Am J Transl Res 2018;10(6):1722-1729
www.ajtr.org /ISSN:1943-8141/AJTR0051720

Original Article

Repair mechanism of mesenchymal stem cells derived from nasal mucosa in orbital fracture

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Received May 28, 2016; Accepted April 19, 2017; Epub June 15, 2018; Published June 30, 2018

Abstract: Objective: Mesenchymal stem cells (MSCs) derived from nasal mucosa are featured by high division and differentiation capacity, with large nuclei, obvious nucleoli and weak cytoplasmic basophily. Imaging examination, typically CT scan, is the gold standard for the diagnosis of orbital fracture. Methods: We isolated MSCs derived from goat nasal mucosa and built the calcification model so as to investigate the repair mechanism of nasal mucosa-derived MSCs in orbital fracture. Expressions of osteogenic markers Runx2, OCN, OPN and BSP were detected using western blot. Results: Nasal mucosa-derived MSCs were successfully isolated and passaged. Nestin was detected by immunofluorescence assay in the cells of the third generation. It was further confirmed that the isolated cells were nasal mucosa-derived MSCs. As indicated by alizarin red staining, the calcification model in nasal mucosa-derived MSCs was successfully built. The relative expressions of Runx2 and OCN reached the highest level after osteogenic induction for 7 d, and the expressions of OPN and BSP were also high. But at 10 d, the expressions of all markers declined somewhat. At 14 d, the expressions of OPN and BSP reached the peak, but without significant differences compared with those at 7 d. Conclusion: The present study suggested that the repair effect of nasal mucosa-derived MSCs in orbital fracture is achieved by facilitating the expressions of osteogenic markers Runx2, OCN, OPN and BSP. However, the pathways of actions are unknown and further studies are required to elucidate the concrete mechanism.

Keywords: Nasal mucosa-derived mesenchymal stem cells, orbital fracture repair, Western blotting

Introduction

Mesenchymal stem cells (MSCs) derived from nasal mucosa are featured by high division and differentiation capacity, with large nuclei, obvious nucleoli and weak cytoplasmic basophily [1, 2]. In addition to mitochondria, the cytoplasm also contains a small amount of rough endoplasmic reticulum (rough ER), free ribosomes, Golgi apparatus, lysosomes and fatty granules [3]. Mesenchyme is a type of colorless, transparent liquid which possesses high division and differentiation capacities. MSCs are present in the connective tissues of adult animals [4-6]. Orbit is situated in the middle of the skull towards the front and vulnerable to fracture caused by an impact [7-9]. Orbital fracture refers to the fracture or displacement of craniofacial skeleton that forms the orbit. Many important structures, including eyeballs, optic nerves, extraocular muscles and other vessels and nerves, are found within the orbit. Therefore, orbital fracture may also cause damage to these structures [10-14]. We isolated nasal mucosa-derived MSCs from goat and built the calcification model. We aimed to analyze the repair mechanism of nasal mucosa-derived MSCs in orbital fracture.

Materials and method

Animals and cells

Goat was provided by the animal center of our hospital. The bone marrow stem cells were purchase from Cyagen Biosciences Company.

Isolation, purification and identification of nasal mucosa-derived MSCs and building of the calcification model

The head of the goat was cut off and the facial skin was disinfected with 75% ethanol. The

