# INFLUENCE OF LOW OXYGEN CONDITION OF BONE MARROW MESENCHYMAL STEM CELL

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## ABSTRACT

The state of low oxygen levels known as hypoxia in humans is considered a dangerous condition is apparently a normal physiological condition and required by the stem cells as they are in the body. Mesencyimal Stem Cells (MSCs) require physiologically optimal conditions of low O2 tension of 1-3% in the bone marrow. The purpose of this study was to reveal the difference between in vitro culture of MSC in normoxia condition (20% O 2 concentration) with hypoxia condition (1% O2 content) especially in terms of viability, pluripotent properties, and MSC proliferation ability of the culture it produces. This research is an explorative laboratory research invitro on Bone Marrow Mesenchymal Stem Cells (BMSCs) culture using hypoxia condition. The study design used Randomized Control Group Post-Test Only Design. This research was conducted for 2 months. There was a significant difference in mean slow proliferation based on the number of Least-like CFU-Cs between the control group, treatment group 1 and treatment group 2, the mean percentage of the number of cells expressing the OCT4 coding gene on immunofluorosense examination between the control group, 1 and the treatment group 2 and the mean percentage of cell numbers expressing the OCT4 coding gene on the immunofluorosense examination between the control group, the treatment group 1 and the treatment group 2 showing p <0.01. There was a significant difference of percentage of non-absorbing color cell number of trypsin blue (viable cells) between control group, treatment group 1, and treatment group 2 showing p value <0.05. This suggests that the precondition of culture with normoxia provides an opportunity for cells to adapt and proliferate before being conditioned in hypoxic cultures. Cultures with hypoxic conditions and preconditions of normoxia are the best culture conditions because they produce cells that are capable of maintaining pluripotency properties while still having better proliferation and viability capability compared with direct hypoxia conditions.

**Keywords: Hypoxia, normoxia, bone marrow, mesenchymal stem cells.**

## INTRODUCTION

The progress of world health science, causing life expectancy level stay longer. In the last decade the attention and research in the field of stem cells (stem cells) has advanced very rapidly. In addition, there is rapid increase in the use of stem cells in the treatment of diseases that are not possible to be

treated again either conservatively or operatively (Kelly & Porucznik, 2014). In the field of orthopedics, stem cell therapy can be used primarily for bone defect transplantation, in non-union cases, spinal cord injuries and peripheral nerve injuries, in the treatment of osteonecrosis, and, more recently, for tissue engineering purposes for patients with congenital anomalies.

The state of low oxygen levels known as hypoxia in humans is considered as a dangerous condition, is apparently a normal physiological condition and required by stem cells like when in the body (niche). Stem Cells (MSCs) require physiologically low optimal O2 tension of 1-3% in bone marrow (Chow et al., 2010), 10-15% adipose tissue (Bizzari et al., 2006) and 2- 9% in almost all body tissues (Gruber et al., 2010), whereas in the seminiferous tubules testes required 0.2 to 1-6% gradation for spermatogenesis (Wenger and Katschinski, 2005).

Although it can be taken from multiple sources, MSC concentrations in tissues are very low, and impossible to isolate the 50-2x108MSCs (the amount usually used in clinical trials) from the donor for each therapy. Thus, in vitro conventional culture to proliferate MSC has become an inevitable choice. (Haque et al., 2013).

Based on the above conditions, low O2 tension (hypoxia) is requiered to support a conducive microenvironment during in vitro culture to remain viable during transplantation. This results in the necessity of culture more cells, and cause viability and the potential for stem cells to decline. Hence, it is required a conducive condition for stem cells during culture, through the provision of hypoxic conditions to form Quiescence cells. However, the oconcentration and optimal duration of hypoxia conditions that can be given to in vitro culture of BMSCs in order for Quiescence cells to be achieved are remain unknown .

The role of hypoxia in maintaining Quiscence cells begins with the occurrence of resistance to the expression of the enzyme Prolyl hydroxylases (PHDs), so that Hypoxia inducible factor-1α (HIF-1α) is released and unbound by van Houple Lindau factor (vHL). Further HIF-1α will be bound to Hypoxia Inducible Factor-1β (HIF-1β) to form Hypoxia Inducible Factor-1 (HIF-1) complex. HIF-1α and HIF-1β bonds occur in specific DNA sequences known as Hypoxia reponsive elements 5'-TACGC-3 '(HRE). Complex of HIF-1α and HIF-1β bonds in HRE occurs early in hypoxic exposure and thus causes cell cycle arrest genes expression. This inhibits the expression of p21, resulting in cell cycling inactivation and resistance of senescence and exhausting cells. This is supposed to make the proliferation of cultured stem cells slower, thus Quiscence Cells can be maintained (Arai & Suda, 2008).

