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Mesenchymal stem cells derived from human adipose tissue exhibit significantly higher chondrogenic differentiation potential compared to those from rats

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Abstract.
BACKGROUND: Osteoarthritis is a prevalent joint disease affecting both humans and animals. It is characterized by articular cartilage degeneration and joint surface eburnation. Currently, no effective pharmacological treatment is available to restore the original function and structure of defective cartilage.
OBJECTIVE: This study explores the potential of stem cell-based therapy in treating joint diseases involving cartilage degeneration, offering a promising avenue for future research and treatment. The primary aim was to compare the characteristics and, more importantly, the chondrogenic differentiation potential of human and rat adipose-derived mesenchymal stem cells (AD-MSCs).
METHODS: Rat adipose tissue was collected from Sprague Dawley rats, while human adipose tissue was obtained in the form of lipoaspirate. The mesenchymal stem cells (MSCs) were then harvested using collagenase enzyme and subcultured. We meticulously evaluated and compared the cell morphology, percentage of cell viability, population doubling time, metabolic proliferation, and chondrogenic differentiation potential of MSCs harvested from both sources. Chondrogenic differentiation was induced at passage 3 using the 3D pellet culture method and assessed through histological and molecular analysis.

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RESULTS: The findings revealed that human and rat AD-MSCs were phenotypically identical, and an insignificant difference was found in cell morphology, percentage of cell viability, metabolic proliferation, and population doubling time. However, the chondrogenic differentiation potential of human AD-MSCs was evaluated as significantly higher than that of rat AD-MSCs.
CONCLUSION: The current study suggests that research regarding chondrogenic differentiation of rat AD-MSCs can be effectively translated to humans. This discovery is a significant contribution to the field of regenerative medicine and has the potential to advance our understanding of stem cell-based therapy for joint diseases.

Keywords: Adipose tissue, chondrogenic differentiation, mesenchymal stem cell, lipoaspirate, osteoarthritis

1. Introduction

Osteoarthritis is a common chronic arthritis disease affecting the knees, hips, and hands [1]. A survey reported that more than 242 million people globally are affected by osteoarthritis [2]. The etiological factors behind its prevalence are hereditary causes, aging, obesity, and injury [3,4]. In osteoarthritis, the articular cartilage in joints gradually degenerates [5]. Once cartilage is damaged, it has limited intrinsic self-healing potential due to the lack of lymphatic drainage, vascular supply, and innervation [6]. Consequently, individuals suffering from osteoarthritis experience joint pain, stiffness, and swelling, which can potentially lead to disability [7]. Currently, treatment options are limited; joint replacement surgery remains the primary intervention, but its accessibility is restricted to the elite population in developing countries [8].

Regarding the treatment of osteoarthritis, stem cell-based therapy emerges as a cost-effective strategy for cartilage damage [9]. Among various types of stem cells, mesenchymal stem cells (MSCs) stand out as an ideal source for tissue repair due to their capacity for easy expansion and differentiation in laboratory settings. Consequently, MSCs hold promise for repairing cartilage lesions and mitigating joint degeneration-related issues. [10]. MSCs are the most recognized multipotent stem cells obtained from various mesoderm-derived tissues. These sources include Wharton's jelly, bone marrow, umbilical cord blood, and adipose tissue [11,12]. Harvesting adipose tissue by liposuction surgery treatment makes it the best source for MSCs as compared to the more invasive method for bone marrow [13]. Previous studies indicate that AD-MSCs exhibit a more extended culture period and greater proliferation capacity compared to bone marrow-derived MSCs [14,15]. Consequently, adipose tissue emerges as a superior and alternative source of MSCs relative to bone marrow [16]. AD-MSCs possess specific properties that make them attractive for cell-based therapies. These include self-renewal capabilities, multi-lineage differentiation potential, robust in-vitro proliferation, and ease of accessibility [17].

