Chinese Journal of Laboratory Diagnosis
Issued on 25 Aug 2016 Vol. 20, No. 8 P.1240

Effects of KAATSU Training on proliferation and differentiation of goat bone marrow mesenchymal stem cells

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Abstract: [Objective] To explore the effects of KAATSU Training on proliferation and differentiation of goat bone marrow mesenchymal stem cells. **[Methods]** 60 Boer goats were randomly divided into experimental group and control group, the experimental group was given KAATSU Training twice a week, non-KAATSU Training twice a week for the control group. 6 months later, we got the goat bone marrow and then separated and absorbed the white cloud layer which mainly contained the mononuclear cell in the upper-middle interface with the method of percoll-density gradient centrifugation, cultured and observed the cell morphology and the proliferation rate; the cells of the two groups were induced into cardiomyocyte like cells by the 5-azacytidine. The cells which had been differentiated were detected with the expression of the cardiac specific antigen α-actin by immunofluorescence assay. **[Results]** The cells isolated from the bone marrow in the white cloud layer were adherent, generated and grew well. In addition, the cells which induced by 5-azacytidine could express the cardiac specific antigen. The bone marrow mesenchymal stem cells of the experimental group were small and round, and their proliferation rate was faster than the control group, though the shape of the cells in the control group were polygonal or triangular, and the proliferation rate were slow. **[Conclusion]** It has been succeeded both in separation and cultivation of the bone marrow mesenchymal stem cells, and also induced the proliferation of turning into cardiomyocyte like cells in vitro. The bone marrow mesenchymal stem cells in pressurization motion for a long period of time were easier to proliferate than the cells in non-pressurization motion, but the differentiate capability were low.

Bone marrow mesenchymal stem cells (BMSCs) are from the mesoderm and are pluripotent stem cells with high capability in proliferation, self-renewal and multi-directional differentiation potential. Further studies have demonstrated that BMSCs can differentiate into cardiomyocytes, neurons or neuroglial cells. Upon in vivo transplantation, these cells can migrate to injured sites (mostly to ischemic or anaerobic sites) and repair respective tissues. Cell transplantation has provided brand-new treatment strategy to irreversible heart diseases. BMSCs are currently considered as one of the most ideal seed cells for cell transplantation, and are often used as carrier cells in gene therapy. Allogeneic BMSC transplantation may trigger immunologic rejection, while autologous stem cells

are of limited quantities. It is therefore crucial to look into how autologous stem cells could proliferate and be release to the bloodstream, especially in large mammals. In recent years, the number of studies focused on small animals such as mice/rat or rabbit is relatively high, but few studies and report investigate into BMSCs in bigger animals like goats. Thus, it is important to study the in vitro directed differentiation of BMSCs from goat as a big animal.

1. Test Material and Method

1.1 Test Material

Experimental animals: healthy Boer goats, 5-azacytidine (sigma, USA); Primary antibodies: anti-alpha skeletal muscle actin antibody (abcam, UK); Secondary antibodies: Dylight 488 affinipure donkey anti-mouse IgG(H+L) (EarthOx, llc, USA), 0.25% pancreatin (pancreatic enzyme) (GIBCO, USA), IMDM (GIBCO, USA), Fetal Bovine Serum (FBS) (Hyclon, USA).

1.2 Test Method

1.2.1 KAATSU Training

60 Boer goats in their 24^{th} to 30^{th} month were selected with their range of body weight from 30 to 35 kilograms, and randomized into experimental group and control group with 30 goats in each group. KAATSU training equipment, invented by Dr. Yoshiaki Sato in Japan, was used. KAATSU belt was tied on the root of experimental goats' left hind limbs, with pressure range set to 60-100 mmHg. Upper limit of pressure was set such that it does not trigger limping in goats. The goats in both groups undergo two rounds of walking training every week for 15 minutes each time. For the rest of the time, they were captivated and bred with the same diet.

