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Original Article

in vitro Study of Primary Isolation and Culture of Adipose-Derived Stem Cells and Induction of Chondrogenic Differentiation

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Abstract

Articular cartilage has constrained potential to restore. The mesenchymal stem cellular remedy has presented new treatment possibilities for this circumstance. The experiment aimed to verify the chondrogenic differentiation capacity of rat adipose tissue-derived mesenchymal stem cells (AD-MSCs) *in vitro* inside the presence or absence of transforming growth factor-beta (TGF-β1). Rat's subcutaneous adipose tissue minced into a small piece (2-3 mm^3) was aseptically collected from the subcutaneous fat under anaesthesia and then digested with collagenase type I (1 mg/mL). Spontaneous chondrogenesis occurred in both AD-MSCs pellet cultures and was similar in both TGF-β1 treated. The untreated pellet cultures were collected after 21 days. Histological assessment for evaluating the level of proteoglycan by alcin blue staining and immunohistochemistry for detecting the presence of collagen type II. A monoclonal antibody directed against collagen type II. Adipose-derived stem cells (AD-MSCs) isolated from rats were immunophenotyped for the expression of MSCs cell surface markers and was performed by Flow cytometer, which demonstrated AD-MSCs highly expressed CD73 (99.69±2.6%), CD90 (98.11±0.3%), and week expression CD44 (17.15±0.3%). The result of histological staining showed the presence of extracellular matrix (ECM) in the hyaline cartilage. This staining indicated a deposit of "acid mucopolysaccharides" in the proximity of the cells. Additionally, most cells are rounded cells stained positive for the presence of the cells encompassed by extracellular matrix (ECM), which were like chondrocytes as seen from the magnified view, lightly pink stained nuclei, and nuclear fast red stain. However, the immunohistochemistry method demonstrated that the presence of TGF-β1 decreased the levels of collagen type I and increased the levels of collagen type II. In conclusion, subcutaneous adipose tissue-derived stem cells can be used in cartilage tissue engineering.

Keywords: Rat, Adipose Tissue, Chondrogenic, Mesenchymal Stem Cells

1. Introduction

Mesenchymal stem cells are non-hematopoietic cells that reside in the bone marrow and other skeletal tissues. Studies have shown that these cells are capable of a variety of non-mesenchymal neurons, such as neurons, lungs, kidneys, and even skin, which is called the plasticity of mesenchymal stem cells. Researchers could differentiate rabbit bone marrow by subcutaneous grafting (1), and Friedenstein, Piatetzky-Shapiro (2) provided conclusive evidence of mesenchymal stem cells in the bone marrow. Specific to adherent cells is that it is called the colony-forming unit (f-CFU). Friedenstein, Chailakhjan (3) also showed that f-CFU could form bone and cartilage-like colonies in a culture medium. Friedenstein was developed by later scientists (4, 5). Mesenchymal stem cells are considered a

suitable source for treating many diseases due to their self-renewing properties and ability to differentiate into skeletal tissues. These cells' efficacy in treating the genetic disease imperfect osteogenesis, improving hematopoiesis in cancer patients undergoing treatment, bone regeneration, repairing necrotic tissue in patients with myocardial infarction, and the treatment of joint diseases have been well demonstrated (5, 6).

Recently, fat-derived mesenchymal stem cells have received a great deal of attention in soft tissue regeneration. These cells can differentiate into cartilage, bone, muscle and fat in the presence of differentiating factors and suitable culture conditions. Multipotent fat-derived mesenchymal stem cells maintain their ability *in vitro* over several passages and increase and maintain cartilage-building ability by cell proliferation and proliferation. Compared to similar cells derived from other tissues, including bone marrow, these cells have many features and benefits, including the easy access of these cells from the patient in quantities.

Relatively abundant using liposuction method. They are now considered a means of treating tissue defects with the individual's stem cells (4).