Besides osteoarthritis, MSCs provide promising therapeutic strategies to treat multiple diseases that currently do not have effective pharmacological treatments. Due to their immunomodulatory, anti-inflammatory, anti-apoptotic, and immunosuppressive effects, MSCs are being explored to mitigate the impact of SARS-CoV-2 infection [18]. According to a recently published review, MSCs are being used in many clinical trials for the treatment of different diseases related to dermatological, musculoskeletal, neurological, cardiovascular, respiratory, renal, gastroenterological, and urological conditions [19]. Essentially, stem cells have the potential to repair or regenerate almost any tissue or organ. Previous studies confirm the safety and significant health benefits of stem cell therapy [20]. In summary, MSCs promise to advance precision medicine and address unmet medical needs.

Under specific physiological or experimental conditions, MSCs exhibit the ability to differentiate into specialized cells [11]. Consequently, MSCs hold promise for therapeutic applications in tissue repair [21]. By leveraging their ability to differentiate into specialized cells, this study explored and compared the

characteristics and chondrogenic differentiation potential of MSCs derived from human adipose tissue with those from rat adipose tissue.

2. Materials and methods

2.1. Sample collection

Rat adipose tissue was collected from the lower abdomen of adult male Sprague Dawley rats ($n = 5$), obtained from the animal house of the local institute. All rats were treated according to guidelines approved by the local ethical committee for animal care. The human adipose tissue was collected in the form of lipoaspirate, which was donated by a patient at a cosmetic surgery center with written informed consent. Lipoaspirate is the medical waste product of cosmetic liposuction treatment that is routinely discarded. The lipoaspirate was harvested by experienced clinical staff in a sterile tube containing 1X PBS and antibiotics (penicillin and streptomycin), then transported to the laboratory within one hour and processed immediately.

2.2. Stem cell isolation

MSCs were isolated and subcultured following a modified version of the previously reported protocol [22]. Briefly, adipose tissues were thoroughly washed four to five times with 1X PBS until all the blood containments were washed off. The washed adipose tissues were finely minced using sterile scissors. The minced tissues were then digested with a collagenase type 1A solution prepared in 1X PBS and incubated in a water bath shaker for one hour to ensure proper digestion. The liquid fat layer and floating adipose tissues were discarded after centrifugation. The resulting pellets were washed by suspending them in culture media to inhibit the enzymatic activity of collagenase type 1A. The pellets were then resuspended and incubated in a lysis buffer for 10 minutes to lyse red blood cells, followed by centrifugation, after which the supernatant was discarded. The pellets were washed twice with 1X PBS. Cells were counted using a hemocytometer and cultured in flasks containing DMEM base media supplemented with 10% fetal bovine serum and antibiotics. The culture flasks were incubated undisturbed at 37 °C with 5% CO_2 for three days. The exhausted media was replaced with fresh media every three days until the cells reached approximately 80% confluence, which took about two weeks. These cultures were referred to as primary cultures.

2.3. Subculturing

Every time the cultures reached 80% confluence, they were subcultured. The exhausted media was aspirated, and the attached expanded cells were gently washed twice with filtered 1X PBS to remove unattached or dead cells. 1X trypsin EDTA (ethylene diamine tetra acetic acid) was added, and the flasks were gently tapped. This was followed by incubation for 4 minutes to completely detach the cells from the flask surface. An equal volume of culture media was added to neutralize the trypsin EDTA, and the mixture was centrifuged. The resulting pellets were resuspended in a fresh culture media, and the cells were counted using a hemocytometer. The cell suspensions were cultured in flasks and incubated at 37 °C with 5% CO_2 for three days to allow the cells to adhere to the bottom surface. The exhausted media was replaced with fresh media every three days until the cells reached approximately 80% confluence, which took about two weeks. These cultures were referred to as the first passage (P1).

2.4. Morphological analysis

Crystal violet staining was conducted to visualize the morphology of MSCs according to a previously reported procedure [23] with some modifications. Briefly, equal aliquots of cell suspension from both samples were taken and washed three times with 1X PBS. The cells were then fixed with 4% paraformaldehyde and stained with 0.5% crystal violet solution, followed by 20 minutes incubation. The excess stain was removed by washing the cells three times with 1X PBS. Finally, the cells were observed under a light microscope.