Quiscence cells are hardly influenced by culture time in hypoxic conditions. After 48 hours under low O2 tension conditions, the role of HIF-1α will be replaced by HIF-2α with different target genes. Target genes in in vitro culture, hopefully will lead to the expression of pluripotency gene such as

OCT4, SOX2, NANOG. How does LTM of BMSCs be adaptive to slow proliferation and optimal ROS content with pluripotency and undifferentiated of stem cells before transplantation in hypoxic conditions of in vitro cultures has not been described. Based on the description above, it is necessary to conduct a study that discloses the difference between in vitro culture of MSC in normoksia condition (20% O2 level) with hypoxia condition (O2 content 1%) especially in terms of viability, pluripotent properties, and MSC proliferation ability of the culture it produces.

**METHODS**

This research was an explorative laboratory research invitro on Bone Marrow Mesenchymal Stem Cells (BMSCs) culture using hypoxia condition. The study design used Randomized Control Group Post-Test Only Design. This research was conducted for 2 months at Institute of Tropical Disease (ITD) Unair, and Tissue Bank RSUD Dr Sutomo, Surabaya. The implementation of this study lasted from January 2015 to February 2015

The BMSCs experimental unit was taken from 3 healthy male rabbits then propagated in invitro to 21 culture plate. A total of 21 culture plate were divided into 3 groups: control group (P0) of 7 culture plate given normoxia condition with 21% oxygen concentration; the treatment group 1 (P1) of 7 culture plate was given normoxia condition for 24 hours then transferred to hypoxia condition with $O_2$ concentration, and treatment 2 (P2) of 7 culture plate conditioned hypoxia condition (concentration of $O_2$ 1%).

The results of the conditioning of each group were observed for viability and proliferation, as well as the potential plurality properties of pasase 4. Observation of slow proliferation, based on the effect of cell mobilization after scratch test; The observation of viability was based on penthanil blue staining where dead cells absorb blue; Pluripotency observation, based on the expression of gene encoding $OCT_4$ and $SOX_2$.

**RESULTS**

The slow proliferation analysis is an analysis of quiescence cells performed on passage four after treatment, ie, by observing the speed of colonized polyphenation at culture. Observations were performed microscopically using inverted microscope with 400X enlargement, based on slow proliferation of least like CFU-Fs (the smallest number of colonies after hypoxia (02 1%) treatment in the 4th passage (after attaching to the bottom of the petri dish) and the mononucleated cell was found in the buffy coat of aspiration and bone marrow as the treatment group 1, compared with the group cultured with normoxia (02 21%) followed by hypoxic culture, and compared with normoxia culture as control The data obtained were CFU-Fs

interpreted in a quantitative and qualitative Quantitative is done through statistical analysis directly with Anava Factorial with various concentrations of 02 (21, 21-1% and 1%) while qualitatively expressed in macroscopic description form presented descriptively.

Table 1 The least-like number of CFU-Fs in Pasase-4

| No. Sample | Control Group (P0) | Treatment Group (P1) | Treatment Group (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 Mean Σ Least-like CFU-Cs in Pasase-4

| Group | Number of Sample | 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 |

We can see the least-like number of CFU-Fs in tables 1 and 2 shows that control group has the highest average (33.286 ± 1.70). While the least-like number of CFU-Fs was found in the treatment group 2 (P2), which was 6,571 ± 0.72

Table 3 Statistical Test Results Average Comparison of Slow Proliferation by Σ Least-like CFU-Cs (One Way Anova with post hoc test)

| Group | Mean ±SD | Sig. |
|---|---|---|
| Control (P0) | 33.286 ± 1.70 [a] | P<0,001 |
| Treatment 1 (P1) | 16.857 ± 1.03 [b] | |
| Treatment 2 (P2) | 6.571 ± 0.72 [c] | |

Table 3 shows the value of P <0.05, which means there is a significant difference in mean slow proliferation based on the number of Least-like CFU-Cs between the control group, treatment group 1, and treatment group 2.

Figure 1. shows the picture (shape) like CFU-Fs microscopically after the treatments were given different $O_2$ concentrations.

