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Isolation and identification of adipose tissue derived-derived mesenchymal stem cells from cats

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Abstract Mesenchymal stem cells derived from adipose tissue (AD-MSCs) have shown promise in the field of regenerative medicine. This work aimed to evaluate the discovery of pre-committed mesenchymal lineages in cat adipose-derived stem cells that create multipotent stromal cells in response to inductive extracellular signals. A cat's inguinal area yielded three grams of fat subcutaneous tissue, which was carefully extracted to produce mesenchymal stem cells (MSCs) that would be enlarged by a fourth passage. Active mesenchymal stem cell proliferation was seen in the fourth passage. Additionally, the study showed that cat MSCs had the innate capacity to stimulate AD-MSC identification using immunocytochemistry examination. Cats' AD-MSCs were consistently positive for CD90 and CD44 at passage three, according to immunophenotypic analysis. Nevertheless, it was consistently discovered that MSCs lacked the hematopoietic specific markers CD34 and CD45.In conclusion, mesenchymal stem cells may be extracted from cat adipose tissue using the direct plating technique, which works well. These cells are an important source of stem cells that might be used for the replacement of organs.

Keywords: Adipose tissue, cat, immunocytochemistry, stem cells

Introduction Adipose-derived stem cells (ADSCs), which have distinct and variable lineage potential, are generated from adipose tissue. Adipose tissue has several applications and is simple to procure. It may be encouraged to differentiate into several types of MSCs and markedly expanded in vitro (1,19). Adipose tissue (AT) contains a significantly higher number of mesenchymal stem cells (MSCs) than bone marrow MSCs (1% vs. 0.01%), compared to other tissues including the placenta, dental pulp, umbilical cord, and dermis (2,20). MSCs may be identified by looking at their morphology and phenotypic traits. Cellular morphology was a key factor in the in vitro identification of MSCs (3,17). MSCs exhibit a variety of phenotypic markers, none of which are regrettably exclusive to MSCs. the common name for molecules found on the surface of cells (4,16,22). Previous studies have shown that AD-MSCs lack the hematological markers CD45 (pan-leukocyte marker), CD34 (hematopoietic progenitor and endothelial cell marker), CD14, and CD11 (monocyte and macrophage marker). They also lack the adhesion molecules CD56 (neuronal cell adhesion molecule 1), CD18 (leukocyte function-associated antigen-1), and CD31 (platelet/endothelial cell adhesion molecule), in addition to the stimulatory molecules CD80, CD86, or CD40. In addition, they have the ability to express adhesion molecules such as CD29, CD166 active leukocyte cell adhesion molecule, CD106 (vascular cell adhesion molecule), lineage differentiation regulator, CD90, CD71, and Stro-1 (5,18). In this study, MSCs were identified using primary antibodies, also known as CD markers, which are generated from adipose tissue. The purpose of this work was to demonstrate the in vitro differentiation of adipose-derived stem cells into distinct mesenchymal lineages.

Material and Methods

Ethical Approval

The University of Baghdad's College of Veterinary Medicine's Research Ethics Committee examined and approved the experimental design and techniques used in this work in compliance with ethical standards for animal welfare (ethics number 603 P.G .dated on March 18, 2024).

Experimental animals

In the current study, one healthy local male cat aged 18 ± 6 months and weighing 1.8 ± 0.2 kg was enrolled. The animal was provided commercial food and water, and it was housed in individual cages. The animal was allowed to settle into its assigned cage for fifteen days prior to the surgical surgery. For five days, intramuscular (IM) injections of the broad-spectrum antibiotic ceftriaxone (22 mg/kg) were administered twice a day. An antihelmintic injection of 0.2 mg/kg Ivermectin (Ivomec, Holland) 0.4 ml/kg SC was given on the first day and day 14 of acclimatization.

