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by Erma Safitri

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Hypoxic Preconditioning For Viable and Self Renewing Mesenchymal Stem Cells (Mscs) As the Regeneration of Spermatogenesis Process

[1,2]Erma Safitri, [1]Suzanita Utama, [2,3]Candra Bumi, [2,4]Sri Wigati Mardi Mulyani, [5]Endang Retnowati, [2,6]Purwati, [7]R. Heru Prasetyo, [1]Mas'ud Hariadi, [8]Aulani'am, [2,9]Ferdiansyah Mahyudin, [2,10]Fedik Abdul Rantam

[1] Department of Veterinary Reproduction Faculty of Veterinary Medicine Airlangga University
[2] Stem Cells Research Divison of Institude Tropical Disease (ITD), Airlangga University
[3] Department of Epidemiology and Biostatistics Public Health, Jember University
[4] Department of DentoMaxillo Facial Radiology, Faculty of Dentistry, Airlangga University
[5] Department of Clinical Pathology, Faculty of Medicine, Airlangga University
[6] Department of Internal Medicine, RSUD Dr. Soetomo Surabaya
[7] Departement of Parasitology, Faculty of Medicine, Airlangga University
[8] Department of Biochemistry Faculty of Life Sciences, Brawijaya University
[9] Departemen of Orthopedic & Traumatology RSUD Dr. Soetomo Surabaya
[10] Department of Vet. Microbiology and Virology Faculty of Vet. Med. Airlangga University

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ABSTRACT

The aim of this research was to obtain MSCs that were viable and self renewing for spermatogenesis process by a treatment of hypoxic precondition in vitro culture. In this research, hypoxic precondition was the use of 1% O_2 concentration which was compared to those of culture under normoxic (21% O_2) condition. Flowcytometric analysis showed that in MSCs culture under 1% O_2 concentration, the level of $CD90^+$ and $CD34^-$ were not altered (remained undifferentiated), meanwhile under 21% O_2 concentration, cells have experienced alteration (became differentiated), that was indicated by the down regulation of $CD90^+$ and up regulation of $CD34^-$. Immunocytochemical and immunofluorescence analysis showed that under 1% O_2 concentration, MSCs culture expressed transcription factors, such as OCT4 and SOX2, meanwhile under 21% O_2 concentration, the transcription factors OCT4 and SOX2 (self renewal function), were not expressed. In conclusion, this research showed that hypoxic preconditioning with 1% O_2 concentration very supported MSCs to remain viable before transplantation for spermatogenesis disorder, because the cells still undifferentiated and self renewal capacity was maintained.

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INTRODUCTION

Cell transplantation therapy of mesenchymal stem cells (MSCs) from bone marrow provides a very promising solution for the regeneration of spermatogenesis process in oligospermic patient (Kilani, 2009). However, the low viability of the transplanted MSCs for the regeneration of normal testis function to produce spermatozoa caused the limitation of efficacy of this therapy (Tang *et al.*, 2005; Kenichiro *et al.*, 2005). Studies on stem cell by Suzuki (2004), Geng (2003) and their co-workers revealed that 93-99 % of the stem cells injected died three to four days after injection which indicated that microenviroment in the degenerative tissue or body organs of patients were not conducive for the viability of the stem cells. The estimated mechanism accounted for the decreasing survivability of stem cells was the high amount of the stem cells underwent differentiation and senescence (not self renewing) prior to transplantation to patients. Therefore the retainment of undifferentiated state and self renewal capacity of MSCs before implantation were very important for stem cells' viability and subsequently the efficacy of stem cell therapy.

Therefore, preconditioning stem cells with hypoxia (1% O_2) during in vitro culture, which is an adjustment to the in vivo niche of the stem cells, need to be conducted in an attempt to increase viability after transplantation to oligospermic patient.

Corresponding Author: Erma Safitri, Department of Veterinary Reproduction Faculty of Veterinary Medicine Airlangga University
E-mail: rma_fispro@yahoo.com

Research Methods:

Procedure of rabbit MSCs isolation and culture:

Rabbit was premedicated and general anesthetized. MSCs from bone marrow was harvested by an aspiration at the middle of femur bone below the condylus. Aspirate contained MSCs from bone marrow was placed in heparinized tubes. Sample in tube was placed in thermos maintained at 4°C during transportation to be processed in stem cell laboratory.

