Effect of Adipose-Derived Mesenchymal Stem Cell Repair in Nicotine Model Rat: A Femoral Bone Diaphyseal Defect Study

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Abstract

Background: The major function of the bone in the skeletal system is to provide structural support to the body and its vital organs. Many patients suffer from the disability to restore bone lesions following bone fractures during crashes or accidents. The use of mesenchymal stem cells such as adipose-derived mesenchymal stem cells (ADMSCs), along with collagen scaffolds, and its transfer to the lesion site can be valued as one of the available treatment options.

Objectives: In the current paper, a study was conducted on the level of mesenchymal stem cell repair from the rat adipocytes, where it was evaluated in bone defects.

Methods: In this study, mesenchymal stem cells were isolated from the rat adipocytes and their stem cell lines were determined with the standard cell tests. The isolated cells were differentiated in the next step and transferred to the two main groups: nicotine modeled and non-modeled (non-nicotine) along with collagens. The repair of the defect caused by a 2 mm drill in the diaphyseal region of the rat bone was evaluated after four weeks using radiographic examination and histopathologic staining.

Results: Radiographic data analysis indicated that bone density was much higher in the non-nicotine group than in the nicotine group. Histopathologic staining showed that bone formation was higher in the non-nicotine group than in the nicotine group. The new bone formation was about 80% and 60% in the non-nicotine and nicotine groups with differentiated osteocytes of ADMSCs, respectively.

Conclusions: Adipose-derived mesenchymal stem cell transplantation is effective in bone defect repair and nicotine plays an important role in the bone repair process as an inhibitory agent.

Keywords: Adipocyte Mesenchymal Stem Cell, Diaphyse, Nicotine, Rat

1. Background

The major function of the bone in the skeletal system is to provide structural support to the body and its vital organs. Bone is also considered to be the main source of minerals, as it provides a suitable leverage system for muscle contraction. Taking these effects into consideration, the consequence of bone loss in body homeostasis can be easily imagined (1, 2). Wars, diseases, accidents, and natural disasters among other types of events can cause damage to the bone tissue, including the skull. The most common cause of mortality in accidents is a hit on the head. Minimal treatment can spontaneously recover the majority of bone damages. However, sometimes spontaneous healing cannot be performed in some cases such as misplaced bone fusion, complete bone loss because of a tumor, and lesion site infection, necessitating further treatment. Orthopedists use a variety of methods to fully repair the damaged bone, such as bone grafting, herbal drugs (antibacterial compounds), or metal implants (2-5). Mesenchymal stem cells can differentiate into bone marrow cells. This seems promising since these cells could be used in the cell therapy of large bone lesions in the near future. Some researchers believe that if the cell therapy strategy is used in bone lesions, the cells should be well-differentiated (6, 7).

The adipose and connective tissues contain different adipocytes retained by collagen fibers. The cytoplasm of these cells is made up of approximately 90% fat and stromal-vascular cells including smooth muscle cells, endothelial cells, fibroblasts, and pre-adipocytes. Pre-adipocytes are located in the vascular-stromal region of the tissue. The appearance of these cells before differenti-
ation is pseudo-fibroblastic. Morphological and biochemical changes in these cells take place due to differentiation, in such a way that the cells become spherical with accumulated fat vacuoles. Pre-adipocytes, owing to their good ability to isolate, cultivate, expand, and differentiate into different cell types, are thought to be beneficial cellular sources (1, 8-10).