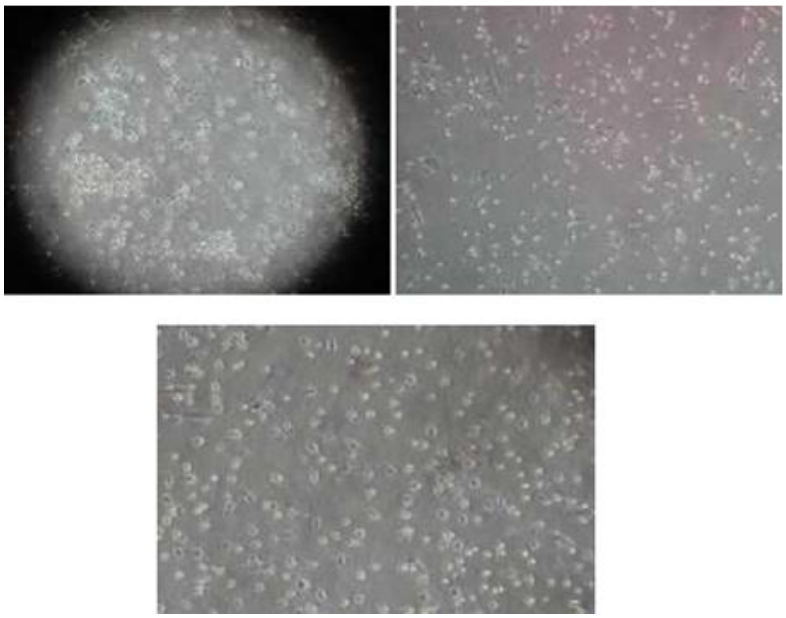


Figure 1. Formed like CFU-Fs after cell attached to petri, using inverted 2000x microscope. A. Normoxia, B Normoxia-Hypoxia, C Hypoxia

Figure 1. It appears that in the treatment of normoxia: the number of CFU-fs is much more and the cell is actually already attached to the dish. In addition, the distance between cells that form a colony is more tenuous. As for the treatment of hypoxia either directly or indirectly: the number of colonies fewer and many cells were still floating (not attact) and colony-forming cells gather more dense and colored a bit yellowish.

**Pluripotency MSC Analysis**

Pluripotency analysis of MSC can be seen by expression of $OCT_4$ phenotype by immunofluorence method. This characterization was aimed at obtaining the expression of gene encoding $OCT_4$ and $SOX_2$ that were immunoflurated in the treatment group of hypoxia one and two but not expressed on 21% $O_2$ normoxia. This showed that $OCT_4$ and $SOX_2$ were expressed as transcription factors so that MSCs were pluripotent and self-renewal. Nevertheless, between treatment 1 and 2 despite the relatively same percentage of cells, there were a significant difference in the number of cells. This suggests that the 24-hour period of normoxia also provides an important role to keep the cell in the petri dish and increase the number of cells with pluripotency properties. Immunofluorescence results from $OCT_4$ and SOX2 at various concentrations of $O_2$ (21, 21-1, and 1%) at passase 4, can be seen in the figure below.

Table 4 Number of Cells Expressing $OCT_4$ Encoding Gene on Immunofluorosense Examination.

| Sample | Control Group (P0) | | | Treatment Group (P1) | | | Treatment Group (P2) | | |
|---|---|---|---|---|---|---|---|---|---|
| | OCT 4 (+) | Cell Total | % | OCT 4 (+) | Cell Total | % | OCT 4 (+) | Total Sel | % |
| 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 Number of Cells expressing $OCT_4$ encoding genes from Immunofluorescence in Section 4.

| Group | Number of Sample | 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 |

It can be seen in table 4 and table 5 that the highest average percentage of cell number expressing $OCT_4$ encoding gene on immunofluorosense examination is in treatment group 2 (P2), that is 82.86 ± 2.65. While the mean percentage of cell numbers expressing the $OCT_4$ coding gene on immunofluorosense examination was at least found in the control group (P0), 1.32 ± 0.55.

Table 6 Statistical Test Results Average Comparison of Slow Proliferation based on $OCT_4$ expression (One Way Anova with post hoc test)

| Group | Rata-Rata± SD | Sig. |
|---|---|---|
| Control (P0) | $1,32 \pm 0,55^a$ | |
| Treatment 1 (P1) | $77,98 \pm 2,04^b$ | P<0,001 |
| Treatment 2 (P2) | $82,86 \pm 2,65^c$ | |

Table 6 shows the value of P <0.001 which means that there is a significant difference in percentage of the average percentage of cell numbers expressing the $OCT_4$ coding gene on immunofluorosense examination between control group, treatment group 1, and treatment group 2.

Table 7 Number of Cells Expressing the $SOX_2$ Encoding Gene on Immunofluorosense Examination

| No Sample | Control Group (P0) | | | Treatment Group (P1) | | | Treatment Group (P2) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $SOX_2$ | Cell Total | Total | $SOX_2$ | Cell Total | Total | $SOX_2$ | Cell Total | 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 8 Average Percentage of Number of Cells Expressing $SOX_2$ Coding Gene from Immunofluorescence Inspection in Pasase-4

| Group | Number of Sample | 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 |

In table 7 and table 8, it can be seen that the treatment group 2 (P2) has highest average percentage of cell number expressing gene encoding SOX2 on immunofluorosense examination is in, that is 92,92 ± 4,31. While the mean percentage of cell numbers expressing the SOX2 coding gene on immunofluorosense examination was at least found in the control group (P0), which was 2.48 ± 0.74.

