VIABILITY OF RABBIT ADIPOCYTE STEM CELLS CULTUREDUNDER DIFFERENT OXYGEN CONCENTRATIONSIN VITRO

by Erma Safttri

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ORIGINAL ARTICLE

VIABILITY OF RABBIT ADIPOCYTE STEM CELLS CULTURED UNDER DIFFERENT OXYGEN CONCENTRATIONS *IN VITRO*

Erma Safttri[1], Pudji Srianto[1], Thomas Valentinus Widiyatno[1], Willy Sandhika[2] and R. Heru Prasetyo[*2]

[1]Faculty of Veterinary Medicine; [2]Faculty of Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia

ABSTRACT

The aim of this research was to determine if rabbit adipocyte stem cells (r-ASCs) can still be viable before transplantation when these are cultured *in vitro* under hypoxic preconditioning (3% O_2 concentration). Samples were divided into two groups, one under hypoxic preconditioning and the other in hyperoxia (20% O_2 concentration). Observations were made through flow cytometry, immunofluorescence and immunocytochemical analyses. Flow cytometric analysis showed that in r-ASCs hypoxic culture, the levels of CD90+, CD44+ and CD45- were unaltered, but changed under hyperoxic culture, indicated by the down-regulation of CD90+ and CD44+, and up-regulation of CD45-. Meanwhile, immunocytochemical and immunofluorescence analysis showed that under hypoxic preconditioning, r-ASCs culture expressed quiescent cells with p63 as marker, but the latter remained unexpressed in hyperoxic culture. In conclusion, hypoxic preconditioning with 3% O_2 concentration supported r-ASCs in sustaining viability before transplantation in rabbit.

Key words: hyperoxia, hypoxic preconditioning, rabbit, r-ASCs, viability

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INTRODUCTION

Sustaining the viability of stem cells is necessary before transplantation for use in several diseases, especially in cases involving degenerative tissues. This viability is what makes stem cells become adaptive and useful in achieving successful transplantation therapies.

The results of the study by Geng (2003) showed that the viability of mesenchymal stem cells (MSCs) injected in rats was very low, 99% of the injected MSCs were already undergoing apoptosis after day 4. This remarkably low viability may be associated with the damaged microenvironment of the tissue or organ, failing to confer resilience to the stem cells. Increasing the viability, then, of transplanted stem cells is essential in improving the success rate in stem cell therapy.

The adaptability and viability of MSCs from bone marrow can be achieved by subjecting them to hypoxic preconditioning *in vitro* culture before transplantation (Safitri *et al.*, 2013; Safitri *et al.*, 2014a). Hypoxic preconditioning has proved that MSCs from bone marrow did not only confer viability and adaptability but also allowed stem cells to express quiescence *in vitro* as observed *in vivo*. Stem cell quiescence *in vivo* is known to inhabit the system long-term (Safitri *et al.*, 2014b).

The long-term maintenance of MSCs

*FOR CORRESPONDENCE:
(email: rheru_prasetyo@yahoo.co.id)

depends on the interaction with their optimal niches, especially specific microenvironments (Arai and Suda, 2008). The specific microenvironments with optimal niches is critical to ensure lifelong tissue maintenance and to protect the stem cell pool from senescent cells (premature exhaustion) (Tsai *et al.*, 2011), and apoptosis (Wang *et al.*, 2008) under various stresses.

This study was conducted to determine the viability of rabbit adipocyte stem cells (r-ASCs) subjected to hypoxic preconditioning *in vitro* with 3% oxygen concentration. This study serves as a new approach in sustaining viability in ASCs, based on the unaltered expression of CD90+, CD44+, and CD45- and quiescent cells expression with p63 as marker.

MATERIALS AND METHODS

Animal studies were performed in accordance with the procedures approved by the Animal Care and Ethical Clearance Committee of the Faculty of Veterinary Medicine, Universitas Airlangga. Experiments also conformed with the National Research Council's guidelines (239-KE) through an ethical seminar. The research was conducted at an experimental laboratory within the Institute of Tropical Diseases and Veterinary Medicine Faculty, Universitas Airlangga.