Some studies have shown that stem cells can be a significant hope for regenerative medicine and many researchers have studied these cells in many animal models such as planarians. The fatty tissues contain high levels of adult adipocytes, pre-adipocytes, fibroblasts, capillary smooth muscle cells, endothelial cells, established macrophages, and lymphocytes. Adipose-derived stem cells (ADSCs) have been isolated recently. Evidence suggests that these cells have the potential to differentiate into organ cells with mesodermic origins. Neurons, pancreatic endocrine cells, liver cells, endothelial cells, and heart muscle cells are all examples of increasing evidence based on the ability of ADSCs to differentiate into non-mesodermic cells (10-14). The surgical procedure is simple and access to subcutaneous fatty tissue and non-complex enzymatic separating process that provides ADSCs is repeatable. Such simplicity and repeatability are more attractive to researchers and clinicians. Also, the growth of ADSCs is easier and faster than that of BMSCs. It is estimated that 1% of the cells in the adipose tissue become mesenchymal stem cells in comparison with 0.001 - 0.002% of the cells from the bone marrow (1, 10, 12, 15). There are more numerous reasons why mesenchymal stem cells are suitable for bone tissue engineering as cell sources. These reasons include rapid and relatively easy separation for the patient, the presence of a high level of mesenchymal stem cells for isolation, immune system moderating properties, the secretion of effective factors in the treatment, the ability to identify and enter to the damaged site, the lack of ethical problems in their use, the low risk of mesenchymal stem cell tumorigenicity, and the power of proliferation and differentiation of mesenchymal stem cells, in addition to the availability of extensive studies on the therapeutic role of mesenchymal stem cells (16-23).

Studies have demonstrated that what can play a vital role in improving the functional activity of the organ is the number of live stem cells in the graft region. It has been shown that free radicals play a major role in reducing the viability of the transplanted cells. One of the most common abnormalities in bone defects is rapid and complete repair. The reduction of bone density by age and non-welding after a fracture not only can disable the person but also may lead to heavy costs for the individual and society. Researchers have always sought to find a suitable treatment for these patients, especially because of the relative limitation of repair in these conditions. Several treatments have been proposed for the rapid repair of bone defects (10, 12, 15, 24).

2. Objectives

In this study, the repair effect of mesenchymal stem cells from rat adipocytes was investigated on bone defects in rats.

3. Methods

3.1. Isolation and Culture of Mesenchymal Stem Cells of Rat Adipocyte

First, male Wistar rats (Rattus Norvegicus) (200 to 250 g) were purchased to isolate and culture the mesenchymal stem cells of the bone marrow. The rats were purchased from the Pasteur Institute (Tehran, Iran). The animals were anesthetized by the inhalation of carbon dioxide. In the beginning, the adipose tissue was taken from the rat testicles, crashed, and washed with sterile salt buffer to isolate fatty stem cells. Then, the adipose tissue was transferred into a solution of salt-buffered saline and 0.3% collagenase (SERVA, Germany) for 45 - 60 minutes at 37°C, allowing the whole tissue to completely digest. After digestion, the enzyme was neutralized by adding cold buffer solution (PBS) (MiltenyiBiotec, Germany) and centrifugation was performed (25-27). The supernatant containing debris and fat droplets was discarded. The resulting pellet was collected and filtered using a diluted salt solution passed through a 70 µm filter. Following filtration, washing with buffer solution was performed twice (400 g/5 min). The cellular precipitate, containing adipocyte mesenchymal stem cells, was used for further experiments. The cells were transferred to a culture container at 30,000 cells/cm². After 48 hours, the floating cells were removed and the attached cells were cultured to reach 90% confluency. After three passages, the resulting cells were frozen and stored for further use.

3.2. Identification of Isolated Mesenchymal Cells

Based on the expression and lack of expression of surface markers, the cells were identified by flow cytometry according to the defined criteria. In this test, CD90 and CD106 were positive markers, while the negative markers were CD34 and CD45. In this study, the bone differentiation test was used to verify the mesenchymal nature of the cells and to evaluate their power of differentiation into osteocytes. After reaching the confluence of the third subculture cells, the cell culture medium was replaced with bone and fat differentiation medium and then evaluated.
for the differentiation using Oil Red and Alizarin Red staining.

3.3. PCR Confirmation of Adipocyte Mesenchymal Cells Differentiated into Osteocytes

The primers were designed using NCBI and Primer 3 software and synthesized by Sinaclon Co. (17). During the PCR assay, primers were bonded on both sides of the target sequence. This allowed for the polymerase activity of DNA polymerase (28, 29). Therefore, the matching of the primer sequence with the template was of particular importance. The specifications of the primers are shown in Table 1.