Cartilage tissue engineering based on fat-derived mesenchymal stem cells has provided a robust solution for expanding three-dimensional cartilage tissue by combining it with suitable biomaterials and optimal growth factors (7).

The β-TGF factor has been widely used to induce the process of differentiation of fat-derived cartilage-derived mesenchymal stem cells (4). Three-dimensional cell differentiation usually occurs on three-dimensional scaffolds in a special differentiation medium. In addition to external growth factors, various scaffolds act as a three-dimensional protective matrix to properly grow cells and tissues (7). To be used. Three-dimensional culture systems are valuable for their ability to differentiate fat-derived progenitor cells into cartilage because of their ability to maintain natural cell phenotypes (8).

Mesenchymal stem cells (MSCs) are a populace of multi-powerful stem cells that can proliferate and differentiate into numerous types of mesodermal tissues, including BM, cartilage and skeletal muscle (9). MSCs are an appealing source of stem cells for the regeneration of broken tissues in medical packages (10) because of their traits as undifferentiated cells, their potential to self-renew with high proliferative potential, and their capability to distinguish into the mesoderm (11). Among various person stem cells, adipose-derived stem cells (ADSCs) can differentiate into not most effective adipogenic, endotheliogenic, chondrogenic, cardiogenic, myogenic, and osteogenic cells *in vitro* below the unique way of life conditions (12). In addition, adipose tissues are plentiful and comparatively without problems acquired, *in vitro* chondrogenic differentiation (13) or differentiation in the presence of growth elements which includes remodelling boom thing-β (TGF-β), bone morphogenetic proteins (BMPs) or insulin-like increase factor-1 (IGF-1) (14). Therefore, ADSCs as a donor show many blessings, for they may be easily acquired in ubiquitously allotted adipose tissue with little damage to the patient and are expandable *in vitro* (15). Therefore, in the present study, we assessed isolating and culturing the adipose tissue-derived stem cell and assessed chondrogenic differentiation in the pellet using growth factor.

2. Materials and Methods

2.1. Experimental Animals Design

This study was performed using the tissue instructions of the Tissue Culture unit National Center of Hematology, Al-Mustansiriyah University. Ten Wister rats that were clinically healthy with an average weight of 250±20 g were used. Rats were kept separately in fibreglass cages, and commercial pellets were used as feed. Water and feed were *ad libitum*. Rats were kept in sterile cages for 15 days for adaptation. The animal shelter had 12-hour dark-light periods and a temperature of 23±2.0ºC. During all stages of the experiment, the animals were treated according to the principles of keeping and using laboratory animals—high-dose injections of pentobarbiturate killed adult

male rats. Under completely sterile conditions, white adipose tissue fragments were removed from the abdominal inguinal region by vertical incision, followed by isolation, and culture in a container containing isotonic solution was transferred.

2.2. AD-MSCs Isolation and Culture

The adipose tissue sample in the culture room and under the laminar hood was cut into small pieces with sterile scissors. The fat sample was washed several times with Phosphate-Buffered Saline (PBS) solution to remove blood cells and debris, and the supernatant was discarded.

The remaining white adipose tissue was transferred to the falcon tube at the bottom of the plate, and 5 to 6 ml of 0.1% collagenase enzyme was added to it and placed in a 37 °C hot water bath (Memmert) for 1.5 to 3 hours (Figure 1A). (During this time, shaking the sample tube helped further break down adipose tissue). After the enzymatic decomposition step, the tubes containing the sample were taken out of the hot water bath and centrifuged at 2000 rpm for five minutes. This operation can be repeated about three times. The oil and adipose tissue were gently removed and discarded, keeping the solution at the bottom of the tube containing the deposited fat cells. The cells were transferred to a culture flask containing DMEM with 10% FBS. Finally, a flask containing the cell was placed in an incubator at 37 ° C and 5% CO.