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Isolation and cultivation of AD-MSCs

Xylazine (2%) 1 mg/kg b.w. (Aleppo-Syria) and ketamine (10%) 10 mg/kg b.w. (Alasan TM, Holland) were given to the cat as part of general anesthesia. via intramuscular injection (6). The inguinal region's skin underwent aseptic surgical preparation, which was followed by a clean skin incision. Three grams of fat subcutaneous tissue were taken out. The removed tissue was placed in sterile 50 mL conical tubes and immediately filled with phosphate buffered saline (PBS) pH 7.2. The incision made during surgery was closed as per standard procedure, and the animals received injectable ceftriaxone (LDP, Spain) at a dose of 20 mg/kg for five days as a systemic antibiotic.

The University of Baghdad's College of Veterinary Medicine's stem cell facility was used for all processing steps involving (AD-MSCs) in order to eliminate any leftover blood. The fat sample was then cleaned using Dulbecco Phosphate Buffer Saline (DPBS) (Gibco TM, USA) after being moved to a biosafety cabinet II. Next, the AT was very finely chopped using a sterile knife. a fifty-milliliter tube was filled with the chopped tissue. Next, 10 mL of collagenase I (2 mg/mL) (Gibco TM, USA) was added, and the mixture was incubated at 37 °C with 10 mL per 3 grams of tissue. Following digestion, this material was diluted 1:1 with 10% fetal bovine serum (Gibco TM, USA) and Dulbecco's Modified Eagle Medium (DMEM) (Gibco TM, USA). Subsequently, the cell solution was filtered through 40 µm cell strainers in 50 mL conical tubes to remove any remaining undigested fragment. After that, the filtrate material was centrifuged for seven minutes at 700 g (g-force). To resuspend the pellet, remove and discard the lipid layer that develops on top. Then, add one milliliter of basal medium and three milliliters of erythrocyte lysis solution (155 mMNH 4 Cl, 10 mM KHCO3, 0.1 mM EDTA) to the pellet. Give it five minutes to get to room temperature (RT). After adding 10 mL of DPBS, the mixture was centrifuged at 700 g for seven minutes. Re-suspend the pellet in 10 mL of media after extracting the supernatant, then seed it into a tissue culture T-75 Flask (Thermo Scientific TM). Then, for three or four days, the cell culture flasks were kept at 37 °C in a humidified environment with 90% and 5% CO2 (Sanyo, Japan). Non-adhering cells were eliminated once 80% of the cells were confluent, with the media being changed every three days for a total of twelve days. The culture was declared passage zero (P0) after 12 days. In order to get homogenous stromal stem cells, the monolayer cell cultures were subcultured at this point. After discarding the preceding medium and rinsing the monolayer two or three times with PBS pH 7.2, the cell was harvested as the subsequent. After incubating for two to three minutes, the cell culture was separated by adding three milliliters of trypsin with EDTA (0.25%) and phenol red (Gibco TM, USA) solution. To speed up the dissociation of the cells, the flask was gently shaken. Then, 5-8 mL of DMEM (Gibco TM, USA) containing 10% fetal bovine serum, an antibiotic-antimycotic (100×) 1% (Gibco TM, USA), phenol red solution, and EDTA (0.25%) were added to halt the trypsin's activity. The cell solution was then cultivated in two T-75 (Thermo Scientific TM) culture flasks at 37 °C, 5% CO2, and 90% humidity.

Immunocytochemistry analysis of AD-MSCs

The following primary antibodies, or CD markers, were utilized in this investigation to identify MSCs: anti-cat CD90, anti-cat CD44, anti-cat CD34, and anti-cat CD45. Following the manufacturer's instructions, MSCs were immunocytochemically treated (Elabscience biotechnology Company). The fundamental workings of this diagnostic test rely on the particular (primary antibody) attaching itself to the appropriate antigen on the target cell's surface, followed by the secondary antibody attaching itself to the primary antibody. The biotin molecule's attachment to the secondary antibody was aided by the horseradish peroxidase enzyme present in the avidin-biotin combination. After MSCs were suspended in DMEM growth fluid and dispersed using trypsin-PBS, they were re-cultured in eight-well multi-well tissue culture slides with DMEM supplemented with 20% FBS. The cells were then allowed to establish an adherent monolayer on the plates by incubating them at 37˚C for one to three days. After the media was aspirated, the cells were fixed for ten minutes with 4% paraformaldehyde. The components were kept at room temperature (20–25 ºC) in a humid chamber for each process. The cells were exposed to 1% hydrogen peroxide for ten to fifteen minutes in order to inhibit the synthesis of endogenous peroxidase. This was followed by three five-minute PBS washes to reduce background staining the cell slice was covered with an aliquot of 1.5% blocking serum and allowed to rest for an hour. After that, the cell slice was given three five-minute PBS rinses. Cell sections were subjected to either an hour-long treatment at room temperature or an overnight