2.5. Percentage viability assessment

The Trypan blue exclusion assay was performed every time before subculturing up to 7 passages. The test followed a previously employed method [24] to evaluate cell viability based on membrane integrity. Briefly, equal aliquots of cell suspension from both samples were mixed with an equal volume of 0.4% trypan blue dye and incubated for 4 minutes. After incubation, the dyed cell suspensions were loaded into a hemocytometer, and a total cell count was performed under a microscope. The percentage of viable cells relative to the total cell yield was calculated using the following formula.

$$\text{Percentage of living cells} = \left(\frac{\text{Number of dead cells}}{\text{Total number of cells}} \times 100\right) - 100$$

2.6. Population doubling time

The cells were counted every time before subculturing, and the time required to reach 80% confluence was recorded for each passage. The population doubling rate was calculated by inputting the data into an online population-doubling software (http://www.doubling-time.com/compute.php).

2.7. Metabolic proliferation assessment

To assess metabolic proliferation based on mitochondrial metabolic activity, the Thiazolyl Blue Tetrazolium Bromide (MTT) assay was employed from passages 1 to 7, following the most cited protocol [25] with minor modifications. Briefly, equal aliquots of cell suspension from both samples were cultured in 96-well plates, leaving the last six wells as a negative control containing only culture media. The plates were incubated at 37 °C with 5% CO_2 for 24 hours. After incubation, MTT solution (5 mg/ml in 1X PBS) was added to each well, including controls. The plates were wrapped in aluminum foil and incubated for three hours until a purple precipitate appeared. Dimethylsulfoxide (DMSO) was added to each well, including controls, to solubilize the formazan crystals. The plates were wrapped in aluminum foil and incubated overnight at room temperature. Absorbance was measured for each well, including controls, at a 570 nm wavelength in a microtiter plate reader.

2.8. In-vitro chondrogenic differentiation

Chondrogenic differentiation was induced according to the protocol previously used by various research groups [26,27] with some modifications. In the present study, chondrogenic differentiation was carried out using a 3D pellet culture method, considered the most efficient for inducing chondrogenic differentiation. Briefly, equal aliquots of cell suspensions were taken at passage three and transferred to a polypropylene

conical tube. The cell suspensions were centrifuged, and the supernatant was aspirated. The pellets were then resuspended in a defined chondrogenic differentiation media and centrifuged for 15 minutes. The tubes were placed immediately in an incubator at 37 °C with 5% CO_2 in a standing position for 28 days, with loosened caps to permit gas exchange. The exhausted media was replaced every three days with fresh chondrogenic differentiation media.

2.9. Histological analysis

Tissue preparation and staining for histological analysis were conducted as elaborated in previous studies by two different groups of researchers [28,29] with some modifications according to the specific circumstances.

2.9.1. Sample preparation

After terminating the chondrogenic culture, the media was aspirated, and the chondrogenic constructs were washed twice with ice-cold PBS. The constructs were then fixed by incubating in PBS containing 4% paraformaldehyde solution. The solution was aspirated after fixation, and the constructs were rinsed with PBS. The constructs were treated with ascending grades of ethanol and clarified by incubating them twice in 100% xylene. Finally, the constructs were placed into a mold filled with melted paraffin and allowed to cool at room temperature until the paraffin solidified. Using a rotary manual microtome, 5 µm thick sections were precisely cut from a solid block of the paraffin-embedded construct. The middle section of the constructs was selected, mounted on a silane-coated slide for staining, and dried at 60 °C in an incubator. The sections were deparaffinized by incubating them three times in 100% xylene and rehydrated by incubating them in descending grades of ethanol. Finally, the sections were rinsed with deionized water.