facial skin was stripped under sterile conditions, and the brain tissues were removed. The nasal bone was cut open in superior direction along bilateral nasal cavities to expose bilateral nasal cavities and sinuses. The nasal mucosa was removed with ophthalmic forceps and placed in PBS. The blood was washed off the nasal mucosa using serum-free DMEM/F12 for 3 times, with nasal mucosa placed in DMEM/F12 containing 1% penicillin and streptomycin. The nasal mucosa was cut into pieces and centrifuged, with supernatant discarded. After digestion with 2 mg/ml type II collagenase, the cells were gently blown with a straw to prepare the cell suspension. Then the digestion was terminated by adding the above-mentioned culture medium, with the collagenase removed by centrifugation. The cell concentration was adjusted to 1×10^6 cells/ml by adding the culture medium. The cells were inoculated to a 6-well plate and incubated in a humidified 5% CO_2 incubator at 37°C. The suspending cells were removed using a straw 3 days later, and the fresh culture medium was replenished for the first time. After that, the culture medium was replaced once every 3 d. Cell passaging was performed by digestion when the cells covered the bottom of the culture flask. The cells of the third generation were inoculated to a 24-well plate for subsequent detection of nestin by immunochemistry [15, 16]. The coverslip with cells grown on it was washed with PBS (0.01 M) for 3 times, 3 min each time. The cells were then fixed in 4% paraformaldehyde for 15 min and washed with PBS for 3 times, 3 min each time. Onto the coverslip 0.5% TritonX-100 was added to incubate the cells at room temperature for 15 min. This was followed by washing with PBS for 3 times, 5 min each time. The cells were further incubated with blocking serum at room temperature for 60 min. Excess blocking serum was removed with the filter paper, without washing the coverslip. Diluted primary antibodies (1:500) were added to incubate the cells in the wet box at 4°C overnight. Then the wet box was taken out and rewarming was carried out at 37°C for 30 min. The coverslip was washed with PBS for 3 times, 3 min each time. Excess liquid was removed from the coverslip using the filter paper, and diluted Alexa Fluor® 647- and APC-conjugated secondary antibodies (1:1000) were added to incubate the cells in the wet box at 37°C for 60 min. All procedures starting from the adding of secondary antibodies were carried out in the dark. The coverslip was washed with PBS for 3 times, 3 min each time, and DAPI was added for nuclear stain in the dark for 15 min. Excess DAPI was removed by washing with PBS for 4 times, 5 min each time. The liquid on the coverslip was removed with filter paper. The coverslip was sealed with anti-fluorescent mounting medium. The four corners of the coverslip were sealed with nail polish. The coverslip was observed under the confocal laser scanning microscope.

The purified MSCs were inoculated to the 6-well plate at the density of 1.5×10^4 cells/well. When the cells grew to 95% confluence, 2 ml of osteogenic differentiation medium was added into each well to incubate the cells for 2 weeks. As a result, the calcium ions would precipitate in the form of calcium salts, which reacted with alizarin red to give the red color. Thus calcification before and after induction was observed to determine whether the calcification model was successfully built. Two groups were set up (group A before induction, and group B 2 weeks after induction), with 3 replicates in each group. The cells were first digested, added with complete culture medium to adjust the cell density to $2*10^4$/ml and inoculated to the 24-well plate containing round coverslips. The cell suspension was added dropwise to the round coverslips so that the cells would grow on the coverslips for 30 min. Culture medium was replenished in the culture dish and incubated in a humidified 5% CO_2 incubator at 37°C for 10 h. The coverslips were washed with PBS twice, 2 min each time, and fixed in 4% paraformaldehyde for 20 min, followed by washing again with PBS for 3 times, 2 min each time, and alizarin red staining at 37°C for 10 min. Finally the coverslips were washed with double-distilled water twice, dried and mounted [17].

Western blot detection of expressions of osteogenic markers Runx2, OCN, OPN and BSP

The purified MSCs were inoculated to the 6-well plate at the density of 1.5×10^4 cells/well. When the cells grew to 95% confluence, 2 ml of osteogenic differentiation medium was added into each well to incubate the cells for 0, 3, 7, 10 and 14 days, respectively. The culture medium was discarded, and the cells were washed with PBS once. For every 10^6 cells, 0.1 ml RIPA buffer was added for cell lysis on ice with gentle blowing using pipette tip. Then the culture dish

was slanted gently so that the content would flow to one side of the dish. The cells were transferred to a 1.5 ml centrifuge tube and oscillated violently for 30 s. Centrifugation was then carried out at 12,000 g at 4°C for 5 min, with supernatant discarded. Total protein was extracted and analyzed by SDS-PAGE. The proteins were transferred to the membranes, incubated with primary and secondary antibodies, and added with color development reagent. The osteogenic markers Runx2, OCN, OPN and BSP were detected and the variation of their expressions was observed over time. For color development, equal volumes of solution A and B were combined in the tube and applied to the upper side of the PVDF membrane to incubate the membrane for 2 min. The PVDF membrane was then wrapped in the fresh-keeping film in the dark room. Excess liquid was removed and the gel was pressed on to the fresh-keeping membrane. The gel was exposed for different time depending on the fluorescence intensity. Next the gel was immersed into the color development reagent. Once the bands appeared, the gel was immediately placed into the fixing solution. The gel was washed with flowing water and dried. Finally, the gel was scanned, and the grayscales of the target bands were analyzed by UVP gel image processing system LabWOrks4.

