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THE CHARACTERISTICS OF RAT EMBRYONIC FIBROBLASTS (REFS) AND RAT BONE MARROW DERIVED MESENCHYMAL STEM CELLS (RAT-BMMSCS) FROM THE WISTAR RAT: A COMPARATIVE STUDY

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ABSTRACT

Objective: Rat embryonic fibroblasts (REFs) and rat bone marrow-derived mesenchymal stem cells (rat-BMMSCs) an be used as *in vitro* models for a variety of studies, including for degenerative diseases such as arterial ischemia, tissue engineering and development of induced pluripotent stem cells (iPSCs). Therefore, the further developments of the use of these two cells of great importance.

Methods: The experiments were performed with Wistar rat, those with 15-17 d gestation (aged 32 w) as a REFs source and those aged 12 w as a BMMSCs source. Dulbecco's modified eagle medium (DMEM) was used for both cell cultures but with different media supplements. Proliferation ability was determined for both by calculating population doubling time (PDT). Characterization was performed by differentiation testing into osteocyte, chondrocyte and adipocyte cells by staining with Alizarin Red, Alcian Blue and Oil Red O and by an investigation of specific antigen characteristics using flow cytometry with positive CD90 and CD29 and negative CD34 markers.

Results: Morphologically, the REFs and rat-BMMSCs had the same fibroblasts like shape. PDT was higher for the REFs than the BMMSCs ($p<0.05$), and both could differentiate into osteocytes, chondrocytes and adipocyte. The characteristics of the positive markers (CD29 and CD90) were higher in rat-BMMSCs than in REFs.

Conclusion: In this study demonstrated that the explant method for REFs isolation and flushing method for rat-BMMSC isolation are both effective. It also showed that rat-BMMSC grow faster than REFs, and that both cells have the same differentiation ability as rat-BMMSCs but with different specific surface antigen characteristics.

Keywords: Antigen-specific, Characteristics, Differentiate, Rat-BMMSCs, REFs

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INTRODUCTION

Fibroblast cells and stem cells from rats have been widely utilized for research in medicine and biotechnology. *In vitro* research using these cells has been to determine the mechanism of interaction and the effect of a protein and to produce cells for the fabrication of cells, tissues and organs for regenerative therapy. Research using REFs has been performed in studies on topics such as the use of REFs as a feeder layer for embryonic stem cells (ESCs), the induction of REFs into neuron cells and the development of induced pluripotent stem cells (iPSCs) [1-3]. The use of REFs as a feeder layer plays a role in generating growth factors through paracrine interactions and supports cell proliferation by creating niche interaction [1]. REFs can be produced from rat embryonic muscle tissue, which produces connective tissue in the rat's body [2].

Rat bone marrow-derived mesenchymal stem cells (rat-BMMSCs) can be obtained by centrifuging or rinsing the femur and tibia [2, 3]. Studies have been performed on the utilization of rat-BMMSCs for the treatment of liver cirrhosis in rat models, therapy for osteoporosis from radiation effects in cancer patients and as therapy in rat ischemic stroke models [2-4]. However, before these cells can be applied further, their characterization is required to prevent adverse therapeutic effects and expectations that are not in accordance with the therapy.

REFs and rat-BMMSC can be bought from cell-provider institutions such as the American Type Culture Collection, but there are obstacles in the transport and required time for processing their provision. To improve the availability of these cells in research laboratories, the further development of cell production methods for REFs and rat-BMMSC is of great importance. This study will provide an overview of isolation, culturing methods and comparative differences in the characteristics of REFs and rat-BMMSC.

MATERIALS AND METHODS

Animal

All experiments were performed with female Wistar rat, those with a 15-17 d gestation (aged 32 w) as a REFs source and female Wistar rats aged 12 w as rat-BMMSC source. The animals were housed with adlibitum food and water and were maintained at a constant temperature. Animal housing and experimental procedures were approved by Local Ethics Animal Welfare Commission.

