
4	18	946	1.90	671	738	90.92	265	283	93.64
5	10	783	1.28	692	782	88.49	252	261	96.55
6	19	912	2.08	883	925	95.46	270	278	97.12
7	22	712	3.09	689	813	84.75	253	294	86.05

Table 9. Statistical Results of Average Slow Proliferation Comparison based on OCT4 Expression (One Way ANOVA with post hoc test)

Groups	Mean+ SD	Sig.
Control (P0)	2.48 ± 0.74	P<0.01
Treatment 1 (P1)	90.00 ± 3.79	
Treatment 2 (P2)	92.92 ± 4.31	

Table 11. Average Percentage of Cells Expressing SOX2 Coding Genes from Immunofluorescence Examination in the 4th Passage

Groups	Number of Samples	Mean + SD	Minimum	Maximum
Control (P0)	7	63.67 ± 2.56	60.61	67.95
Treatment 1 (P1)	7	86.71 ± 4.31	80.51	93.27
Treatment 2 (P2)	7	89.29 ± 5.36	80.37	95.80

Table 12. Statistical Results of Average Slow Proliferation Comparison based on OCT4 Expression (One Way ANOVA with post hoc test)

Group	Mean + SD	Sig.
Control (P0)	63.67 ± 2.56	P<0.05
Treatment 1 (P1)	86.71 ± 4.31	
Treatment 2 (P2)	89.29 ± 5.36	

of the number of cells expressing the SOX2 coding gene in the immunofluorescence examination was obtained in the control group (P0), amounting to 2.48 ± 0.74.

Table 9 shows a significant difference in the average percentage of the number of cells expressing the SOX2 coding gene on immunofluorescence examinations among the control group, treatment group 1, and treatment group 2. The data match was based on the similarity significance of cut off value, which must be ≥ 60% (85 % in cultures with 1% of O2 and 85% in cultures with O2 of 21% to 1%), whereas. in normoxic cultures (21% of O2 concentration), the similarity significance of cut off value was less than 60% (= 50%). In **Table 10** and **Table 11**, it can be seen that the highest average percentage of cells that did not absorb color from trypan blue staining (viable cells) was in treatment group 2 (P2), reaching 89.29 ± 5.36. The least average percentage of the number of cells that did not absorb color from trypan blue staining (viable cells) occurred in the control group (P0) with 63.67 ± 2.56.

Table 12 shows that the p-value was less than 0.05, meaning that there was a significant difference in the percentage of the number of cells that did not absorb color from trypan blue staining (viable cells) among the control group, treatment group 1, and treatment group 2. The post hoc test showed that the average percentage of viable cells between the control group and the treatment groups had a significant difference. However, there was no significant difference in the average percentage of viable cells between treatment group 1 and treatment group 2.

The results of this study are limited to proliferation and pluripotency. Many factors can influence the results of the study. Thus, the different factors studied would also contribute different results on the proliferation and the pluripotency of stem cells.

DISCUSSION

In this study, the proliferation still took place in hypoxic cultures in P1 and P2 to form CFU-Fs until the 4th passage. As for the Normoxia group with 21% of O2, the capability to proliferate to form CFU-Fs was faster than P1 and P2 until the 4th passage. However, the formation of MSCs in P1 and P2 groups was faster than in the normoxia group. This condition proves that the treatment of hypoxic conditions causes controlled proliferation. The following is the explanation. Although hypoxic conditions cause slow proliferation concerning the smallest amount of CFU-Fs compared to normoxia, the process of MSC formation in hypoxic cultures is faster than normoxia. This is in accordance with previous research studies on MSCs from rats given hypoxic conditions. In that study, that proliferation was proven to keep occurring after two months after the MSCs rat was cultured under hypoxic conditions of 3%. Meanwhile, in normoxic cultures, there was an acceleration of cellular senescence. As seen in **Fig. 2**, MSC growth in rats was complete within two months of normoxia culture, followed with the cell growth formation and the emergence of senescence cells.

Phenotype identification of OCT4 and SOX2 and BMSCs after being cultured with hypoxia treatment was carried out through the immunofluorescence method. The identification aims to obtain the expression of OCT4 and SOX2 after the treatment of hypoxic conditions before being compared with control (21% of 02). In this study, the expression of OCT4 and SOX2 was detected at the 4th passage under hypoxic conditions (P1 and P2). This finding shows that the treatment of hypoxic conditions causes the emergence of transcription factors OCT4 and SOX2. Therefore, MSCs characterized as multipotency had transformed into pluripotency.