Co-culture of nasal mucosa-derived MSCs and BMSCs

Non-contact co-culture utilizes the effect of paracrine cytokines released by one type of cells on another type of cells, without direct contact between the two cells. With this method, the two types of cells can be easily separated, which makes it easier for subsequent experiment. Millicell cell culture insert (Transwell chamber) is permeable a cup-like device with a permeable membrane at the bottom of the cup (0.1-12.0 um pore polycarbonate membrane insert). In the present experiment, a polycarbonate membrane insert was located in the middle of the Transwell chamber (pore size 3.0 um). The Transwell system was placed into the culture plate, with cell A inoculated to the upper chamber and cell B to the lower chamber. The cytokines secreted by cell B would act on cell A through the membrane. In the meantime, cell A created a microenvironment for the growth of cell B [18].

Specifically, the Transwell chamber with PET membrane was used in combination with the 24-well plate for non-contact co-culture. The log phase nasal mucosa-derived MSCs were harvested and digested with 0.25% trypsin to prepare a single-cell suspension. The cells were counted and the cell concentration was adjusted before inoculation to the Transwell system. BMSCs of the third generation were inoculated to the lower chamber at the amount of 2×10^5 cells. With the upper chamber placed into the wells, the nasal mucosa-derived MSCs were inoculated to the chamber at the amount of 2×10^5. The culture media in the upper and lower chambers were allowed to merge so as to establish the co-culture system. Three groups were set up for the co-culture, and the proportions of nasal mucosa-derived MSCs to BMSCs were 1:4, 1:1 and 4:1, respectively. The cells were added with DMEM/F12 90%+ high-quality fetal bovine serum 10%+ penicillin and streptomycin (final concentration 100 U/ml) and incubated in a humidified 5% CO_2 incubator at 37°C for 0, 3, 7, 10 and 14 d, respectively. The optical cell proportion and co-culture duration were determined by observing the effect on the growth of BMSCs.

Detection of synergistic osteogenic effect of nasal mucosa-derived MSCs and BMSCs by ELISA and alizarin red staining

Alkaline phosphatase (ALP) activity was detected in the supernatant of co-culture by using ELISA, and the synergistic osteogenic effect of nasal mucosa-derived MSCs and BMSCs was detected by alizarin red staining. After centrifugation at 3000 rpm for about 20 min, the supernatant was collected. The ALP level was detected by sandwich ELISA. The micropore plate was coated with purified goat anti-ALP antibodies to prepare solid-phase antibodies. Into the antibody-coated micropores, samples to be detected/standards and HRP-labeled goat anti-ALP antibodies were added successively to form the antibody-antigen-labeled antibody complexes. After thorough washing, TMB substrate was added for color development. In the presence of HRP, TMB was catalyzed to give blue color, which finally turned to yellow in the presence of acid. The darkness of color was proportional to the ALP level. The absorbance (OD) values were measured at 450 nm wavelength using a microplate reader. The standard