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treatment at 4ºC using 125 µl of diluted primary antibody at a ratio of 1:50. After that, they had three PBS changes and five cleaning minutes. After 1.2 milliliters of biotinylated secondary antibody were incubated for 30 minutes, cell slices were rinsed for five minutes with three different PBS solutions. Following the addition of 650 µl of AB enzyme reagent to the cell slice, PBS was washed twice, resulting in a two-minute rinse period. Add 1 drop (approximately 50 µL) of DAB Concentrate to every 1 mL of DAB Substrate to create the DAB Working Solution. Stir well. Create a fresh solution before utilizing and keep the previously created one hidden. The freshly created DAB Working Solution is valid for four hours. Any unused solution has to be discarded. Tan or brownish yellow is the affirmative signal during this time. To prevent overreaction, the material was seen using light microscopy at a 40x magnification.

Results

Adipose tissue located in the subcutaneous area of the cat's inguinal region was used to create adipose-derived stem cells. After being seeded and allowed to develop in a cell culture flask, they revealed a diverse cell population. The adhering cells showed a range of morphologies, including big, flattened forms and tiny, rounded, spindle-shaped ones. Most of the cells had a fibroblast-like appearance as they multiplied and got closer to the confluence. The tiny, spherical cells that were prominently adhered to the cell layers' surface eventually shrank as a result of multiple passages. Conversely, by passage 4, the fibroblast-like cells had increased in number and homogeneity of shape, purifying to the high level. (Figure 1).

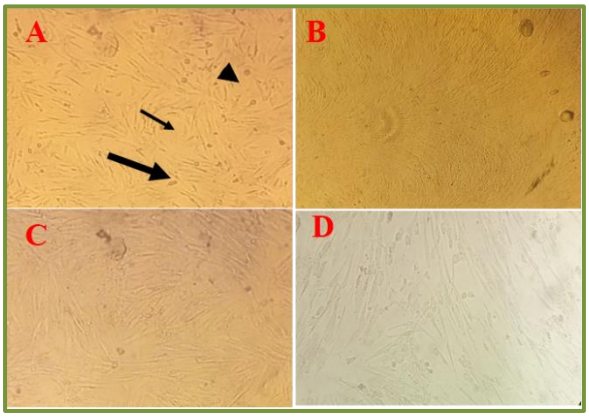

Figure 1: Morphological features of the isolated cells derived from cat adipose tissue (A) passage 0 shows spindle polymorphic cells (thin arrows), round (arrowhead) and polygonal (thick arrow) cells (B) after3 days of p 2, (C) after 5 days of p 3, (D) after 5 days of p 4, fibroblast-like cells (thin arrows) X100.

A mixed cell sample was subjected to a rapid separation of MSCs using an immunocytochemical staining method. According to this research, the majority of adhering AD-MSCs had intense brown staining and were positive for the CD90+ and CD44+ markers. On the other hand, these cells did not express CD34- or CD45+, which were brightly blue-stained using Harris Hematoxylin stain. (Figure 2).