2.9.2. Staining and visualization

Toluidine blue solution was employed according to a standardized protocol for histological analysis. This basic thiazine metachromatic dye has a high affinity for acidic tissue components. The middle section of the constructs was stained by incubating them in a 0.1% toluidine blue solution at pH 4.0 for 5 minutes, then rinsed with distilled water to remove excess stain. The sections were then dehydrated using an ascending ethanol series and clarified in three consecutive steps with 100% xylene. A small drop of permount mounting medium was applied to the stained sections, which were then covered with a coverslip and visualized under the microscope.

2.10. Gene expression analysis

The chondrogenic constructs were minced and digested into chondrocytes by incubation with the collagenase II enzyme. Gene expression analysis was performed on chondrocytes as an experimental group and MSCs as a negative control group. Total RNA was isolated from four types of samples (rat AD-MSCs and chondrocytes, human AD-MSCs and chondrocytes) using a TRIzol kit according to the manufacturer's protocol. RNA quantification was then performed using a nanodrop spectrophotometer.

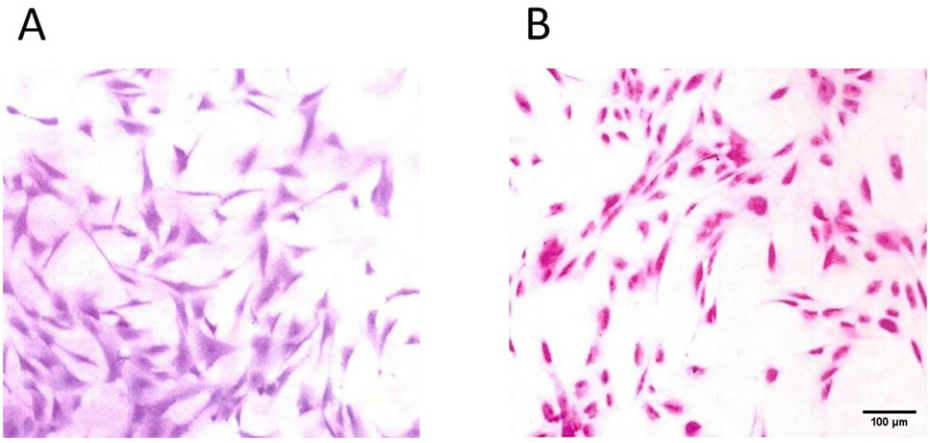

Fig. 1. Crystal violet staining. (A) human and (B) rat AD-MSCs showed fibroblast-like morphology.

2.10.1. Primer designing

Primers were designed from mRNA sequences of two cartilage-specific genes (ACAN, COL2A1) and one housekeeping gene (GAPDH) as a positive control. The primers were designed to amplify two consecutive exon regions flanked by an intron.

2.10.2. Reverse transcriptase PCR

Reverse transcriptase PCR was performed using a superscript™ IV one-step RT-PCR kit according to the manufacturer's instructions. Three sets of previously designed primers were individually tested with all RNA samples. Both cDNA synthesis and PCR amplification were carried out in a single reaction tube using gene-specific primers. Reverse Transcriptase PCR products were analyzed using a 1.5% agarose gel electrophoresis and visualized under a gel documentation system.

2.11. Statistical analysis

The data were expressed as mean ± standard deviation (SD). We conducted two-tailed Student's *t*-tests using the SPSS program, with statistical significance set at ($P < 0.05$).

3. Results

3.1. Morphology analysis

A total of 45 ml of adipose tissue was collected from five Sprague Dawley rats (8–10 ml per rat). For the human sample, experienced clinical staff at the cosmetic surgery center provided more than 45 ml of adipose tissue in the form of lipoaspirates exclusively for research purposes, out of which 45 ml was proceeded. Based on the crystal violet staining experiment results, human and rat AD-MSCs exhibited a fibroblast-like shape and were phenotypically identical (see Fig. 1).