Isolation and culture of r-ASCs

Adipose tissue was first aspirated from the peritoneal region of one 6-month old male rabbit (New Zealand strain). Aspirate was stored and kept at 4°C and transported to the experimental laboratory at the Institute of Tropical Diseases, Universitas Airlangga for *in vitro* culture. Adipose tissue was first washed and separated from the mixture of stomach fluid, blood and fats, and further separated using a sterile separator funnel. Samples were washed with sterile saline solution and heated at 37ºC repeatedly until clear. The funnel was reversed for 4-5 min then closed; its position was kept still for 3-5 min to allow for separation. For tissue digestion, the same volume as that of the sample was prepared, along with warm sterile saline buffer containing 500 CDU/ml (equivalent to 0.5 Wunsch units/ml) of collagenase. Washed fat was poured from the separatory funnel into a sterile bottle which can hold four times the aspirate volume. Saline buffer or collagenase mixture was added, covered and placed on a warm shaker that was prewarmed at 35-38ºC for 20±5 min. Frequency and amplitude of the shaking was set at a point that would allow mixing but prevent the separation of the floating adipose tissue from the collagenase liquid.

Tissue was then transferred into a sterile glass separatory funnel and allowed to settle for 5-10 min to begin phase separation. The next stage of the stopcock was opened, and the non-floating fraction, through a sterile 265 mm filter, was obtained in a sterile beaker. Aliquots of the non-floating solution were then collected in a beaker glass and placed in a 50 ml centrifuge tube and mixed at 400 g for 5 min at room temperature with low to medium speed. Supernatant aspirate (top layer) was slowly poured in a tube without pellets from the cell. Finally, the suspension was filtered in a 100 mm cell strainer and collected in a sterile tube (Rantam *et al.*, 2009).

In vitro r-ASCs culture in hypoxic and hyperoxic preconditioning

Samples were centrifuged, then supernatant and cell suspension were aspirated and transferred into a 100 µl stromal inoculation of the cell medium placed in a plate. About 500 mg of adipose tissue were added with stromal medium until the container was full. Seventy-two hours after plating, aspirate was added in the medium. Next, the cell was washed with pre-warmed PBS (1% antibiotic added in solution), doing an up and down pipetting to gently clear the cells from tissue and blood cell fragments. Fresh stromal medium was added, just until well capacity of culture plate was reached. The subsequent medium was changed every 2-3 days until the cell has formed confluence of 80-90%, then the cells were harvested. This was done by removing the medium well first and storing it in a sterile tube under sterile media conditions to be used for subsequent cell culture applications. Next, 250-500 µl of cells

were added to the sterile warm PBS, letting the PBS be above the cell for 2 min. Furthermore, PBS was mixed with 500 µl trypsin/EDTA solution (0.5%) and incubated for 5-10 min. At this point, observation under the microscope showed that 90% of the cells were removed. Five hundred µl stromal medium were then added to fill the serum in solution. This was used to neutralize trypsin reaction.

The medium which contains the cell suspension was transferred from the well into a sterile tube, centrifuged at 300 g for 5 min. Small amounts of aspirate of supernatant and cell suspension were mixed with stromal medium. Furthermore, a counting process was done using a cell dilution aliquot in trypan blue (for a 1:7 dilution 12.5 µl cell suspension was added for every 87.5 µl trypan blue. Cells were counted using a hemocytometer. After this, cells were placed back in the appropriate culture plate cells (Rantam *et al.*, 2009). Passage was conducted thrice, then cells were subjected to two preconditioning treatments: (1) hypoxic chamber with only 3% O_2 placed in an incubator with 5% CO_2; and (2) hyperoxic environment with 20% O_2 concentration.

Flow cytometry observation of CD90+ and CD44+ expression

Flow cytometry analysis was utilized to determine the expressions of CD90+ and CD44+. First, fat-free aspirate was centrifuged at 6000 rpm for 15 min at 4°C. The precipitate was mixed with cytoperm/cytofix two times the amount of cell number obtained. This mixture was centrifuged again and formed a supernatant and a pellet. BD wash was added to the pellet at four times the amount of cell number obtained from the first centrifugation. Mixture was centrifuged and added with lysis buffer at the amount twice that of the first obtained cell number. Subsequently, labeled antibody was added to each sample, arranging five tubes at once and processing in a similar manner. Wash tube was added with single staining with CD90 FITC and double staining with CD44 PE and CD45 PerCP. Finally, all samples were stored in 4°C in a darkroom and were analyzed with flow cytometry for 1 h (Macey, 2007).