Table 9 Statistical Test Results Average Comparison Percentage of Cells Expressing Gene $SOX_2$ Coding from Immunofluorescence Inspection in Pasase-4.

| Group | Mean ± SD | Sig. |
|---|---|---|
| Control (P0) | $2,48 \pm 0,74^a$ | |
| Treatment 1 (P1) | $90,00 \pm 3,79^b$ | Sig < 0,01 |
| Treatment 2 (P2) | $92,92 \pm 4,31^c$ | |

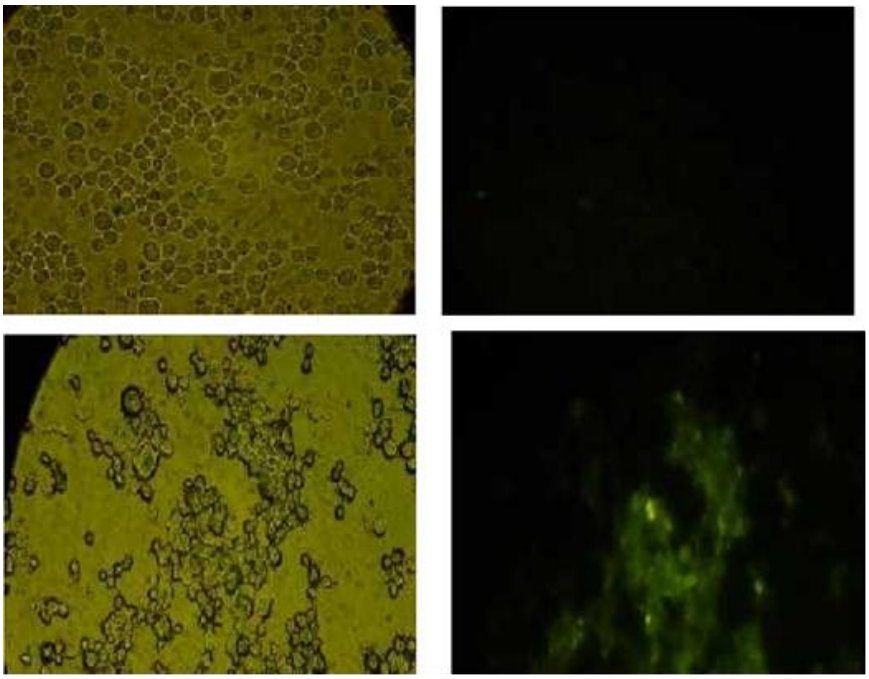
Table 9 shows the value of P <0.01, which means that there is a significant difference in the percentage of the average percentage of cell numbers expressing the $SOX_2$ coding gene on immunofluorosense examination between the control group, treatment group 1, and treatment group 2.

Data compatibility is based on the similarity significance value cut of 60% (85% in $O_2$ culture 1% and 85% in culture $O_2$ 21 to 1%), whereas in normoksia culture ($O_2$ 21%) similarity significance value cut of <60 % (= 50%).

**Expression of phenotype $OCT_4$ and $SOX_2$ by Immunofluorescence**

The characterization of $OCT_4$ and $SOX_2$ of BMSCs by phenotype after culturing by treatment of hypoxic conditions was done through the

Immunofluorescence method. The characterization was aimed to obtain $OCT_4$ and $SOX_2$ expression after treatment of hypoxic condition compared with control ($O_2$ 21%). In this study the expression of $OCT_4$ and $SOX_2$ emerged after treatment of hypoxia conditions, whereas in controls $OCT_4$ and $SOX_2$ were not expressed. This suggests that the treatment of hypoxic conditions causes the emergence of transcription factors so that MSCs become pluripotent in addition to MSCs of bone marrow characterized by multipotency, the presence of hypoxic conditions causes BMSCs to be pluripotency. Nevertheless both transcription factors are expressed with different cell numbers in the treatment of hypoxia with direct $O_2$ 1%, with an incubation of $O_2$ 21% for 24 hours then continued $O_2$ 1% to passage 4. This suggests that the incubation is 24 hours after isolation at normoxia before treatment of hypoxic conditions also plays a role in the development of MSC, where many cells have not been attached when petri is directly conditioned hypoxia. As for the concentration of $O_2$ 21% $OCT_4$ and $SOX_2$ are very small expressed. Immunofluorescence results from $OCT_4$ and $SOX_2$ at various treatments, can be seen in Figure 2