Isolation and culture of REFs

The female Wistar rats were anesthetized with ketamine: xylazine in a 2: 1 ratio intraperitoneally. The stomach was disinfected with 70% alcohol prior to the removal of the uterus. The uterus was taken to a biosafety cabinet to remove the fetus, and then the fetus sterilized with absolute iodine for 15 to 20 seconds and then washed 3 times with phosphate buffer saline (PBS). The sterilized fetus was then prepared to obtaining its carcass by separating the head, limb and tail organs. The carcass of the rat was then skinned, and the internal organs in the abdomen and chest were lifted clean. The muscles were cut using scissors and tweezers with a size of 2 to 5 mm and accommodated in PBS. The laying of explants used tweezers or 1000 µl micropipette. The glass containing explants were incubated at 37 °C and 5% CO2 levels for 3 h or until the explants were attached to the bottom of the plate. An amount of 1.5 ml of fibroblast culture media added, namely high glucose dulbecco's modified eagle medium (DMEM; Gibco), 10% bovine fetal serum (FBS; Gibco), 0.1% nonessential amino acid (Gibco), 0.1% Insulin Transferrin Selenium (Gibco), 1% Antibiotic-antimycotic (Gibco 15,240,062, Carlsbad, USA) on explants. Within 2 d, replaced the culture medium.

Isolation and culture of rat-BMMSC

The bone marrow was obtained from the tibia and femur bones of the rats. After removal of the peripheral muscle and tendon, both bones were soaked in betadine and PBS twice. Each bone was cut at both extreme ends and then flushed from one end of the bone and directly accommodated in a 12 well plate dish. The culture medium was put into a 3 ml syringe with a 22-gauge needle. The culture medium consisted of DMEM/F12+1% GlutaMAX (Gibco), 10% FBS and 1% Antibiotic-antimycotic. The sample was then incubated at 37 °C and 5% CO2. After 24 h of culturing, the medium was replaced. Later culture medium replacements were provided once every 2 d. 1st passage was carried out after 7 d of culturing with trypsinization, using 0.05% trypsine ethylenediaminetetraacetic acid (EDTA) (Gibco, Carlsband, CA, USA) and planting in a 25TC flask with cells density 6x10 [5].

Determination of population doubling and population doubling time

Growth rate was determined by calculating population doubling (PD) and population doubling time (PDT). To calculate PD and PDT, REFs and rat-BMMSCs from the 4th passage. PD is the ability of cells to multiply and was calculated by the following formula; (log10 (number of harvested cells)-log10 (number of cells planted)/log10 (2), while PDT was calculated with Δt/PD, where t is the time of the culture. In this study, Δt = 6 d (104 h). The analysis was performed by a T-test using SPSS 16, Inc., Chicago, IL, USA.

Immunophenotyping with flowcytometry

Characterization was done at the 3th or 4th passage both of cells. The preparation of the REFs and rat-BMMSCs suspension was performed with trypsinization using 0.05% trypsin EDTA, with inactivation using DMEM 10% FBS. A total of $2x10^5$ cells per tube (eppendorf tube) were centrifuged at a speed of 1500 rpm for 5 min. After removing the supernatant, 50 µl PBS 3% FBS trituration and 5 µl antibodies CD34, CD90-FITC and CD29-PE were added and the sample covered with aluminum foil for 15-30 min at 4 °C. Next, 1 ml PBS 3% FBS was added, and the sample was transferred to a tube for analysis using BD Accuri C6 Plus, BD Life Sciences, San Jose, CA, 95131, USA.

Chondrogenic, adipogenic, and osteogenic differentiation and staining of REFs and rat-BMMSCs

Multipotent line induction was performed post 3th passage. Induction was carried out with an induction medium for osteocytes, adipocytes and chondrocytes (Gibco, Carlsbad, CA, USA). Cells that had undergone 70% confluent were induced with the induction medium for 14 d, with medium replacements every 2-3 d.

Staining was done by fixing both of cells using 4% paraformaldehyde for 30 min at room temperature. After discarding the fixation solution, the sample was washed with PBS twice, followed by the addition of Alizarin (Sigma-Aldrich, United States) staining solution for osteocytes, Oil red O (Sigma-Aldrich, United States) for adipocytes and Alcian Blue (Sigma-Aldrich, United States) for chondrocytes for 30 min. Discard the staining solution and rinse with water 2 times. Observations were made under an inverted microscope.

RESULTS

In this study, the REFs were isolated using a primary explant culture technique and the ratBMMSC were isolated using a flushing technique. After 2 d of culturing, there were fibroblast cell grow around the explants (fig. 1A). After 24 h of culturing, the rat-BMMSCs had a heterogeneous population (fig. 1B). After the 2nd passage, the population of rat-BMMSCs was again homogenus. The morphology of both the REFs and rat-BMMSCs revealed no marked differences between these two populations (fig. 1C and 1D): they were both spindel-shape and adherent type of cells.