After stem cell isolation, the medium was changed every four days. For this purpose, the solution thrown into the flask was discarded. The fresh DMEM medium, 10% of which was FBS (Figure 1B), was then added to the culture flask and transferred back to the incubator. At this stage, cell growth was evaluated under an inverted light microscope. The cells were spherical at this stage.

After the regular change of culture media, the cells reached the density stage. To transfer the cells to a larger flask, cell passage was performed. After three cell passages, flow cytometry was used to confirm the mesenchymal nature of the cultured stem cells.

2.3. Phenotypic Characterization of AD-MSCs by Flow Cytometer

For flow cytometry, conjugated antibodies, Anti-Human-CD34-FITC, Anti-Human-CD90-FITC, Anti-Human-CD31-RPE, Anti-Human-CD105-RPE with negative control (Dako Corporation, Glostrup, Denmark) was used (isolated cells in the third passage). First, cells attached to the bottom of the flask were isolated by Trypsin/EDTA, and cell count was performed using a Neubauer slide. In the next step, in a dark environment, an appropriate concentration (ratio 1:10) of the mentioned labelled antibodies was added with negative control and kept at room temperature for 20 minutes. After washing with PBS, flow cytometric analysis was performed by FACSort (Becton Dickinson, San Jose, CA) (Figure 1C).

First, the primary culture medium was discarded, and the cells were incubated with a differentiation medium, including DMEM containing 10 mM beta-glycerol phosphate, 0.2 mM ascorbic acid and 7 to 10 M dexamethasones for 21 days. The differentiation medium was changed every three days. To evaluate the differentiation, staining with Alizarin red was performed to detect calcium stores.

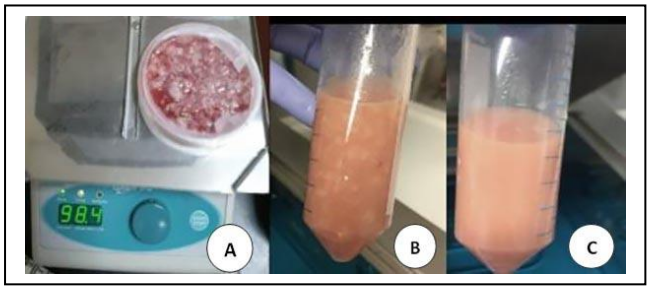

Figure 1. Isolation and cultivation of AD-MSCs by using (**A**) enzymatic digestion for Collection of adipose tissue, (**B**) Discarding the fat layer and supernatant after tissue digestion and centrifugation. (**C**) Pellets after discarding fat layer and culture with 3 ml media

2.4. Chondrocyte Differentiation

To differentiate cells into cartilage, the supernatant was emptied, and the cells were combined with cartilage-forming medium containing DMEM, Transforming Growth Factor-β3 (TGF-β3) (10 ng),

Bone Morphogenetic Sigma-Aldrich, St. Louis, MO (10 ng), protein-6 (BMP-6); Sigma-Aldrich, St. Louis, (1%), Insulin-Transferrin-Selenium (ITS), 0.1 μM dexamethasone, 50 μM 2-phosphorus ascorbate, 1% penicillin-streptomycin and 1% Fetal Bovine Serum (FBS) were incubated for 21 days. Specific blue toluidine staining was used to evaluate the differentiation into cartilage (Figure 2).

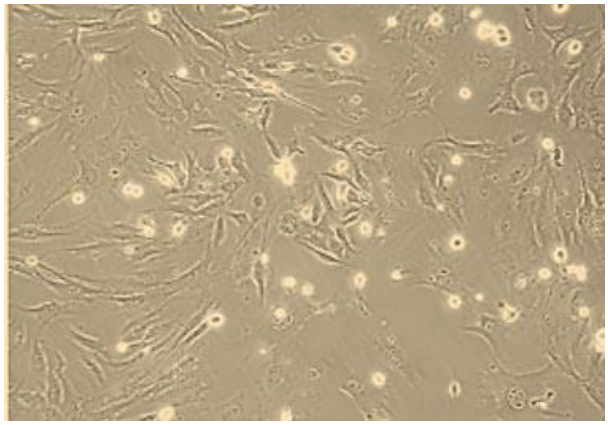

Figure 2. Isolation, morphological of AD-MSCs Outgrowth of stem cells from adipose tissue after 3 days (×400)