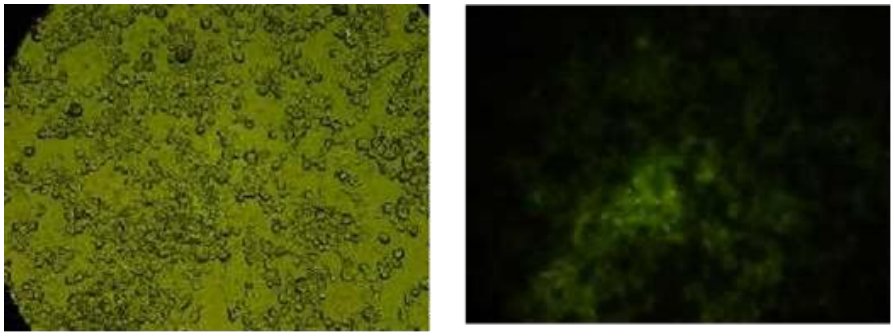


Figure 2 Negative expression of $OCT_4$ in the control group (left unfiltered, right with green filter, no visible green glow). Positive expression of $OCT_4$ in treatment group 1 and 2 (left unfiltered, right with green filter, positive $OCT_4$ glow green) (Top to bottom)

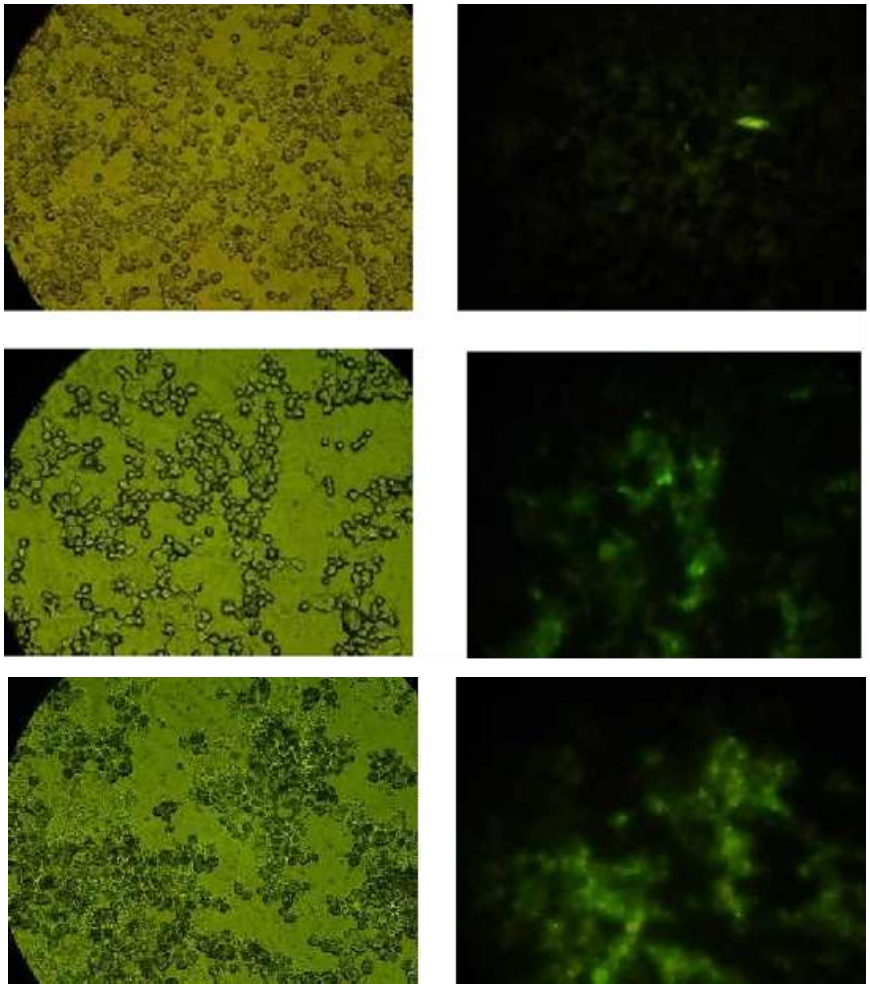


Figure 3 Negative Expression of $SOX_2$ in the control group (left unfiltered, right with green filter, no visible green glow). Positive expression of $SOX_2$ in treatment group 1 and 2 (left unfiltered, right with green filter, positive $SOX_2$ green) (Up and down)

**Number of Viable Cells After Painting with Tripan Blue**

Table 10 The number and percentage of non-absorbing cells from Tripan Blue (viable cells)

| Sample | Control Group (P0) | | | Treatment Group (P1) | | | Treatment Group (P2) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Viable | Cell Total | Total | Viable | Cell Total | Total | Viable | Cell Total | 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 |

Table 11 Average percentage of non-absorbing cells from Tripan Blue (viable cells)

| Group | Number of Sample | 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 |

In table 10 and table 11, it can be seen that the treatment group 2 (P2) has the highest average percentage of non-absorbing color cell number of tripan blue (viable cells), that is 89.29 ± 5.36. While the mean percentage of non-absorbing cell counts from the tripan blue (the viable cell) was obtained at least in the control group (P0), which was 63.67 ± 2.56.