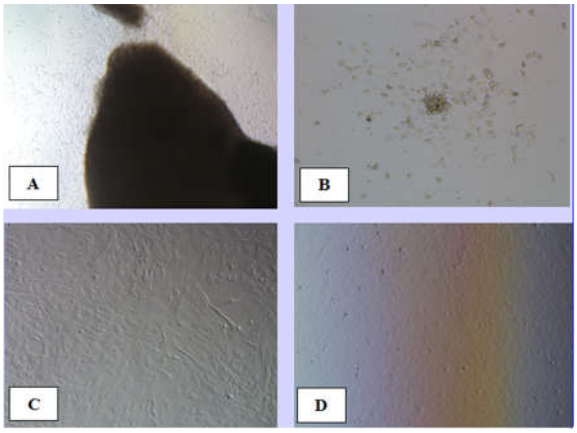

Fig. 1: Rat embryonic fibroblast (REFs) on the second culture day (A), rat-bone marrow-derived mesenchymal stem cells (rat-BMMSCs) after 24 h of culturing and after replacing culture medium (B), REFs (C) and rat-BMMSCs (D) from fourth passage, fifth culture day. Both cells have a spindle-shaped morphology (magnification x40)

Table 1: PD and PDT of the REFs and rat-BMMSCs

	PD time (h)	PD
REFs	60,64 ± 4,47*	1,85 ± 0,08*
Rat-BMMSCs	36,55 ± 5,53*	3,81 ± 0,39*

Abbreviations: PD, population doubling; PDT, population doubling time; rat-BMMSCs, rat bone marrow-derived mesenchymal stem cells; REFs, rat embryonic fibroblast., *p< 0.05

Average PDT (in hours) and PD in the REFs cultures during 4th passage were lower than that of the rat-BMMSCs (table 1). This difference was statistically significant. Furthermore, during 6 d of culturing, the rat-BMMSCs doubled on average almost four times and the REFs doubled fewer than two times.

We analyzed cells of the appropriate size (forward scatter) and granularity (site scatter) in the region P1 (fig. 2A and 2D, fig. 3A and 3D). A triple immunofluorescence technique was performed to validate the results of the flow cytometric analysis. Isotype was shown in fig. 2B and 2C and fig. 3B and 3C. Then the cells selected for

analysis (population E1 and R1) that were positive for both CD90 and CD29 (fig. 2E and 3E). In population E1 there was a>95% expression of CD90 (fig. 2F), but R1, 100% population were expressed positive for CD29 (fig. 3F).

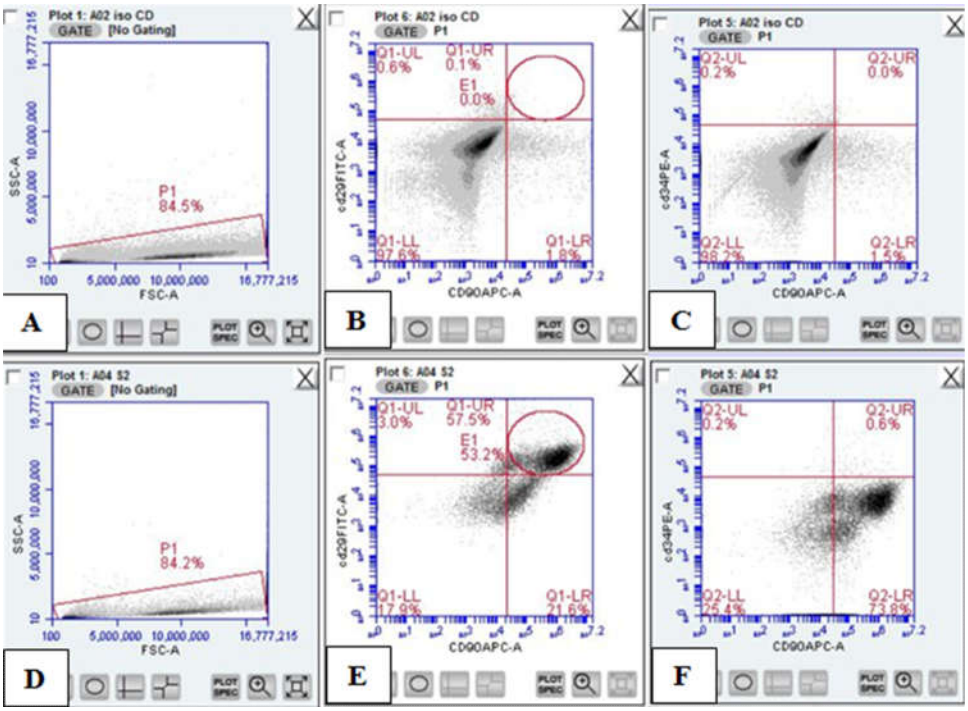

Fig. 2: Analysis of the phenotype of rat embryonic fibroblast (REFs) by flow cytometry: A and D) size (forward scatter) and granularity (side scatter) of cells; B and C) isotype of the CD45, CD29 and CD90 antigen (P1); E) expression of the CD29 and CD90 antigens in the population (Q1-UR) from REFs after the fourth passage; F) expression of the CD29 and CD90 antigens in the population of CD34-negative cells (E1)