Sample was transferred into 15 ml sterile blue capped tubes and then the tube was rinsed twice with 5 ml sterile Phosphate Buffered Saline (PBS). PBS was added up to a total volume of 10 ml. The diluted sample was loaded over a same volume of Ficoll in a separate 15 ml tube. Centrifugation at 1600 rpm was performed for 15 minutes at room temperature. After centrifugation, the cells were collected from Ficoll-PBS interface using sterile pasteur pipette and transferred into a 15 ml tube. The cells were then resuspended in PBS up to a total volume of 15 ml. The tube was inverted gently 5 times to homogenize the suspension. The suspension was then centrifuged again at 1600 rpm for 10 minutes. Supernatant and floating cells were discarded and cell pellet was resuspended in 6 ml of α MEM media. Mononucleated cells were plated in 10 cm^2 plates at 2.10^7 and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours to let the cells adhere. After 24 hours, media and non-adherent cells were discarded. Adherent cells were rinsed twice using 5 ml of PBS. Ten ml of fresh α MEM media was then added into dish and the dish was returned into the incubator. Culture was observed daily under an inverted microscope. Every 4 days medium was changed, preceeded by a rinse using 10 ml PBS then 10 ml of fresh α MEM media were replaced. Culture was continued until approximately 75-80% confluence was attained. After confluence, cells were passaged into several dishes for subculture (Rantam *et al.*, 2009). Passage was conducted 3 times, then cells were divided into two hypoxic precondition treatments of 1% in hypoxic chamber inside a 5% CO_2 incubator while another treatment was the use of 21% oxygen (normoxia).

At the fourth day after hypoxic precondition treatments cells were analysed for the expression of surface marker CD90 and CD34 by flowcytometry, and for transcription factors OCT4 and SOX2 by immunocytochemistry and immunofluorescence microscopy. Flow cytometric analysis was performed using a flowcytometer (FACSCalibur) and CellQuest software. Antibodies used were monoclonal FITC-conjugated anti-rabbit CD90 (Biossusa) and PE-conjugated anti-rabbit CD34 (BD). Antibodies used for immunocytochemical and immunofluorescence analysis were polyclonal FITC-conjugated anti-rabbit OCT4/POU5F1 (BioLegend) and polyclonal FITC-conjugated anti-rabbit SOX2 (BioLegend).

Research Results and Discussion:

Flowcytometric analysis showed that under 1% O_2 concentration, the level of $CD90^+$ and $CD34^-$ cells in MSCs culture were not altered (still undifferentiated). Meanwhile under 21% O_2 concentration, cells have experienced alteration (became differentiated), that was indicated by the decrease of $CD90^+$ and the increase of $CD34^-$ cells (Figure 1 - 3).

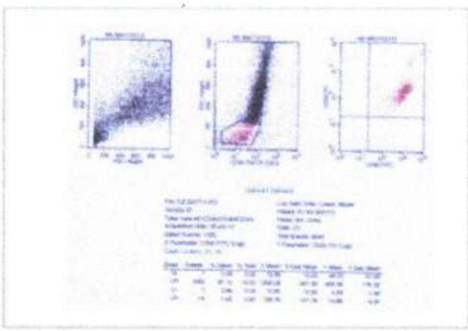

Fig. 1: Flowcytometric analysis of MSCs culture before hypoxic preconditioning (Control) showing positive expression of CD90 (98.66%), and negative expression of CD34 and CD45 (0.68% and 0.68% respectively).

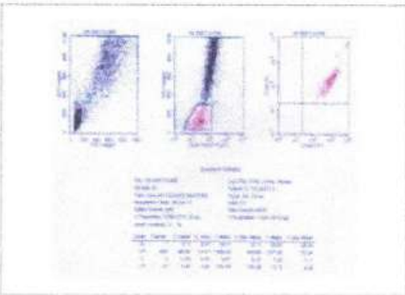

Fig. 2: Flowcytometric analysis of MSCs culture under hypoxic precondition (1% O_2 concentration) showing positive expression of CD90 (99.66%) and negative expression of CD34 and CD45 (0.11% and 0.23%).

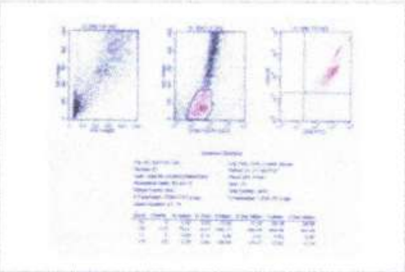

Fig. 3: Flowcytometric analysis of MSCs culture under 21% O2 concentration (normoxia) showing positive expression of CD90 (79.07%) and negative expression of CD34 and CD45 (0.35% and 0.59%).

Immunocytochemical staining showed the expression of transcription factor (self renewal function) such as OCT4 and SOX2 in the MSCs culture. Meanwhile under 21% O_2 concentration, transcription factor OCT4 and SOX2 were not expressed (undetected by immunocytochemistry and immunofluorescence staining) (Figure 4 - 7).