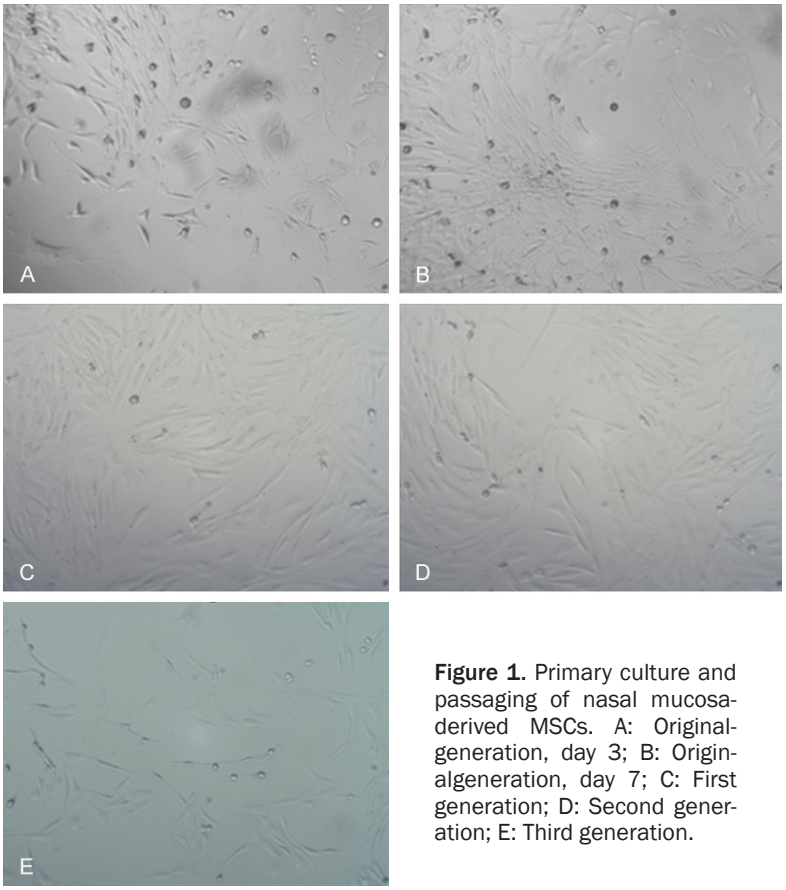

Figure 1. Primary culture and passaging of nasal mucosa-derived MSCs. A: Original-generation, day 3; B: Origin-algeneration, day 7; C: First generation; D: Second generation; E: Third generation.

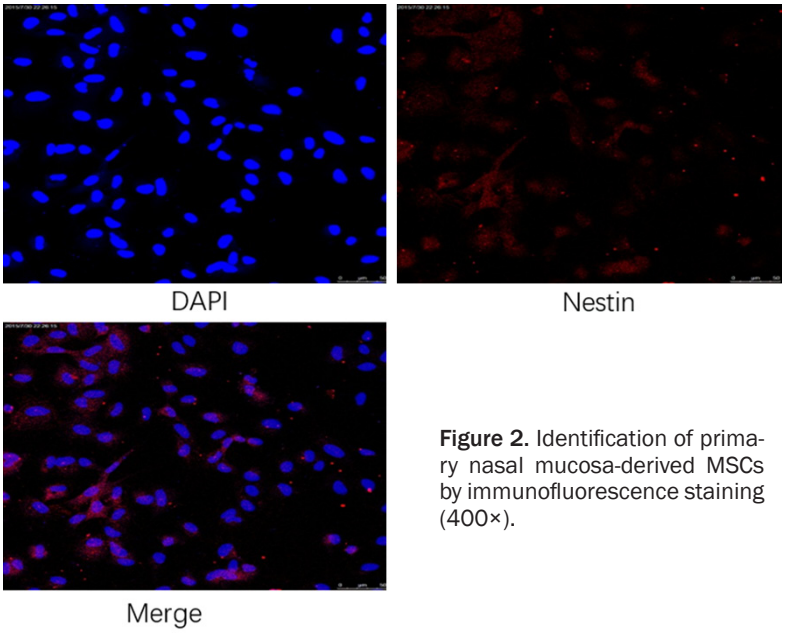

Figure 2. Identification of primary nasal mucosa-derived MSCs by immunofluorescence staining (400×).

curve was plotted and the ALP level in the samples was calculated.

MTT cell proliferation assay of nasal mucosa-derived MSCs and BMSCs after induction

MTT cell proliferation assay is a common method for detecting cell viability. The succinate dehydrogenase in the mitochondria of live cells will reduce exogenous MTT into water-insoluble violet formazan crystals, which precipitate in the cells. But this will not occur in dead cells. Then DMSO is added to dissolve formazan in cells, and the absorbance values are measured at 540 nm wavelength using a microplate reader. Thus the number of live cells is measured indirectly. Within a certain range of cell number, the amount of crystals formed is proportional to the cell number. So far MTT assay has been applied widely in cell viability detection, large-scale screening for anti-tumor drugs, cytotoxicity test and radiation sensitivity test of tumors [19].

After co-culture of BMSCs and nasal mucosa-derived MSCs (1:1) for 14 d, two groups were set up for MTT assay, namely, calcification induction group and non- calcification induction group. Cells of two different treatments were taken, and after the cell concentration was adjusted to 1×10^5 cells/ml, they were inoculated to a 96-well plate with 100 μL per well. Each group had 3 replicates. The 96-well plate was incubated in the 5% CO_2 incubator at 37°C for 24 h, 48 h, 72 h and 96 h, respectively. Into each well 20 μL of 5 mg/ml MTT was added, and the cells