Immunocytochemical analysis

Immunocytochemical (ICC) analysis was performed to determine the expression of p63 as marker of quiescent cells. Before the ICC methods were carried out, cytological preparation and further examination through immunocytochemical technique using monoclonal antibodies p63 was conducted. Observation on p63 expression was done using a light microscope. P63 expression is indicated by the number of cells with brownish chromogen discoloration (Kumar and Rudbeck, 2009).

Immunofluorescence analysis

Immunofluorescence analysis was performed to determine the expression of quiescent stem cells using p63 protein as marker (Safitri *et al.*, 2014b). After hypoxic preconditioning of r-ASCs, indirect immunofluorescence was done to detect p63 protein expression. The r-ASCs cell culture was harvested, placed in a 15 ml tube and fixed with methanol. After 15 min, each culture was coupled with a rabbit anti-p63 protein/ P51A polyclonal antibody reagent, FITC conjugated primary antibodies (bs-0723R-FITC-Biossusa), were washed with PBS, then dropped on an object glass, and analyzed under a fluorescence microscope (Rantam *et al.*, 2014).

Statistical analysis

Expressions of CD90+ and CD44+ with flow cytometry and p63 with immunocytochemical method were statistically analyzed using SPSS 15 for Windows XP with the level of significance set at 0.01 and confidence level at 99%. Comparative hypothesis testing was done by testing data normality with Kolmogorov-Smirnov test, doing homogeneity of variance test, analysis of variance (ANOVA), and post-hoc test (least significant difference test) using Tukey's HSD at 5%.

Phenotype expression results were analyzed by immunofluorescence. Phenotype expressions analyzed were done on p63 expression as the marker for quiescence stem cells.

RESULTS AND DISCUSSION

The level of expressions of CD90+, CD44+, and CD45- in cells analyzed with flow cytometry showed that under hypoxic preconditioning in 3% O_2 concentration, r-ASCs culture were undifferentiated. Meanwhile, under 20% O_2 concentration, cells differentiated, as indicated by the decreased expression of CD90+ and CD44+ (Fig. 1A-1C).

Based on Figure 1, the phenotype showed no significant differences between hypoxic treatments and control, suggesting that stem cells cultured in this study remained undifferentiated. Attaining undifferentiated cells was one of the main objectives of this