Table 10. Number and Percentage of Cells that Did Not Absorb Trypan Blue (viable cells)

Sample No.	Control (P0)			Treatment 1 (P1)			Treatment 2 (P2)		
	Viable	Cell Total	%	Viable	Cell Total	%	Viable	Cell Total	%
1	348	634	60.61	624	714	87.39	237	265	89.43
2	388	734	61.24	669	831	80.51	216	253	85.38
3	401	751	63.12	587	683	85.94	226	243	93.00
4	433	702	67.95	682	752	90.69	228	238	95.80
5	397	744	62.75	693	743	93.27	238	272	87.50
6	407	688	64.33	656	789	83.14	247	264	93.56
7	416	713	65.73	584	679	86.01	176	219	80.37

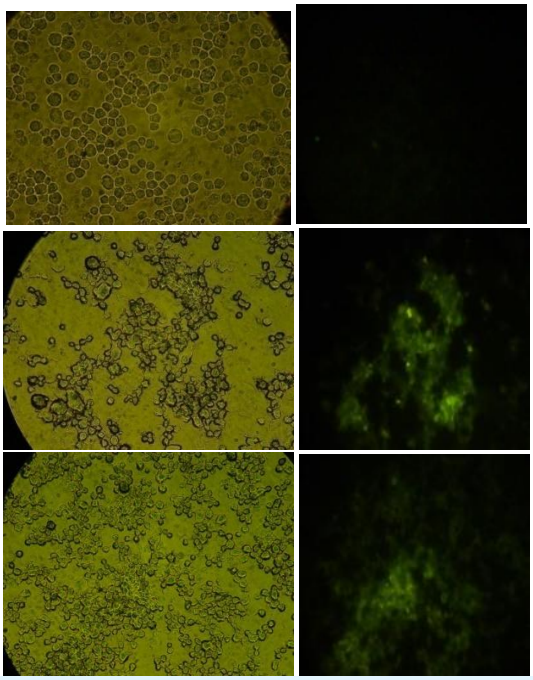

Fig. 2. Negative expression of OCT4 in the control group (left without filter, right with a green filter, no green glow). Positive expression of OCT4 in treatment group 1 and 2 (left without filter, right with a green filter, OCT4 positive glowing green) (top to bottom)

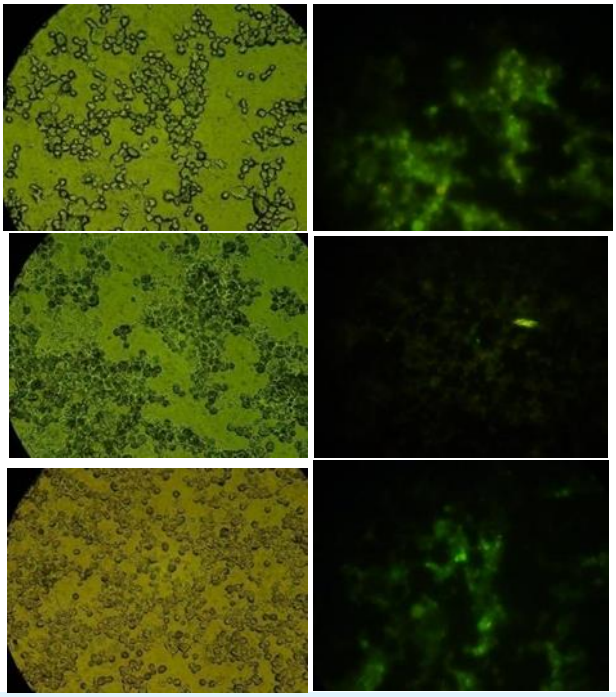

Fig. 3. Negative expression of SOX2 in the control group (left without filter, right with a green filter, no green glow). Positive expression of SOX2 in treatment group 1 and 2 (left without filter, right with a green filter, positive SOX2 glowing green) (top to bottom)

The results of pluripotency analysis in this study were based on the expression of phenotype OCT4 and SOX2 genes by immunofluorescence and are in accordance with the results of studies which state that after 48 hours of hypoxia administration in stem cell culture, HIF1-α expression would take place and directly becomes upstream regulator and transcription factors OCT4 that are essential to maintain pluripotency. Similarly situation also take place in the other transcription factors such as SOX2 and Nanog, where both are also regulated by HIF1-α (Forristal, et al. 2010. Covello, et al. 2006.

The viability of cultured cells was analyzed by trypan blue staining where the dead cells (not viable) would absorb the color. The calculation results indicated the cells that absorbed the most trypan blue (not viable) were in the control group, followed by P1 and P2 groups. This finding shows that the normoxia culture condition results in many dying cells due to the inability to adapt to high oxygen conditions.

The death of the cell in normoxic conditions was due to mitochondrial dysfunction and caspase-dependent apoptosis (Nie, et al. (2011. The cells that were cultured under normoxic conditions would undergo nuclear apoptosis and chromatin condensation. Normoxia conditions would increase the activity of caspase 3 which would initiate the process of proteolysis and cell apoptosis. In addition, hypoxic conditions also resulted in mitochondrial dysfunction, marked by the release of cytochrome c into the cytosol, which indicated the damage in the mitochondrial membrane, and subsequently, the cytochrome c would also initiate apoptosis.

According to this study, the cells cultured under hypoxic conditions also experienced apoptosis. However, the number of cells that experienced death in the group cultured under normoxic preconditions before hypoxia was not as many as those directly cultured under hypoxic conditions. This finding shows that the precondition of culture with normoxia provides the cells the opportunity to adapt and proliferate before being conditioned in hypoxic culture.

CONCLUSION

Hypoxic culture conditions at 1% of O2 concentration result in cells undergoing slow proliferation, compared to normoxic conditions. Hypoxic culture conditions at 1% of O2 both directly and with normoxic preconditions, are capable of maintaining the mesenchymal stem cell pluripotency, characterized by the expression of OCT4 and SOX2 proteins. In terms of cell viability, hypoxic culture conditions at 1% of O2 concentration result in a lesser decrease in cell viability than the cultures with normoxia.

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