2.5. Histological Staining and Immunohistochemistry

Twenty-one days after culturing the cells in plate 24 cells, the samples were fixed in 4% paraformaldehyde after 20 rinses with PBS buffer for 20 minutes. After washing them with distilled water, toluene blue dye was added to them. The samples were then examined under a light microscope. Twenty-one days after cell culture on the scaffold, the samples were prepared from scaffolds with paraffin blocks after washing, fixing, dewatering and clarification. The 5-micron sections were stained with Toluene blue. The samples were then examined under a light microscope.

After preparing the slides with 5-micron sections, clarifying and hydrating, the tissues were fixed in Acetone to evaluate the immunohistochemistry of cartilage formation. The slides were recovered in the microwave for 20 minutes. The slides were washed in saline buffer (TrisTBS). The tissue hydrogen peroxide activity was neutralized with an H_2O_2 solution in 70% ethanol for 5 minutes at room temperature. Collagen II (Antibody Anti Collagen) was incubated overnight at 4 °C. After washing the slides in TBS buffer, again with Goat anti-Rabbit IgG H&L (HRP) at room temperature for 2 hours. After washing in TBS buffer, the samples were incubated with DAB substrate for 10 minutes and washed with running water for 1 minute. The samples were examined using a light microscope.

2.6. Statistical Analysis

Data were analyzed using SAS software (V. 9.1). The mean values were compared through the least significant difference (LSD) and Tukey test at 5%.

3. Results

3.1. Morphology of Adipose Tissue-Derived Mesenchymal Stem Cells

In the experimental examination, after 48 to 72 h, small heterogeneous cell colonies were seen; the cells were flattened and spherical formed and started to attach to the covered surface of a plastic lifestyle flask with the formation of colonies (Figure 2). After 10 days, the cells and colonies accelerated markedly, and colonies were progressively elevated in size and interface to form a monolayer of adherent cells. The flasks had been confluent. These cells displayed small rounded, spindle formed, and huge flatted morphology after 12 days of culture at (P1). Then on (P2), after two days, submit way of life AD-MSCs indicated excessive confluence of spindle-formed cells and the cells aligned themselves alongside their longitudinal axis. Following 5 days, the AD-MSCs at P3 tradition established a combination of large, flat, spindle formed; round, and polygonal-formed cells; following P4, indicated fibroblastic morphology spindle-fashioned with multiple nucleoli, which are an ordinary morphology of (AD-MSCs). Also, cells stored their morphology while subculturing past passage 4 (Figure 3).

3.2. Characterization of AD-MSCs

The isolated AD-MSCs were analyzed by flow cytometry to determine the expression of cell surface markers. The analysis indicated that the AD-MSCs highly expressed CD73 (99.69±2.6%), CD90 (98.11±0.3%) illustrated in (Figure 4) and weak expression CD44 (17.15±0.3%), while not expressing the hematopoietic stem cell marker CD45 (0.47±0.3%) (Figure 5).

3.3. Histological analysis of MSCs

AD-MSCs were trypsinized for unattached cells from the flask. The cultured in the TGF-β1 treated pellets should have a spherical shape with a diameter of 1mm with a cell count of 5×10^6 for each test (Figure 6). The result of Hematoxylin-eosin (H&E) staining demonstrated the flattened round cells and a homogenous chondrocyte distribution. The rounded cells' darkly stained nuclei and compact cartilage tissue were clear (Figure 7). Also, they used alcian blue to check the presence of extracellular matrix (ECM) in the hyaline cartilage. This staining indicated a deposit of "acid mucopolysaccharides" in the proximity of the cells.