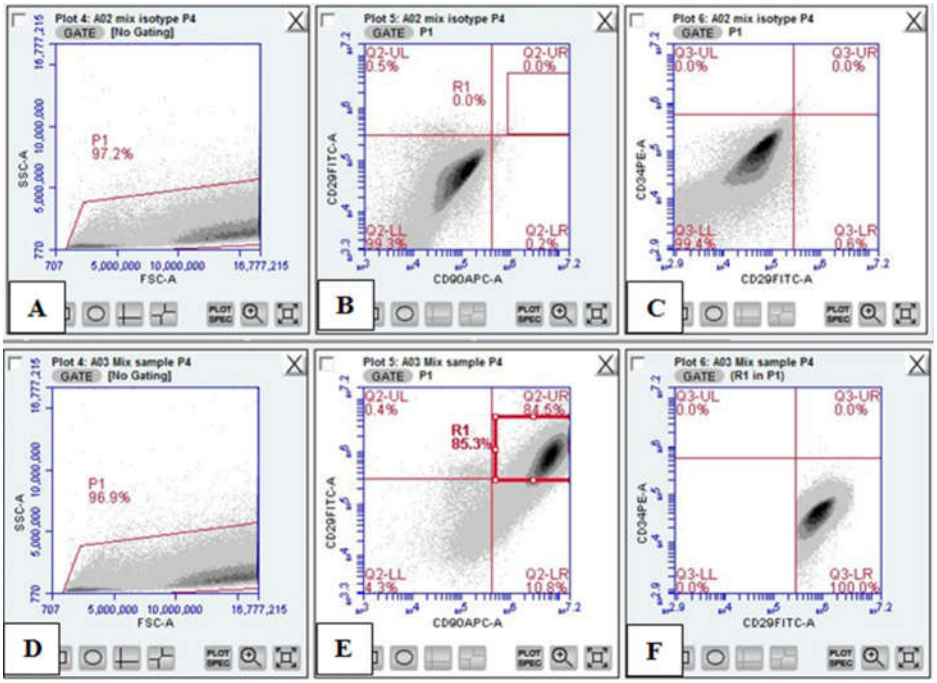

Fig. 3: Analysis of the phenotype of ratBMMSC by flow cytometry: (A and D) size (FSC) and granularity (SSC) of cells; (B and C) isotype of CD45, CD29 and CD90 antigen (P1); E) Expression of CD29 and CD90 antigens in the population (Q2-UR) from ratBMMSC after the 4th passage, (F) expression of CD29 and CD90 antigens in the population of CD34-negative cells (R1)

The REFs and rat-BMMSCs were cultured in chondrogenic, adipogenic and osteogenic media. After differentiation for 2 w, the Alcian Blue staining of cartilage speroid, Oil Red O staining of lipid droplets in the cells and Alizarin Red staining of the mineralized matrix were observed using inverted microscopy (fig. 4A-4C and fig. 5A-5C).

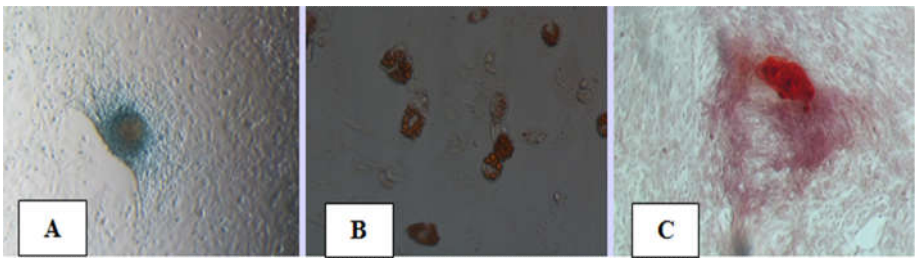

Fig. 4: Staining of the rat embryonic fibroblast (REFs) with Alcian Blue (A), Oil Red O (B), and Alizarin Red (C) (magnification 100x)