1.2.2 Bone marrow is extracted from the goats and BMSCs were separated and cultured.

After 6 months, BMSCs were extracted from the left hind limbs of the goats from femur and tibia under sterile condition. Diaphysis was dissected to flush the bone marrow out using IMDM culture fluid. Bone marrow extracted is treated by density gradient centrifugation using Percoll cell separation fluid in density of 1.082 g/ml, at the speed of 500 x g/min, for 25 minutes. Middle mononuclear cell layer was extracted and thoroughly rinsed with 0.01M PBS and culture medium was added to prepare mononuclear cell suspension fluid. Number of cells was counted and cells are seeded in perfect medium at the concentration of $1.0x10^6$/ml. (contains IMDM in the volume fraction of 10% PBS, and 100U/ml of Penicillin Streptomycin) Cell culture is then placed in a 37°℃ incubator, and non-adherent cells were removed in 24 hours. Solution is exchanged once every 3 days.

1.3 Passaging subculture of Goat Bone marrow mesenchymal stem cells (BMSCs)

When the fusion status of cells reached 80-90%, PBS was added to the culture to rinse and cells were digested using 0.25% trypsin at 37°℃. Once cells began crimping, IMDM culture containing 10% Fetal Bovine Serum (FBS) was used to terminate digestion, and cells were transferred to centrifugal tube and centrifuged for 5 minutes at 1200r/min. After that, the cell culture was transferred to 2 culture plates to continue cultivation. Solution is exchanged once every 3 days, and the aforementioned procedures are repeated once the percentage of adherent cells has attained 80-90% fusion to trigger repeated cell passaging.

1.4 Growth Curve

BMSCs were cultured using IMDM culture containing 10% Fetal Bovine Serum, and observed every 24 hours under inverted microscope to count the number of cells. Each sample was counted for 3 times and the average was used for calculating the average across all samples in experimental group and control group respectively. Upon 12 days of consecutive observation, growth curve was plotted using number of survived cells (ten thousand/ml) against culture time (days).

1.5 Inducing specific differentiation of goat BMSCs into myogenic cells

Goat MSCs passaged and cultured up to the 2^{nd} generation is extracted and 10μmol/L 5-azacitidine was added for induction. After 24 hours, fresh IMDM culture containing 10% Fetal Bovine Serum (FBS) was exchanged as culture medium, and upon 14 days of culturing, expression of the cardiac specific antigen α-actin was assessed using immunofluorescence assay.

1.6 Immunofluorescence Assay

Induced cells are extracted and culture medium is disposed. 4% formaldehyde was used for fixation under room temperature for 30 minutes. The cells were then rinsed 3 times using phosphate buffer solution (PBS) for 15 minutes each time. 1% (vol/vol) Triton was used to prepare a 5% (wt/vol) BSA antibody dilution and this antibody dilution is used in the ratio of 1:100 to dilute the rat anti-alphα-actin antibody. Diluted antibody is added to the cells and incubated for 4 times overnight. On the following day, the cells were rinsed 3 times using phosphate buffer solution (PBS) for 15 minutes each time. Antibody dilution is then used in the ratio of 1:200 to dilute the secondary antibody (donkey anti-mouse IgG), after which the secondary antibody is added to the cells and incubated for 1 hour in room temperature, avoiding light. The cells were then rinsed 3 times using phosphate buffer solution (PBS) for 15 minutes each time. 5% glycerine was used for mounting the cells onto a slide and observed under florescence microscope.

2. Results

2.1 During the conduct of the study, 9 and 10 goats respectively from the experimental group and control group died and left the study due to disease. The actual numbers of goats completing the study were 21 in the experimental group and 20 in the control group.