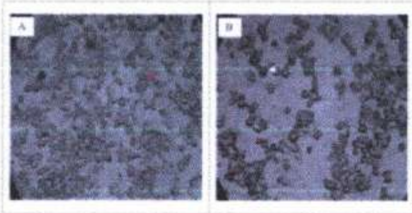

Fig. 4: Immunocytochemical analysis of MSCs culture under hypoxic precondition (1% O_2 concentration). **A.** Positive expression of OCT4 (red arrow head). **B.** Positive expression of SOX2 (yellow arrow head).

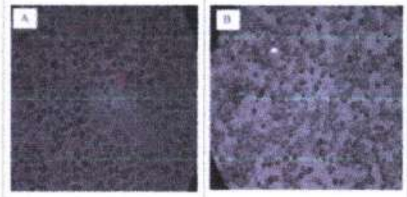

Fig. 5: Immunocytochemical analysis of MSCs culture under 21% O_2 concentration (normoxia). **A.** Negative expression of OCT4 (red arrow head). **B.** Negative expression of SOX2 (yellow arrow head).

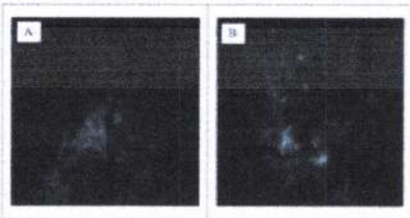

Fig. 6: Immunofluorescence analysis of MSCs culture under hypoxic precondition (1% O_2 concentration). **A**. Positive expression of OCT4 showing green fluorescence. **B**. Positive expression of OCT4 showing green fluorescence.

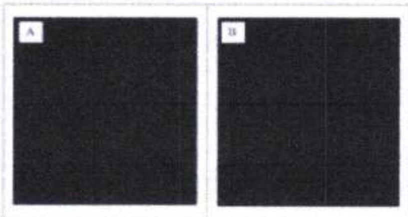

Fig. 7: Immunofluorescence analysis of MSCs culture under normoxic (21% O_2) condition. **A**. Negative expression of OCT4. **B**. Negative expression of SOX2.

Based on the results of this research, it could be explained that hypoxic precondition of 1% O_2 concentration caused the inhibition of Prolyl hidroxylases (PHDs) enzyme expression which caused the formation of Hypoxia Inducible Factor-1 (HIF-1) complex. This HIF-1 complex caused cell cycle arrest gene expression. Furthermore, 48 hours after cell cycle arrest genes expression, caused the expression of transcription factor (pluripotency genes) like OCT4, SOX2, NANOG and REX-1. Transcription factor is a component that is capable to stimulate stem cells to proliferate to be theirselves (self renewal), therefore these cells are always young and do not undergo senescence (aging).

Transcription factor which is the pluripotency genes (capable to differentiate into whatever cells needed by the damaged body) was estimated to be activated by Hypoxia Inducible Factor 2α (HIF 2α) after the initiation by Hypoxia Inducible Factor 1α (HIF 1α). The pluripotency genes which were observable through the expression of transcription factors such as OCT4 dan SOX2 in this research could influence MSCs to be always self renewing themselves.

Therefore hypoxic preconditioning using 1% O_2 concentration for MSCs culture can support to mantain viability and self renewal potency until transplantation to oligospermic patient with spermatogenesis disorder. This is caused by the remain undifferentiated and self renewing stem cells.

Hypoxic preconditioning using 1% O_2 concentration caused HIF-1α release from van Houpel Lindau (vPL) which was then accumulated in nucleus. The high level of HIF-1α would inhibit Reactive Oxygen Species (ROS) that acted as free radical. The inhibition of ROS would inhibit the expression of protein genes P53 and P21. Therefore, cell cycle arrest genes were sensitized, which ended up with the slow proliferation and maintenance of stem cells. This maintenance was also supported by the reduced ROS by the role of HIF-1α therefore p53 gene expression was inhibited. The inhibition of p53 gene expression caused an inhibition of the opening of mitochondrial membrane pt pore. Therefore, cytochrome C that acted as apoptotic protease activing factor-1 (APAF-1) caused inhibition of the release of various caspases (Caspase 9 and Caspase 3) as apoptotic cascade. The inhibition of P53, cytochrome C and caspases would cause the inhibition of cell death of the cultured stem cells. Meanwhile, the decreased P21 caused an inhibition of the active cycling cell which prevented cell senescence process from happening.

Conclusion:

From the research results, it could be concluded that hypoxic preconditioning with 1% O_2 concentration was very supportive to MSCs in maintaining cells viability before transplantation for spermatogenesis disorder therapy, because cells remained undifferentiated and had the potency for self renewal.

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