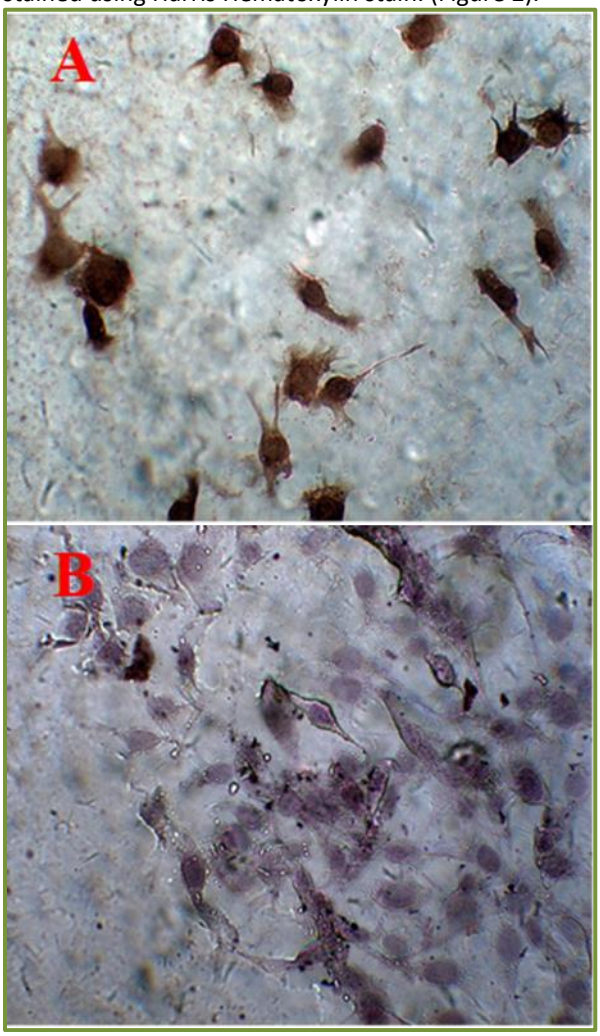

Figure 2: Immunophenotypic analysis of AD-MSCs of cats at the second passages reveal under light microscope 10×, (A) shows that the MSCs positive response for CD90 and CD44 marker and stain with brown color DAB stain. (B) shows that the MSCs negative response for CD34 and CD45 marker and stain with blue color using counter stain hematoxylin.

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Discussion

Within three to four days of incubation, AD-MSCs were attached to tissue culture plastic surfaces, according to this study. The cells were tiny, rounded, spindle-shaped, and massive, flattened, resembling fibroblasts. Fibroblast-like cells proliferated. Similar observations have been made before (7,14). AD-MSCs are a broad population of undifferentiated cells that may be created from a range of organs and can grow into many cell lineages when given the right culture conditions and stimulation (8,9,15). The MSCs were characterized by the surface antigens cluster differentiation. When using the immunocytochemistry staining approach to investigate phenotypic traits, the results for the CD34 and CD45 markers were negative. Conversely, adherent cells stained with brown-colored DAB stain were found to be positive for CD09 and CD44. These results in agreement with (10,21) who showed that MSCs had CD105 positivity. When the DAB reaction exhibited a brown granular stain in the cell cytoplasm, it was considered to be positive for the surface antigen CD105. Because MSCs were counter stained with blue hematoxylin (hematoxylin), they tested negative for CD-34. The expression profile of these markers indicates that MSC function as a single cell population since every marker under examination was consistently expressed in a way that was consistent with those of current MSC markers. demonstrating that the study's cell samples shared traits with MSCs (11). Several studies showed that these markers were expressed in different places on skin fibroblasts and stromal cells (12). Because fibroblasts deteriorate with age and eventually pass away, fibroblast contamination in MSC cells impairs the stem cell's capacity to differentiate. Furthermore, employing these MSC preparations for therapeutic reasons might be risky since some of the fibroblasts there have the capacity to proliferate and become malignancies (13). The MSC surface markers, CD44, CD90, and CD105, lacked specificity. These indicators were also seen in positive fibroblasts MSCs, and fibroblasts lacked the endothelial cell marker (CD31) as well as the hematopoietic cell markers (CD34, CD45). The findings of this study may aid in the development of sequential growth factor delivery devices by tissue engineering researchers.

Conflict of interest

There is no conflict of interest in this study as stated by the authors.

Acknowledgment

Not applicable.

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