Table 12 Test Results of Statistical comparison of viable cell average

| Group | Mean ± SD | Sig. |
|---|---|---|
| Control (P0) | 63,67 ± 2,56[a] | |
| Treatment 1 (P1) | 86,71 ± 4,31[b] | p< 0,05 |
| Treatment 2 (P2) | 89,29 ± 5,36[b] | |

Table 12 shows the value of p <0.05 which means that there is a significant difference in the percentage of non-absorbing color cell number of trypsin blue (viable cells) between control group, treatment group 1 and treatment group 2. Post-hoc test shows that the mean percentage of viable cell counts between the control group and the treatment group had a significant difference. However, there was no significant difference between the percentage of the number of viable cells between treatment group 1 and treatment group 2.

**DISCUSSION**

**Slow Proliferation Analysis**

Slow proliferation in this study was observed in passage 4. Observation was based on the number of least-like CFU-Fs. There was significant difference (p <0.01) between normoksia 02 21% culture, with incubation normoksia 21% 24 hours later hypoxia 1 %, and directly with 1% $O_2$ culture. This is in the opinion and Koif et al. (2007), which states that slow proliferation can be determined by the smallest number of colony forming

fibroblastasts (like CFU-Fs) units in the control hypoxia control group ($O_2$ 2 1% condition).

It was also found that in hypoxic cultures P1 and P, it was still able to proliferate to form like CFU-Fs until the fourth passage. As for the 21% Normoxia group 21%, proliferating ability forms like CFU-Fs faster than P1 and P2 up to the 4th passage. However, the formation of MSCs in the P1 and P2 group colonies was faster than in the normoxia group. This proves that the treatment of hypoxic conditions leads to controlled proliferation. The explanation is as follows: that, although hypoxic conditions cause slow proliferation based on the smallest amount of CFU-Fs compared to normoxia, but the process of forming MSCs in hypoxic cultures is faster than normoxia

In the normoxia group, it is the opposite, where the amount of like CFU-Fs is very high and significantly different with both hypoxic treatments, but slow to form MSCs. The results of this study are supported by research by Cipolleschi et al. (1993), which states that environmental oxygen conditions (18%) result in an increase in the number of stem cells that become progenitors. This means ironically, according to Cipolleschi et al. (1993), contrary to the fact that normoxia cultured stem cells are capable of proliferating to form CFU-Fs until the 7th day of the culture process (day 7 CFU-FS) but once the mesenchymal is formed, it rapidly becomes progenitor compared to the oxygen environment low (1%). In cultures with a 1% hypoxia condition in the CIpolleschi et al. (1993) causes a decrease in all progenitor types, suggesting that the stem cells in the hypoxic culture are preserved as mesenchymal (not transformed into progenitor cells as in normoxia).

According to Adam and Scadden (2006), slow proliferation is a process of proliferation and a population of stem cells present in the body in order to maintain its existence through its ability to reproduce itself slowly. This ability can be done repeatedly, even allegedly unlimited, but can also be maintained for a relatively long time in vivo. In vitro, however, in the cell culture process, it is generally stimulated to continuously proliferate through a series of passages until cell proliferation becomes limited and until it eventually enters a permanently stalled growth state known as replicative senescence (Duan et al., 2005) . According to Duan et al. (2005), proliferation may also be stalled early due to some exposure to agents that can damage cells. Exposure to such agents, such as: ultraviolet, gamma radiation, treatment with histone deacetylase inhibitors, pharmacological agents, and oxidative stress (hyperoxia). This early stalled proliferation is known as premature senescence.

The results of pluripotency analysis were based on the expression of the phenotype of $OCT_4$ and $SOX_2$ genes immunofluorescence. This result is in accordance with Covello et al. (2006) and Forristal et al. (2010), which states that after 48 hours of hypoxia in stem cell cultures will result in expression of HIF1-α directly upstream regulator and an essential

$OCT_4$ transcription factor to maintain pluripotency. Likewise with other transcription factors such as $SOX_2$ and Nanog, both are also governed by HIF1-α (Forristal et al., 2010) (Figure 7). According to Boyer et al. 2005) says that all three transkniption factors such as $OCT_4$, $SOX_2$ and Nanog retain stemness and suppress genes that cause differentiation. $OCT_4$ and $SOX_2$ are the 2 factors and 4 transcription factors introduced by Takahashi and Yamanaka (2006), which were first obtained in the iPS cells program.