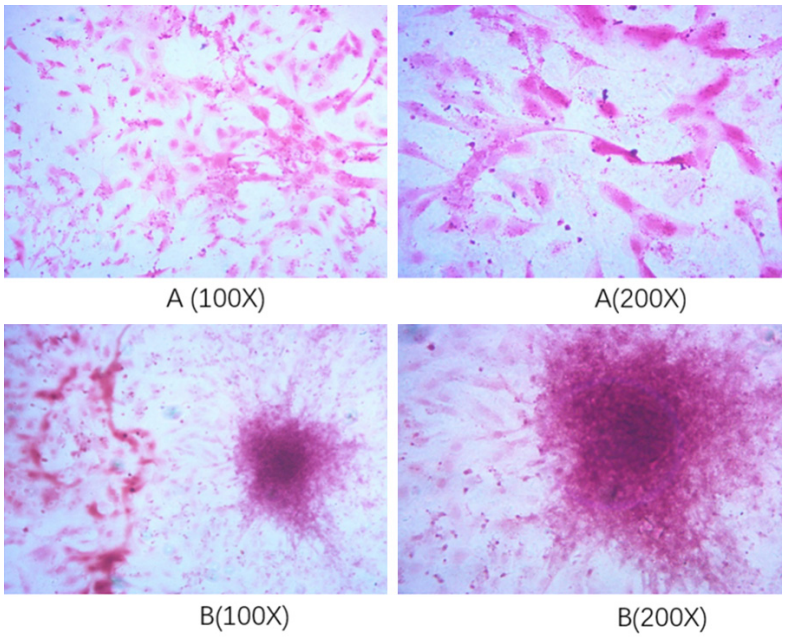

Figure 3. Alizarin red staining of nasal mucosa-derived MSCs (after 2 weeks of osteogenic induction).

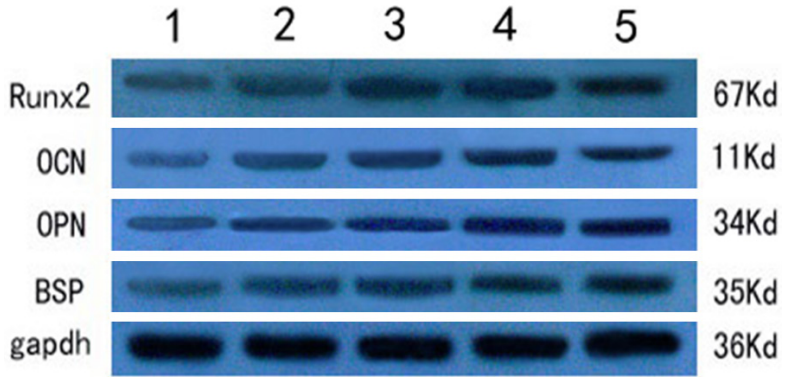

Figure 4. Expressions of Runx2, OCN, OPN and BSP by Western Blot detection. Note: 1. Nasal mucosa-derived MSCs; 2. Nasal mucosa-derived MSCs+ induction for 3 d; 3. Nasal mucosa-derived MSCs+ induction for 7 d; 4. Nasal mucosa-derived MSCs+ induction for 10 d; 5. Nasal mucosa-derived MSCs+ induction for 14 d.

were incubated for 5 h. Then the incubation was terminated, the culture medium was discarded, and 150 ul DMSO was added into each well. The plate was oscillated at low speed on a shaker for 10 min to fully dissolve the crystals. The absorbance values were measured at 490 nm using a microplate reader, with the setting of zero wells (culture medium, MTT, DMSO) and control wells (cells, dissolving medium, culture medium, MTT, DMSO).

Result

Culture and identification of nasal mucosa-derived MSCs and building of calcification model

At 3 d after inoculation, the nasal mucosa-derived MSCs adhered to the wall. At early stage, the cells were mostly flat, polygonal epithelioid cells. The cells showed cobblestone appearance and nest-like distribution. As the cells were passaged for several generations, the epithelioid cells disappeared and the spindle-shaped nasal mucosa-derived MSCs proliferated rapidly. When the cells covered the bottom of the flask, they were arranged in basically uniform direction (**Figure 1**). The identification of primary nasal mucosa-derived MSCs is shown in **Figure 2**, and the staining after 2 weeks of osteogenic induction in **Figure 3**.

Results showed that nasal mucosa-derived MSCs were successfully isolated and passaged. Nestin was detected by immunofluorescence assay in the cells of the third generation. It was further confirmed that the isolated cells were nasal mucosa-derived MSCs. As indicated by alizarin red staining, the calcification model in nasal mucosa-derived MSCs was successfully built.