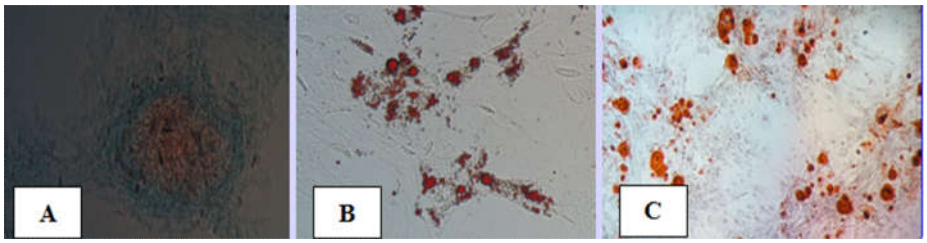

Fig. 5: Staining of the rat bone marrow derived mesenchymal stem cell (rat-BMMSCs) with alcian blue (A), Oil Red O (B), and Alizarin Red (C) (magnification 100x)

DISCUSSION

In this study, we were successful in the isolation, culture and expansion of REFs with a primary explant culture technique. After 2 d of culturing, cell growth began to appear with a spindle shape morphology, which was of fibroblast cells (fig. 1A). This technique is easier and cheaper for producing fibroblast cells as compared to enzymatic techniques [1]. There were also no debris cells or blood cells, such as erythrocytes and red blood cells can become contaminants during culturing. In addition to the expense of enzymatic methods, enzymes can also damage fribroblast cell, thereby reducing cell viability.

The of bone marrow flushing method for obtaining rat-BMMSCs used by Zhang and Chan [3]. They used a cell strainer or nylon mesh to separate bloods clots and bone debris. In this study, we separated impurities by changing the culture medium after 24 h of culturing and the homogeneous rat BMMSCs was obtained after 2nd passage. The rat-BMMSCs were adherent cells that would stick on base of the petri dish, by replacing the medium, then impurities such as blood cells will be wasted (fig. 1B). After a number of passages, the REFs and rat-BMMSCs showed the same morphology fibroblast-like or spindle shape (fig. 1C and 1D).

During 6 d of culturing in the 4th passage, the rat-BMMSCs showed much faster growth than the REFs (table 1). Ichim *et al.* reported that fibroblasts have a shorter dobling time than mesenchymal stem cells (MSCs) [3]. Soundarajan and Kannan stated that fibroblasts are a differentiated cell that have a limited ability to divide before experiencing senescence [4]. In this study, although the REFs and rat-BMMSCs were tested at the same passages, their ability to proliferate was different.

In this study, the rat-BMMSCs took about 38 h to multiply so that in 6 d, the culture was only able to divide 4 times. This was slower than that reported by He *et al.*, who showed 37 times division after 16 d culture [3]. However, Gala *et al.* reported that the rat-BMMSCs produced from Lewis rats at 3 mo old had 10 PD times in a 46-day culturev [1]. The current study used 12-week-old female Wistar rats, while He *et al.* used 4-week-old male Sprague Dawley rats. This demonstrates that culture conditions, type and age of source affect the quality and capability of the cells to proliferate.

The phenotypic characterization of REFs and rat-BMMSCs was analyzed with triple immunofluorescence (CD34-PE, CD90-APC and CD29FITC) using flow cytometric. Flow cytometric can identify the surface Ag expression of both populations cells rapidly. For the rat-BMMSCs, the present study detected a surface marker Ag of CD29 and CD90 more than 95% and they did not appear to express CD34. This is in accordance with the standard/criterion from international society stem cell teraphy. Rat and human CD29 and CD90 are the surface marker of mesenchymal cells, and CD34 is a hematopoietic stem cells marker [9]. Fibroblasts have the same markers as MSCs, especially CD90 so Denu *et al.* (2016) has stated that both cells are probably of the same cell type [4, 5].

To identify the biological property of the REsF and rat-BMMSCs, we induced both for trilineage differentiation: chondrogenic, adipogenic and osteogenic. Both were able to be differentiated after 12 d of induction, and they were then stained with Alcian Blue, Oil Red O and Alizarin Red for quantitative observations. Shperoid cartilage, red lipid droplets and mineralization of the REFs and rat-BMMSCs were observed by microscopy with magnification 100x. This differentiation ability is in accordance with several publications that have stated that both fibroblast cells and MSCs have the ability to differentiate into three types of cells [6-8, 4, 5].

This study has found that the explant method for the REFs isolation and flushing method for rat-BMMSC can be used effectively. This study also found that rat-BMMSCs grow faster than REFs.

CONCLUSION

REFs have the same differentiation ability as rat-BMMSCs but have different specific surface antigen characteristics.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

All authors have none to declare.

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