Additionally, most cells are rounded cells stained positive for the presence of the cells encompassed by extracellular matrix (ECM), which were like chondrocytes as seen from the magnified view, lightly pink stained nuclei, and nuclear fast red stain (Figure 8). To confirm the result, immunohistochemical analysis for COLII revealed its presence in all pellet cultures, highest in the TGF-β1 treated pellets. The immunostaining intensity was lowest compared with cultures without the growth factor (Figures 9 and 10).

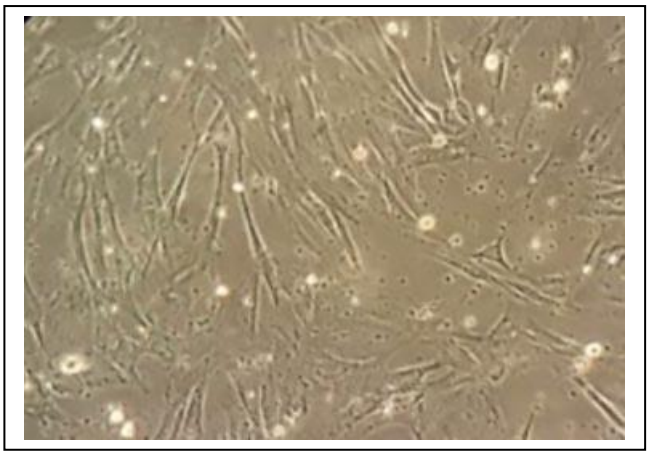

Figure 3. Shows AD-MSCs reach confluences 80% in p4, and the fibroblast-like morphology in monolayer culture (×400)

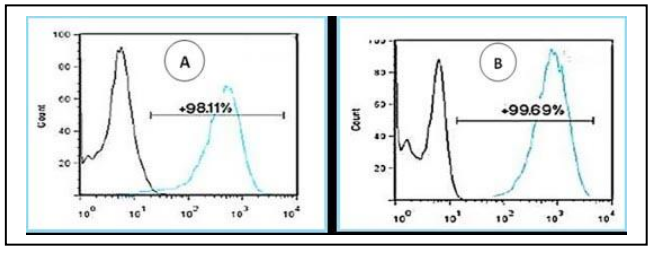

Figure 4. Flow cytometry histogram of the immunophenotype AD-MSCs showed (**A**) the expression cell markers strong CD90 and (**B**) the cell markers positive CD73 expressions

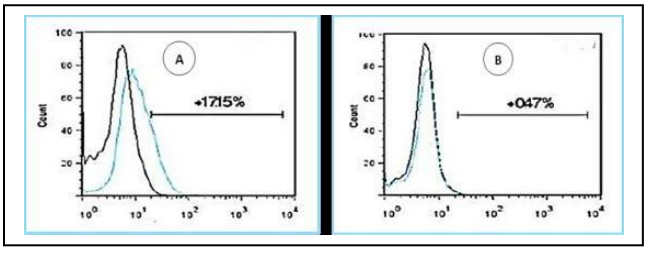

Figure 5. Flow cytometry histogram of the immunophenotype AD-MSCs showed (**A**) the expression cell markers positive CD44 expressions and (**B**) AD- MSCs had a negative tendency to express CD45 marker hematopoietic stem cells

Figure 6. Photographic showed AD-MSCs Outgrowth of stem cells after 21 days with treated TGF-β1in pellet culture media