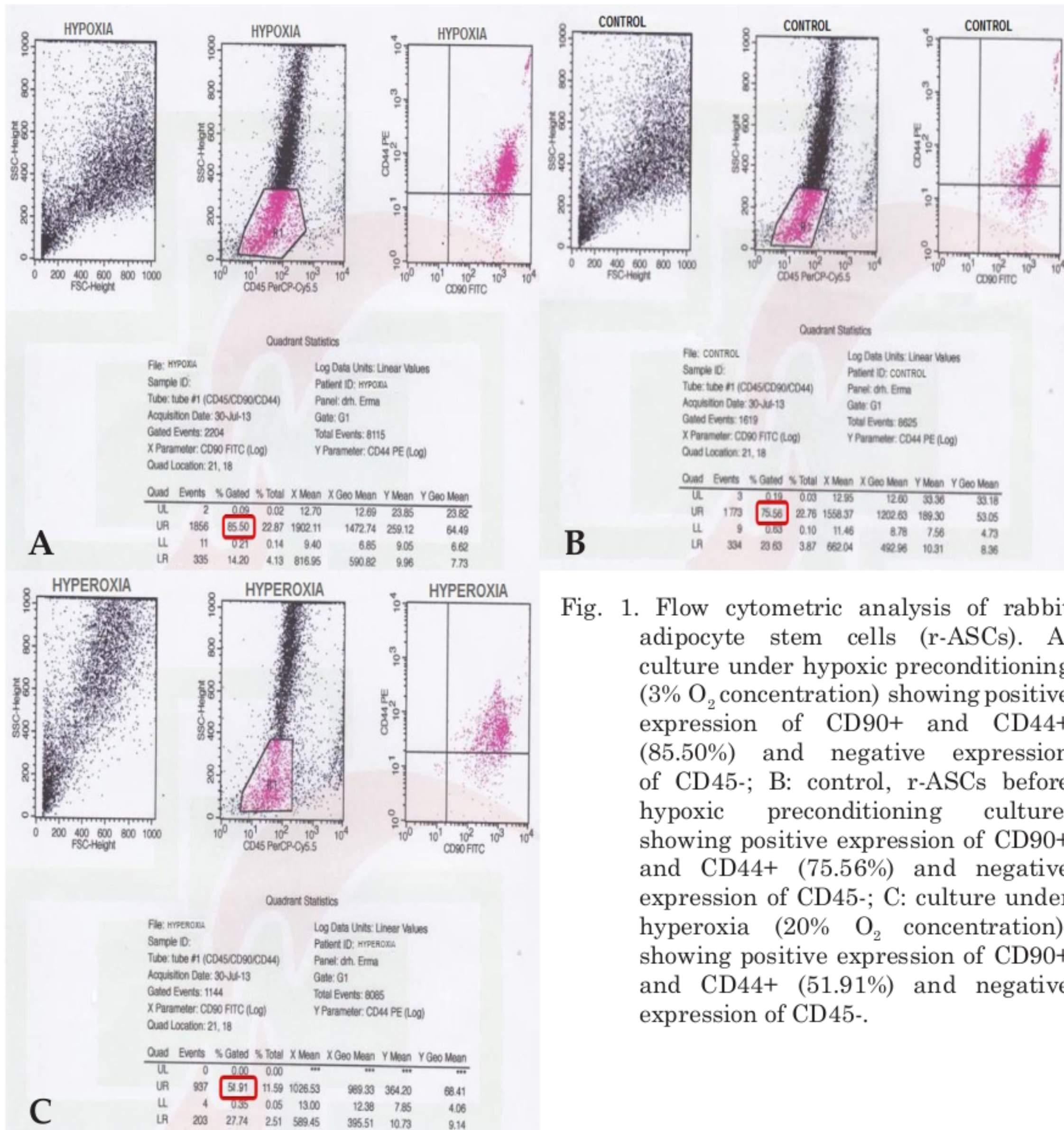

Fig. 1. Flow cytometric analysis of rabbit adipocyte stem cells (r-ASCs). A: culture under hypoxic preconditioning (3% O_2 concentration) showing positive expression of CD90+ and CD44+ (85.50%) and negative expression of CD45-; B: control, r-ASCs before hypoxic preconditioning culture, showing positive expression of CD90+ and CD44+ (75.56%) and negative expression of CD45-; C: culture under hyperoxia (20% O_2 concentration), showing positive expression of CD90+ and CD44+ (51.91%) and negative expression of CD45-.

study, since this is a key characteristic of long-term maintenance in stem cells. The results of this study is consistent with the report of Halim *et al.* (2010), which stated that undifferentiation is one of the unique characteristics of stem cells that sets it apart from other cells of the body.

The existence of this unique undifferentiated stem cells means that the *in vitro* cell culture in this study can survive much longer than the progenitor or the mature stem cells. This is based on the studies by Takubo (2012) and Elliason *et al.* (2010), where they emphasized that long-term maintenance (LTM) must be manifested by stem cell cultures to ensure the viability of transplanted stem cells. LTM, according to both researchers, can be attained when the stem cells are in the G0 phase when transplanted. G0 stage is the stage in which cultured cells are not bound to undergo the rest of the cell division (G1/S/G2/M) (Hermitte *et al*., 2006; Arai and Suda, 2008). According to Morrison and Spradling (2008), Mohyeldin *et al.* (2010) and Suda *et al*. (2011), this stage is characterized by the presence of dormant or quiescent cells, and it is likely to occur if there is a conducive microenvironment for the stem cells.