Hence, for both forms of senescence, whether replicative or premature to occur, a special treatment (an in vivo corresponding niche) is required so that cultured cells can replicate repeatedly and unlimited but do not experience both forms of senescence.

Such special treatment is believed to be achievable if proliferation is manipulated into slow (slow proliferation), as in this study. This is in accordance with the research of Panyukhìn et al. (2008) in MSCs from rat given hypoxic conditions. In the study it can be proven that proliferation remains after 2 months of MSCs rat is cultured in 3% hypoxia, whereas in normoksia culture senescence acceleration occurs. Seen in Figure 2 the growth of MSCs from the rat has been complete within 2 months of normoxia culture followed by the formation of stagnant cell growth and the emergence of senescence cells.

**Pluripotency MSCs Analysis**

Pluripotency analysis was based on the phenotypic expression of the $OCT_4$ and $SOX_2$ genes by immunofluorescence. Identification of $OCT_4$ and $SOX_2$ and BMSCc in phenotype after culturing by treatment of hypoxic conditions is done through Immunofluorescence method. The identification was aimed at obtaining $OCT_4$ and $SOX_2$ expression after treatment of hypoxic conditions compared with control (02 21%). This shows that the treatment of hypoxia conditions leads to the emergence of transcription factors $OCT_4$ and $SOX_2$, so that MSC's characterized by multipotency can be said to be pluripotency.

The condition of hypoxia is a condition that resembles the natural state of the stem cell and the quiscent state, so that the cell with the hypoxic condition remains capable of maintaining pluripotency characterized by the expression of $OCT_4$ and $SOX_2$. Whereas in normoxia, although cells have high poliferation, these cells directly differentiate into progenitor cells, resulting in a loss of pluripotency, characterized by the absence of $OCT_4$ and $SOX_2$ expression.

Based on research Yoshida et al. (2009), it is explained that the relationship between hypoxia, HIF1-α and pluripotency genes such as $OCT_4$, $SOX_2$ and Nanog is extremely crucial to produce induced pluripotent stem cells, since the proven evidence of hypoxia improved efficiency and reprogramming. Zadore et al. (2011) state that transplantation of NSCs is also necessary for the hypoxia precondition environment during in vitro culture process. The effectiveness of replacement therapy and stem cells requires

knowledge and mechanisms that affect the early stem cell development process, such as migration, proliferation and stem cell commitment (Buzanskan et al., 2009; Szymczak el al., 2010) and their dependence on oxygen levels under environmental conditions appropriate. It can not be denied that the functional relationship between hypoxia Inducible factor and sternness with transcription factors together that in vivo, the niche of NSCs is a hypoxic condition of very strong dependence on early developmental processes on oxygen levels.

Described by Szablowska-Gadomska et al. (2011), that the hypoxic culture of NSCs is a pathway stabilization. The explanation is as follows:

In low oxygen tension, α subunits of hypoxia inducible factor become stable and accumulated due to prolil hydoxilase (Ivan et al., 2001) as a HIF factor inhibitor (HIF identified as asparaginyl hydroxylase). Both require the oxygen molecule as the substrate. So when asparaginyl is not hydroxylated, transcriptional co-activators such as P3O / CBP-CREB (cAM P-response clement-binding protein) can interact with CAD. So according to Lando et al. (2002b), complete activity and cell response to hypoxia require stabilization of HIF1-α transcription factors and CAD activation. Furthermore, after HIF1-α is stable, it will be located from the cytoplasma in the nucleus of the cell and dimerisation occurs with HIF1-β. According to Wong el al. (1995), HIFi-β also called ARNT can be a heterodimer with HIF1-α. The occurrence of dimerization between HIF1-α and HIF1-ß requires bHLH domains and some parts of the PAS domain. It is also said that bHLH domains are of great importance for the binding of the HIF1-α + HIF1-β complex in DNA (Bardos and Ascheroft, 2005). The next complex HIF (HIF1-α + HIF1-β.) Is bound to a specific sequence and DNA known as HRE (Hypoxia response elements). The introduction of the HIF1-α + HIF1 -ß complex on HRE ends in the basic domain (N terminal end) (FandreY el al., 2006). Zagoska and Dulak mentioned that HRE is localized in any target gene targeting. On the contrary, normoxia occurs pathway degradation and HIF1-α. The explanation is as follows Hipoxia inducible factor is regulated by oxygen sensitive hydolation of HIF1-α subunit (Tuckerman et al., 2004) HIF1-α contains many proline amino acids in known and proxy hydroxylase domain PHD (prolyl hydroxylase domain ). The PHD catalyzes this reaction through the addition of an oxygen to Pro-402 and / or Pro-564 and converts it to 4-hydroxyproline (Maxwell, 2005; Berra et al., 2006). In this case oxygen and 2-oxoglutarate are required for the PHD activity consisting and 3 isoforms: PHD1, PHD2 and PHD3. The activity of these three equally shows dependent oxygen (Tuckerman el al., 2004). Under conditions of normoxia HIF1-α is uhiquilinuted and degraded through 265 proteasome (KalIio et al., 1999). According to Fandrey et al. (2006), Von Hippel Lindau (pVHL) vital protein suppressor tumor