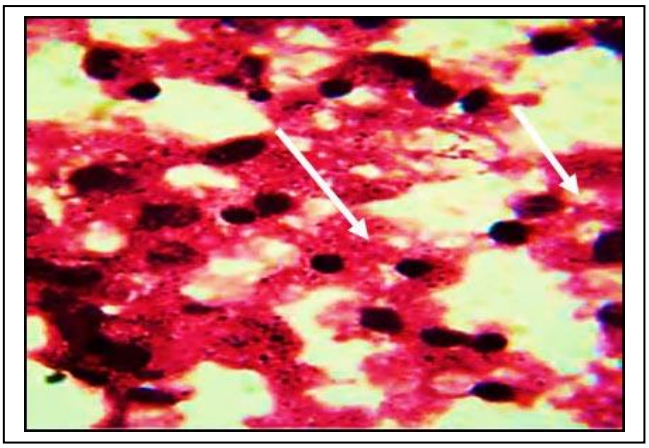

Figure 7. Alcian blue staining of chondrogenesis after the 21st day's culture in media showed Homogenous cell distribution with compact cartilage tissue evident (×200)

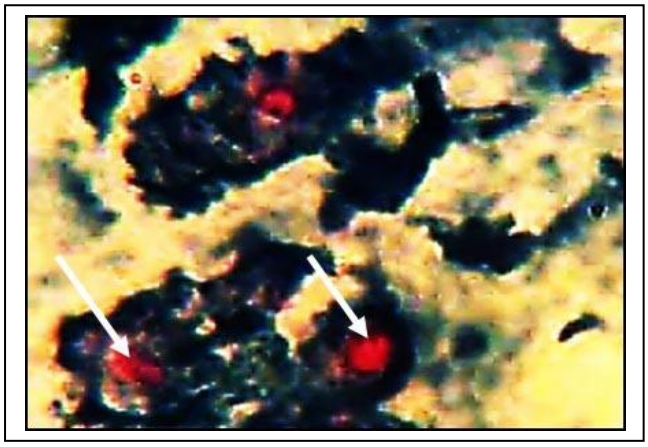

Figure 8. Alcian blue staining showed positively stained nodules of a chondrocyte, and the cell nuclei are stained with nuclear fast red (×200)

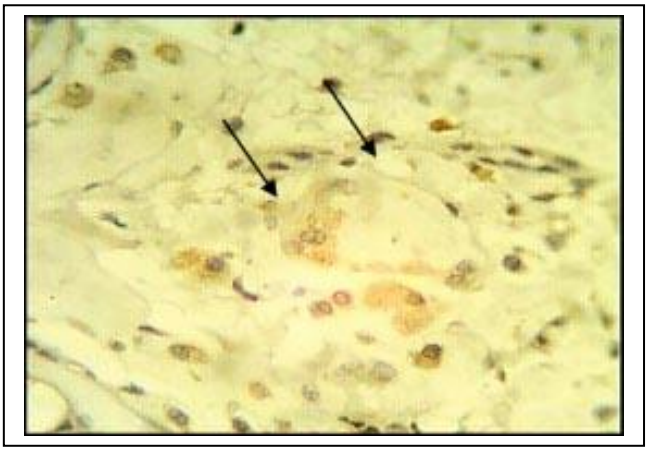

Figure 9. Immunohistochemical staining showed the existence of collagen type II in ECM was highest localized at the pellet treated with TGF-β1 media (×200)

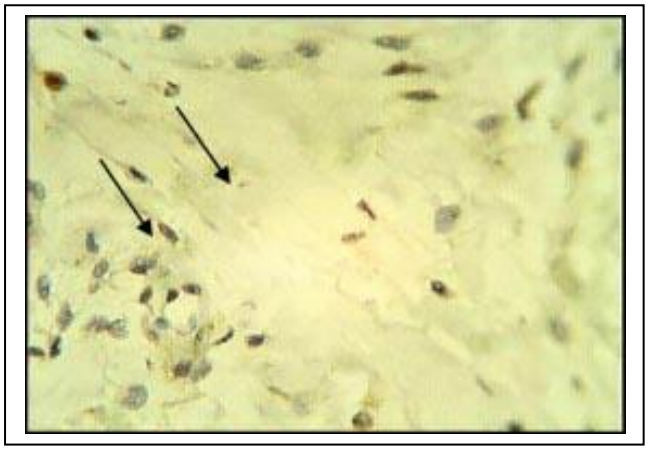

Figure 10. Immunohistochemical staining showed the lowest existence of collagen type II in ECM with cultures without the growth factor (×200)