The unique feature of stem cells is its ability to make it possible for any undifferentiated cell to become any kind of cell upon transplantation. This is the case of stem cells *in vivo*, such that, when under undifferentiated conditions, cells can differentiate into more than one cell type (multipotent/pluripotent). Thus, if undifferentiated cells' viability *in vitro* can be sustained, then cells that need to be transplanted can differentiate into any type of desired cell.

Immunocytochemical staining and immunofluorescence analysis enabled the expression of p63, the viability factor of mesenchymal stem cells. This positive expression was evident in r-ASCs cultured in hypoxic preconditioning at 3% O_2 concentration (Fig. 2A and 2B). Meanwhile, p63 expression was absent in cultured cells subjected to 20% O_2 concentration (Fig. 3A and 3B).

Hypoxic preconditioning at 3% O_2 concentration caused r-ASCs to remain undifferentiated and viable. This feature in stem cells is important to ensure long-term tissue maintenance (Arai and Suda, 2008) and to protect the stem cell pool from premature exhaustion or turning into senescent cells (Tsai *et al*., 2011) and undergoing apoptosis (Wang *et al*., 2008). The lifelong maintenance of stem cells largely depends on the interaction with their specific microenvironment called niche (Arai and Suda, 2008).

In this study, hypoxic preconditioning with 3% O_2 concentration *in vitro* niche resembles the *in vivo* microenvironment. Specifically, under hypoxic preconditioning, inducible factor-1α (HIF-1α), a transcription factor, is stabilized. This means that HIF-1α levels are elevated in stem cells, which regulate their metabolism (Simsek *et al*., 2010; Takubo *et al*., 2010).

The one mechanism relating to the favorability of hypoxic conditions for stem cell maintenance is the oxygen-dependent hydroxylation of amino acids on specific α subunit of the hypoxia-inducible transcription factors (HIFs) (Watt *et al*., 2009). The activity and stability of HIF-1α protein subunits eventually allow their targeting for proteosomal degradation. In this study, as oxygen levels drop below 3%, hydroxylation becomes inhibited, stabilizing the HIF-1α subunits to bind with HIF1ß, and then bind to hypoxia response element (HRE) in target genes, and, in association with transcriptional co-activators, turn these genes on.

Genes activated by HIFs include those encoding proteins involved in cell proliferation, self renewal and survival. Self renewal markers like SOX2 and OCT4 sustain undifferentiated state (Mohyeldin *et al*., 2010).

Being undifferentiated is the basic characteristic of ASCs. In this study, undifferentiated state is indicated by the level of CD90+, CD44+, and CD45- expression in r-ASCs culture. Flow cytometric analysis of r-ASCs culture under hypoxic preconditiong with 3% O_2 concentrations showed positive expression of CD90+ and CD44+ (85.50%) (Fig. 1A), which was not significantly different (P>0.05) with the control (before hypoxic preconditioning). The control group showed positive expression of CD90+ and CD44+ (75.56%) (Fig. 1B). Meanwhile, under