regulates the ubiquitination process through the specilic ubiquion E3 ligase complex, binding pVHL to hydroxylase HIF1-α subunits (Fig. 6.7). Based on the above mentioned literature supporting the results of this study, that the degradation of HIF1-α ends in $SOX_2$ expression in genotype and phenotype.

The results of this study, in the form of pluripotent activity after hypoxic treatment until the 4th passage, through the phenotypes of OCT4 and SOX2, support one of the main focuses on adult stem cell studies in vitro. This is because, in vivo required a balance between differentiation, apoptosis and self-renewal and stem cells. The balance, one of which is regulated by the niche and microenvironment where the stem cells are located. According to Banna et al. (2004), in in vitro feeder-free cultures, the fate of stem cells in addition to being influenced by growth factors, interleukins or serum, in affecting the balance between self-renewal, differentiation and apoptosis, also influenced the conditions given during the culture process, which in this study a 1% hypoxia condition.

Pluripotency is the potential to be able to differentiate into any body cell and all three embryonic layers (ectoderm, mesoderm and endoderm). The potential of these three embryonic layers is generally characteristic of embryonal stem cells (ESCs) (Szablowska-Gadomska et al., 2011). According to Halim et al. (2010), says that compared to progenitor cells that have unipotent properties, stem cells, especially ESCs have much greater potential differentiation. The originated ESCs and the inner cell mass (1CM) in the blastocyst are pluripotent, thus being able to differentiate into different types of body building cells and the three embryonic layers including nerve cells, blood cells, cardiac cells and immune system cells. In this study, MSCs, which are adult stem cells in general, this study of MSCs, which are adult stem cells that are generally characterized as multipotent, can express both genes and phenotypes of OCT4 and SOX2 genes after hypoxia treatment at in vitro cultures. Therefore, it is suspected that in the future MSCs with hypoxia precondition have pluripotent potential. This assumption is reinforced by the opinion of researchers (Takahashi et al., 2007; Takahashj and Yamanaka, 2008; Yu et al., 2007) that efficiency and reprogramming iPS cells is very high in hypoxic conditions (Figure 8)

**The viability analysis of MSCs**

The viability of cultured cells was analyzed by tripan blue painting. Dead cells (not viabel) will absorb the blue tripan color. The calculation results show that most cells absorb tripan blue color (not viable) that is on, control, then P1 and group P2. This suggests that the normoxia culture conditions result in many cells experiencing death, unable to adapt to high oxygen conditions. Cell death in normoxia is due to mitochondrial dysfunction and caspase-dependent apoptosis. (Zhu W, et al., 2006). Cells cultured under normoxia will experience apoptosis of the nucleus and chromatin condensation. The condition of normoxia will increase caspase 3 which will

initiate the process of porteolysis and cell apoptosis. In addition, the hypoxic condition also results in mitochondrial dysfunction characterized by the release of cytochrome c into the cytosol, which signifies mitochondrial membrane damage, and subsequently cytochrome c will also initiate apoptosis.

From this study it was found that cells cultured in hypoxic conditions also experienced cell death, however, the number of cells experiencing death in groups cultured with precursors of normoxia before hypoxia was not as much as that of cells directly cultured under hypoxic conditions. This suggests that the precondition of culture with normoxia provides an opportunity for cells to adapt and proliferate before being conditioned in hypoxic cultures.

## CONCLUSION

Mesenchymal stem cell culture results with this hypoxia condition can be concluded:

1. Hypoxic culture condition at 1% O2 concentration causes the cell to experience slow proliferation compared with normoxia condition.
2. Hypoxic culture conditions at 1% O2 concentrations, either directly or with the preconditions of normoxia, are capable of maintaining pluripotency properties of mesenchymal stem cells, characterized by the expression of the OCT4 and SOX2 proteins.
3. Hypoxic culture condition at 1% O2 concentration resulted in decreased cell viability less than culture with normoxia.
4. Cultures with hypoxic conditions and preconditions of normoxia are the best culture conditions because they produce cells that are capable of maintaining pluripotency properties while still having better proliferation and viability capability compared with direct hypoxia conditions.

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