3.4. Preparation, Surgery, and Grouping

Experiments were all carried out following the instructions of the Animal Ethics Committee of Islamic Azad University. This protocol observes the ethical principles and protection of animals used for laboratory and other scientific purposes. The collagen needed for preparing hydrogel was extracted from the rat’s tail. The extracted collagen (0.04 mg/mL) was first dissolved in acetic acid to prepare the collagen solution. Then, this mixture was mixed with cell culture media (DMEM 1X, 350 µm), NaOH 0.4 N (26 µm) and FBS (10%). As we needed the pH to be close to the body pH, NaOH was added to neutralize the scaffold. In the next step and before injection to the damaged area, 100,000 extracted cells were mixed with it. The present study was conducted on 24 adult male rats weighing approximately 150 - 200 g. All the rats were kept under similar circumstances (12 h light and 12 h dark cycle at 20 - 25°C).

The rats were assigned to two groups: a control group (with no nicotine injection) and a nicotine group. To do experiments on the rats, 200 µL of the mixture of nicotine and saline (1/10 µL dilution) was injected into the control group (three times every 48 hours). Access to water and food was free for both groups. After shaving the thighs, a thorough cleaning was performed in the area using iodine and alcohol. To anesthetize each rat, 90 mg/kg of ketamine and 10 mg/kg of xylazine were used intraperitoneally. After shaving the thighs, the area was cleaned with iodine and alcohol. During the surgery, the rats’ hands and feet were fixed on the specified points on the surgery bed and then, a cut was made onto the skin. For adequate access to the relevant area, the fascia and muscles were cut slowly. Trephine was used to create a defect (2 mm diameter). In this step, the cells of each group were cemented to a critical size for bone regeneration (differentiated osteocytes from the adipose-derived mesenchymal stem). Consequently, the post-care was done (stitching up, disinfecting the wound with tetracycline spray, and injecting amoxicillin (0.1 mg/kg)). The rats were all kept in a similar condition in the animal house (clean cage, suitable temperature, and appropriate nutrition). After four weeks, the rats were sacrificed with a high dose of anesthesia and the specimens were removed.

3.5. Radiographic Examination

Four weeks after the cell transplantation, a radiographic examination of the right thigh femoral bone was performed with a Senographe 600T Senix HF device at a dose of 22 kV and 9 mAs. The test was performed from the anterior-posterior (AP) and lateral (Lat).

3.6. Hematoxylin-Eosin Staining

To do the histopathology test, the samples were immersed in 10% formalin for fixation. Then, all samples were immersed in 10% nitric acid. After decalcification, the sample was sent to the histopathology laboratory. Tissue processing and sectioning (5 microns) were the next steps performed. The specimens were stained using Hematoxylin-Eosin (H&E) and examined and photographed with a light microscope (30-35).

4. Results

4.1. Isolation and Culture of Bone Mesenchymal Cells

As already stated, mesenchymal cells have two main characteristics, i.e., easy isolation and proliferation compared to other types of stem cells and the potential for conversing the different types of cells. Considering these characteristics, mesenchymal cells were selected for culture and evaluation of the damaged tissue repair. To do so, the isolated adipocyte cells were first centrifuged as mesenchymal cells and then were filtered and purified (being cleaned from other impurities using PBS). Since binding to the surface is one of the characteristics of mesenchymal cells, other cells were washed out by intermittent media changes. Having the cells being sub-cultured four times, they were relatively purified; a part of them was then frozen. Flow cytometry showed that the tested cells were cells with very low expression of CD34 and CD45 and high expression of CD90 and CD106 markers, indicating they were mesenchymal stem cell types. The differentiated mesenchymal stem cells to osteocytes and adipocytes were recognized by alizarin red and oil red, respectively. Their differentiation is shown in Figure 1.

4.2. PCR Analysis

The results of the PCR confirmed the expression of specific osteocyte genes differentiating ADMSCs into osteocytes including alkaline phosphatase, osteocalcin, and osteopontin (Figure 1).
Table 1. Primer Specifications

| Primer | Sequence                  | Size (bp) | TM   |
|--------|---------------------------|-----------|------|
| SPP1   | F: 5'CGCAGTTCTCCTGGCTGAAT-3' | 150       | 59.35|
|        | R: 5'ACTGTGTTGCTTAATGCCG-3' | 57.30     |      |
| ALP1   | F: 5'GGACCCTGCTTACCAACTC-3' | 245       | 61.40|
|        | R: 5'AACTTGTCCATCTCCAGCCG-3' | 59.35     |      |
| BGLAP  | F: 5'GAATAGACTCCGGAGCCTAAC-3' | 229       | 61.40|
|        | R: 5'FCCAGTCCTGCGAGCTAGCC-3' |           |      |

4.3. Radiographic Images

The results of radiography showed that the rate of osteogenesis in the non-nicotine group was much higher than that of the nicotine group. It also demonstrated that the level of this density was higher in the differentiated group. This issue was completely true for the nicotine group. The self-repair was evident in the control group without any cell transplantation compared to the nicotine group (Figure 2).