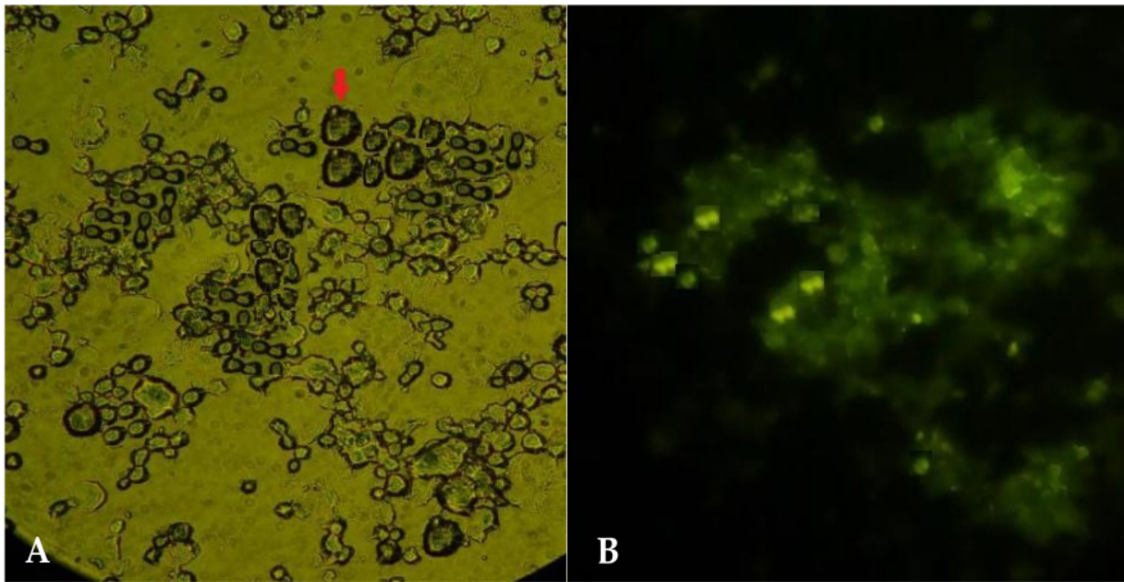

Fig. 2. Rabbit adipocyte stem cells (r-ASCs) cultured under hypoxic preconditioning (3% O_2 concentration). A: Immunocytochemical analysis: positive expression of p63 (red arrow head) expression of p63 (red arrow head); B: Immunofluorescence analysis: positive expression of p63 (green fluorescence).

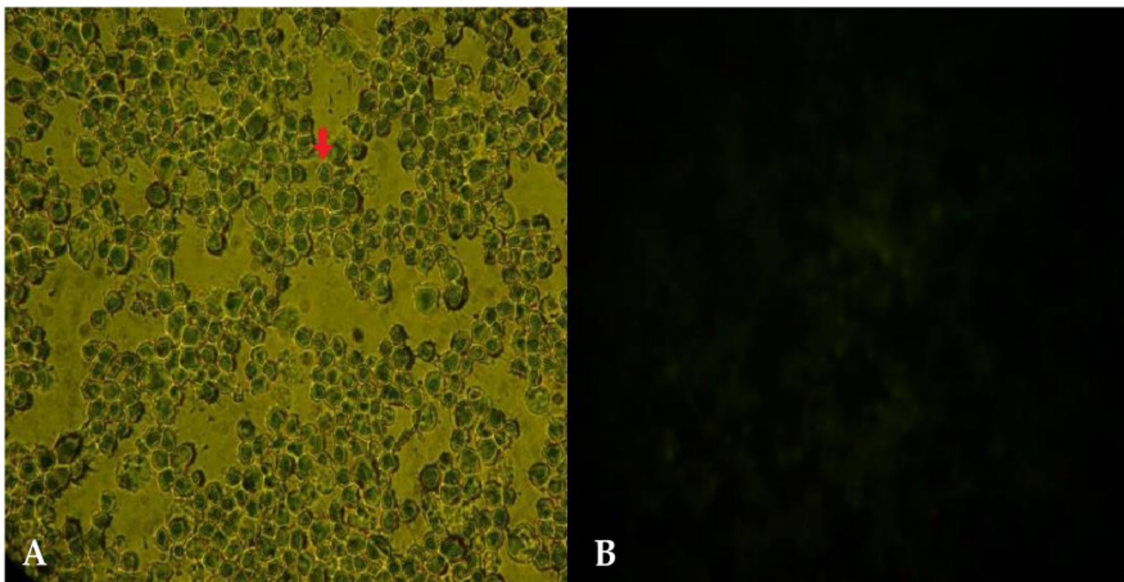

Fig. 3. Rabbit adipocyte stem cells (r-ASCs) cultured under hyperoxia (20% O_2 concentration). A: Immunocytochemical analysis: negative expression of p63 (red arrow head); B: Immunofluorescence analysis: negative expression of p63 (absence of green fluorescence).

hyperoxia (20% O_2 concentration), CD90+ and CD44+ expression was less pronounced (51.91%) (Fig. 1C).

This study shows that hypoxic preconditioning with 3% oxygen concentration enabled the maintenance of viability in r-ASCs before transplantation, as evident in the unaltered expressions of CD90+, CD44+, and CD45- and expression of quiescent cells with p63 as marker.

ACKNOWLEDGMENT

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