2.2 Morphological characteristics of goat BMSCs and the effect of KAATSU Training on differentiation of BMSCs

BMSCs become adherent within 48 hours of culture. The cells are of various shapes; some are round while others are triangular. As the duration of culturing increases, the shape of most cells becomes triangular and the speed of proliferation increases to 1 generation passage in every 3 to 5 days. BMSCs in the experimental group and control group are cultured under the same condition. Upon increase in culture time, the differences in morphology between cells become larger. As seen on the microscope on the 12^{th} day of culture, there are more round cells in the experimental group while less in the control group, and most cells in the control group were triangular. (Figure 1)

2.3 Growth Curve

Based on results from cell counts, the number of cells did not change in days 1-2 of the culture, suggesting that it was the latent period for proliferation. On days 3-9, the number of cells rapidly increased and entered into exponential growth. On days 9-12, cell growth slowed down. (Figure 2)

2.4 Expression of the cardiac specific antigen was assessed using immunofluorescence assay

After 24-hour culturing upon induction by 5-azacitidine, as observed on day 14 using immunofluorescence assay, the cells were in fibrous shape and green color. In other words, the induced cells all had strong expression of cardiac specific antigen α-actin, suggesting that the goat BMSCs are either in the progress or have completed differentiating into cardiomyocytes-like cells. (Figure 3)

Figure 1 Morphology of experimental group and control group BMSCs on day 12 of culturing (x 4 times)

Figure 2 Goat BMSCs growth curve (experimental group VS control group)

Figure 3 Immunofluoresence assay on day 14 after 5-azacitidine induction (x 40 times)

3. Discussion

Mesenchymal stem cells (MSCs) isolated and extracted from goat bone marrow are adherent

and capable of passaging. They differentiate into specific cell types under suitable conditions, suggesting that the culturing of goat BMSCs has succeeded. Upon induction by 5-azacitidine, cardiac specific antigen α-actin is expressed, suggesting that goat BMSCs into cardiomyocyte-like cells is either in progress or completed. The mechanism of goat BMSCs differentiating into cardiomyocyte-like cells upon induction by 5-azacitidine remains unknown. Existing research and studies support the view that since 5-azacitidine is reduces methylation, and therefore leads to demethylation in certain cytosine in DNA, thus induced the differentiation of BMSCs into cardiomyocytes. Based on results as shown above, goat BMSCs have a relatively high speed of proliferation and high potential for directed differentiation, which suggests that the in vitro induction of directed differentiation using BMSCs of large animals under specific conditions is feasible. This serves as reference for clinical application in both animals and human medical treatment. Also, other studies have suggested that the BMSCs have low immunogenicity and allow easy introduction and expression of exogenous genes, which allows its wide application across areas such as tissue engineering, cell and gene therapy. Goats are similar to mammals such as human, bovine, rabbits and rats. This establishes the foundation for future research using goat BMSCs for recombinant gene cloning donor cells selection and research in embryonic growth, and serve as adult stem cell with broad future possibilities for applications. Meanwhile, it was identified that the growth morphology of BMSCs from experimental group versus control group differed during cell culturing. BMSCs in experimental group have undergone repeated passage and become small and round, which is close to the morphology of initial generation cells immediately after isolation from bone marrow, with high potential for proliferation. BMSCs are mainly extracted from bone marrow, which typically is anaerobic. Animals in the experimental group were also put under anaerobic condition for metabolism by KAATSU training. Upon feedback stimulation under anaerobic condition, the Bone marrow stroma cells may have increased power in proliferation, while differentiation potential is limited. On the other hand, control group animals did not receive KAATSU training, and under the environment of normal oxygen concentration, most BMSCs are in triangular shape and readily differentiated into cardiomyocyte-like cells after induction. This suggests that BMSCs tend to incline towards differentiation under normal oxygen concentration, rather than anaerobic condition. The effect of KAATSU training on BMSCs requires further investigation and study. For instance, if further investigation is done on how pressure should be increased in KAATSU training, and what training time and frequency would facilitate cell proliferation and differentiation, it becomes possible to set various KAATSU training conditions to suit clinical needs, enabling control over proliferation and differentiation of BMSCs.

(Submission Date: December 20th, 2015)