4. Discussion

The current examination confirmed the rat adipose tissue-derived stem cell, which revealed muli-lineage capability and chondrogenic deferential capacity of AD-MSCs in the foundation of growth element (TGF-β1). This result considers previous studies (9-11). Characterization of MSCs has been completed via go-with-flow cytometry to decide the expression of cell surface markers. The analysis indicated that the AD-MSCs incredibly expressed CD73, CD90, and weak expression CD44, while now not expressing the hematopoietic stem cell marker CD45 (12). These outcomes were also observed by many other researchers (13-15), who showed these consequences as an affirmation of the undifferentiated state of the cells, those cells stained with brown colouration corresponding DAB reaction.

Moreover, in the general public of our primary tradition, adherent cells have been no longer express CD45 and CD34 markers; this is in agreement with other studies (16); each presence and shortage of these unique markers proved that our cultured cells are to mesenchymal stem cells and prepared for differentiation. The result confirmed that crucial situations for chondrogenic deferential potential are 3-dimensional pelts, increase factor, and high-density cell tradition. The study showed that using the TGF-β family promoted chondrogenic deferential potential (17). In the contemporary, have a look at that applied TGF-β1 brought about chondrogenic deferential in 3 weeks, in addition to our results showed that pellets handled with TGF-β1 were larger whilst in comparison to pellets cultured without TGF-β1, we have got now not located any variations inside the histology of the pellets with and without TGF-β1 remedy. In taking a look at with the aid of locating whilst isolation and culturing human adipose tissue and induction osteogenic, adipogenic deferential by applied increase issue TGF-β3 length for 3 weeks (18). In addition, histological evaluation validated the presence of extracellular matrix (ECM) inside the hyaline cartilage; this staining indicated a deposit of "acid mucopolysaccharides" inside the proximity of the cells.

Additionally, most cells constructed from rounded cells stained high quality for the presence of the cells encompassed by extracellular matrix (ECM), which had been wanting to chondrocytes as visible from the magnified view, lightly crimson-stained nuclei and nuclear fast red stain (19, 20). Several specific collagen kinds are located in articular cartilage; however, ninety–95% of the collagen present is inside the shape of collagen type II fibrils (21); later, in pellet cultured in media 2 (TGF- β1) by myself, the chondrocytes expressed collagen1, a marker for hypertrophic chondrocytes, Experimental research accomplished *in vitro* confirmed that TGF-β1 superior proliferation and chondrogenic differentiation of MSCs. Many authors stress the significance of TGF-β1 on cartilage maturation (22, 23). In addition, in cartilage rehabilitation, cells will always be differentiated *in vitro*, decreasing the occurrence of mobile fusion after *in vivo* transplantation (24).

Rat adipose- tissue carries a population of especially the density of stem cells that may differentiate into chondrogenic cells. However, their differentiation in biomaterials, or inside the presence of boom elements aside from TGF-β1 or their aggregate, desires to be planned. Therefore AD-MSCs might also play a vital position in cartilage tissue engineering.

Authors' Contribution

Study concept and design: S. H. A. A. and Z. K. Z.
Acquisition of data: Z. K. Z.
Analysis and interpretation of data: S. H. A. A.
Drafting of the manuscript: Z. K. Z.
Critical revision of the manuscript for important intellectual content: S. H. A. A.
Statistical analysis: S. H. A. A.
Administrative, technical, and material support: S. H. A. A. and Z. K. Z.

Ethics

The Tissue Culture unit, National Center of Hematology, College of Medicine, Mustansiriyah University, Iraq, approves all the procedures.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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