Western blot detection of osteogenic markers Runx2, OCN, OPN and BSP

Total protein extraction was performed in nasal mucosa-derived MSCs after osteogenic induction for 0, 3, 7, 10 and 14 d, respectively. Osteogenic markers Runx2, OCN, OPN and BSP were detected by Western Blot, and the varia-

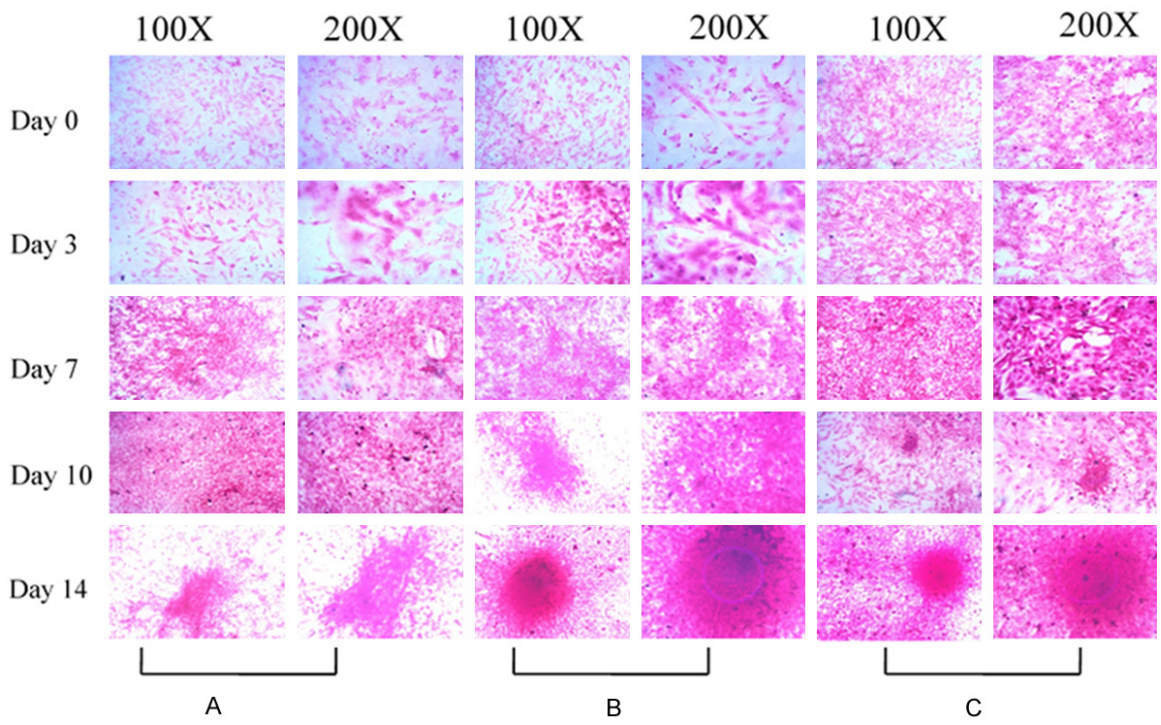

Figure 5. Synergistic osteogenic effect of nasal mucosa-derived MSCs and BMSCs by alizarin red staining. A. Co-culture with proportion of nasal mucosa-derived MSCs to BMSCs being 1:4; B. Co-culture with proportion of nasal mucosa-derived MSCs to BMSCs being 1:1; C. Co-culture with proportion of nasal mucosa-derived MSCs to BMSCs being 4:1.

tion of the expressions over time is shown in **Figure 4**. The relative expressions of Runx2 and OCN reached the highest level after osteogenic induction for 7 d, and the expressions of OPN and BSP were also high. But at 10 d, the expressions of all markers declined somewhat. At 14 d, the expressions of OPN and BSP reached the peak, but without significant differences compared with those at 7 d.

Synergistic osteogenic effect of nasal mucosa-derived MSCs and BMSCs

ALP activity in the supernatant of co-culture was detected by ELISA, and synergistic osteogenic effect of nasal mucosa-derived MSCs and BMSCs was detected by alizarin red staining. The results of alizarin red staining were shown in **Figure 5**.

As indicated by ALP levels in the supernatant using ELISA kit, for each proportion of cells (1:4, 1:1, 4:1), the ALP activity increased with longer co-culture duration. Alizarin red staining showed that the calcification in each proportion was improved with longer co-culture duration.