4.4. Histopathologic Images

According to the histopathologic staining, it was shown that the rate of osteogenesis and bone formation in the canal was more pronounced in the non-nicotine group than in the nicotine group. As shown in Figure 3, the level of regular bone formation was better and more in the group differentiated from adipocytes than in other groups. Comparing nicotine and non-nicotine groups, we observed the regular and continuous bone formation in the non-nicotine group differentiated from adipocytes, which was more distinct than that in the nicotine group with the same situation. The microscopic observations are shown in Figure 4 based on the repair percentage average in the defected area. Samples with severe fractures were
not reported and not entered in the percentage determination.

5. Discussion

The processes and mechanisms needed to be taken and acted to repair the damaged site are often difficult to handle in several cases. These cases include fractures in which wound healing and repair of the lesion are difficult or in diseases such as cancer, infection, osteoporosis, osteoarthritis, etc. Today, mesenchymal stem cells and biodegradable scaffolds are widely used to repair bone defects (2-4).

In this study, mesenchymal stem cells were isolated from the fat tissue of rats. Then, they were identified utilizing adhesion tests, flow cytometry, differentiation into adipocytes and osteocytes with specific staining, and expression of the desired genes by PCR. The tests confirmed the stem cell originality of the cells. Finally, the isolated cells from each group were differentiated into osteocytes (the third sub-culture cells that were placed in osteogenesis differentiation media for three weeks, undergoing morphological changes). They got denser in some places. The reddening of extracellular materials in the osteocyte differentiation culture was the result of staining. Then, they were assembled with the prepared collagen to transfer to modeled and non-modeled animals. Niedzwiedzki et al. treated bone defects in the right arms of rabbits using non-autologous BMSC transplants and considered the left arms as controls. On days 20 and 40 after transplantation, the results of the radiographic analysis indicated that in the experimental organ, the matrix filled the bone defect. Meanwhile, in the control organ, the bone defect was not filled well with the matrix. Accordingly, the results showed a significantly higher bone mineral density in the experimental group than in the control group (36).

For the treatment of pediatric patients with osteogenic defects, Horwitz et al. used non-autologous bone marrow cell transplantation. They found that bone density increased after three months of treatment and that the rate of fracture reduced (37). Shih et al. also indicated that biomechanical tests in the group under the autologous transplantation of stem cells in the demineralized femur distal of dogs showed significant increases in comparison with the allograft treatment group, non-cellular demineralized bone marrow matrix (PDMB) group, and no treatment group (38).
In this study, we evaluated two main groups of nicotine and non-nicotine models. For each major group, a control group was also considered. The differentiated osteocytes were obtained from adipocytes. The cells were transferred to the lesions according to the grouping. After four weeks, radiography results showed that the rate of osteogenesis was much higher in the non-nicot ine group than in the nicotine group. The radiography results also showed that the rate of this density was more in the non-nicot ine group receiving differentiated cells from adipocytes than in the nicotine group. In contrast, the results of histopathologic staining showed that osteogenesis and bone formation were more in the non-nicot ine group than in the nicotine group. In addition, the rate of regular bone formation from adipocytes. The amount of osteogenesis in the control groups toward the center was observed in the margin of the defect, which was more than that in the nicotine control group.

5.1. Conclusions

Autologous graft/transplantation of adipocyte stromal cells in rats with bone defects can lead to the accelerated recovery of the bone. Meanwhile, what basically applying more positive effects on bone defect repair is autologous ADMSCs transplantation.

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Footnotes

Authors' Contribution: All authors done equally.

Conflict of Interests: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Ethical Approval: Experiments were all carried out following the instructions of the Animal Ethics Committee of Islamic Azad University. This protocol observes the ethical principles and protection of animals used for laboratory and other scientific purposes.

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