Effect of calcification induction on the proliferation capacity of nasal mucosa-derived MSCs and BMSCs

MTT cell proliferation assay was carried out after co-culture of nasal mucosa-derived MSCs and BMSCs for 14 days (1:1) for calcification induction group and non-calcification induction group. The effect of nasal mucosa-derived MSCs and BMSCs on the proliferation capacity of BMSCs was analyzed, as shown in **Figure 6**.

As compared with the non-calcification induction group, the calcification induction group showed no obvious differences in the proliferation capacity of nasal mucosa-derived MSCs and BMSCs. All cells proliferated vigorously.

Discussion

In the present study, we found the repair effect of nasal mucosa-derived MSCs in orbital fracture is achieved by facilitating the expressions of osteogenic markers Runx2, OCN, OPN and BSP.

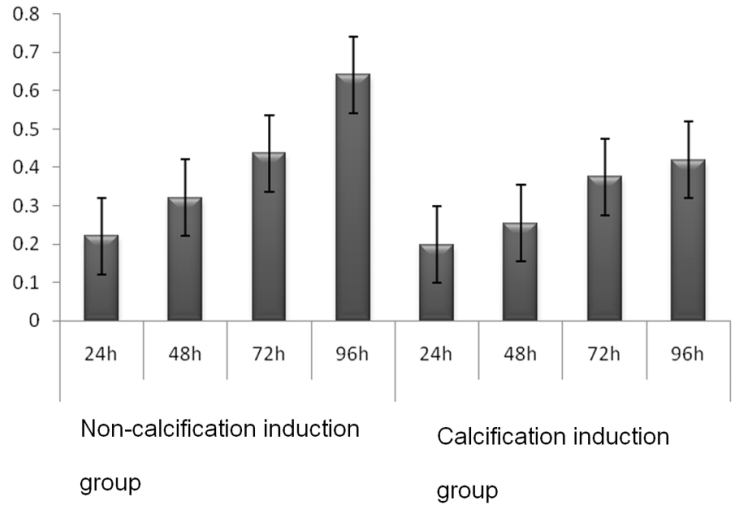

Figure 6. MTT results comparison between the two groups.

Orbital fracture is mainly diagnosed based on trauma history, physical examinations and imaging techniques. CT scan is considered as the gold standard for the diagnosis of orbital fracture, which also determines the range and severity of orbital fracture [20-22]. Surgery is the preferred choice for orbital fracture. Early surgery refers to that performed within 3 weeks after trauma, for the purposes of eliminating and improving functional amblyopia, preventing and correcting enophthalmus, repairing and reconstructing orbital morphology and correcting orbital malformation [23]. Mesenchymal stem cells (MSCs) is one of the adult stem cells which generated from the early development stage of mesoderm. MSCs exist widely in many tissues of human body, and play an important role in immune regulation, reduce inflammation, and repair injury for its characteristic of differentiation and low immunogenicity function [24]. MSCs exist in many tissues (such as bone marrow, umbilical cord blood and umbilical cord, placenta and adipose tissue), with mesenchymal cells to a variety of series (such as bone, cartilage and fat cells) or mesenchymal differentiation matter series of cell potential, and is of the unique ability of cytokine secretion [25]. MSCs can be isolated from bone marrow, adipose tissue, synovium, bone, muscle, lung, liver, pancreas, amniotic fluid and umbilical cord blood at present.

In the present study, we isolated the nasal mucosa-derived MSCs from goat and built the calcification model so as to study the repair mechanism of nasal mucosa-derived MSCs in orbital fracture. After osteogenic induction of nasal mucosa-derived MSCs for 7 days, the relative expressions of Runx2 and OCN reached the highest level, and those of OPN and BSP were also high. As the duration of co-culture of nasal mucosa-derived MSCs and BMSCs increased (0-14 d), the ALP activity in the supernatant also increased, with enhanced calcification. Compared with the non-calcification induction group, the calcification induction group showed no obvious differences in the proliferation capacity of nasal mucosa-derived MSCs and BMSCs.

In conclusion, the repair effect of nasal mucosa-derived MSCs in orbital fracture is achieved by facilitating the expressions of osteogenic markers Runx2, OCN, OPN and BSP. However, the pathways of actions are unknown and further studies are required to elucidate the concrete mechanism.

Acknowledgements

This work was supported by the Shenzhen Science and Technology Innovation Committee Fund of China (JCYJ20140414114853649).

Disclosure of conflict of interest

None.

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