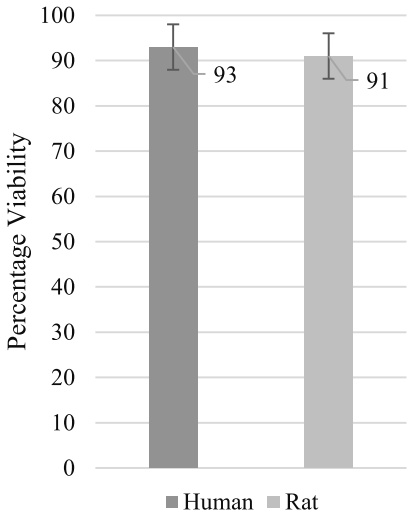

Fig. 2. The percentage viability difference between human and rat AD-MSCs was insignificant.

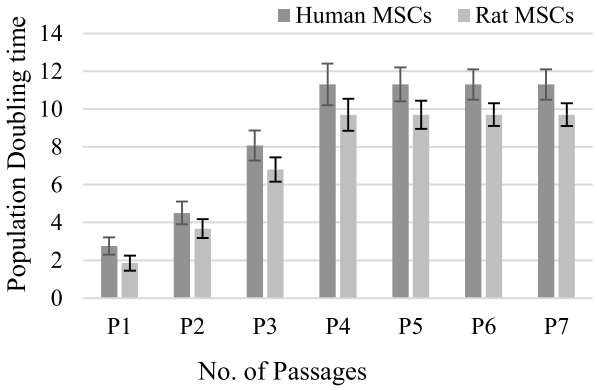

Fig. 3. Population doubling time (y-axis) constantly increased up to passage 4 (x-axis) and became constant for both types of AD-MSCs. The difference was not significant at $p < 0.05$.

3.2. Percentage viability

Non-viable cells were identified by their staining with trypan blue dye, which resulted in a blue cytoplasm appearance. In contrast, viable cells retained no dye and exhibited transparent cytoplasm. The trypan blue exclusion assay results from passages 1 to 7 demonstrated that human AD-MSCs were 93% viable, while rat AD-MSCs were 91% viable (see Fig. 2).

3.3. Population doubling time

The online population doubling software results were plotted as a graph (see Fig. 3). It was observed that the population doubling time consistently increased until passage four and remained constant until passage seven for both types of MSCs. According to statistical evaluation, the population doubling time for human AD-MSCs was insignificantly higher than for rat AD-MSCs.

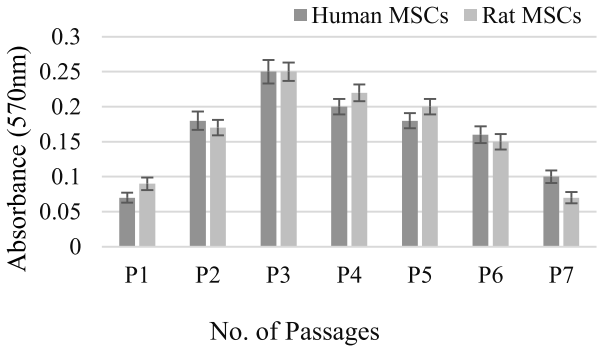

Fig. 4. Mitochondrial activity (y-axis) was constantly increased till passage 3 (x-axis), then started continually decreasing till passage 7 with the same rate in both types of MSCs. The difference was not significant at $p < 0.05$.

3.4. Metabolic proliferation

In-vitro cell viability was assessed using an MTT assay, which measures total mitochondrial activity directly associated with the number of living cells. MTT entered the cells and passed into the mitochondria, where 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (yellow) was reduced by succinate dehydrogenase into an insoluble dark purple formazan product. The formazan crystals were then solubilized with an organic solvent DMSO and measured at 570 nm using a microtiter plate reader. Graphical representation of the results in Fig. 4 showed that both types of MSCs exhibited increased MTT values up to passage three, indicating more formazan formation due to tetrazolium salt breakdown. This correlated with increased mitochondrial dehydrogenase activity and enhanced cell viability. However, in later passages, the MTT value consistently decreased, mirroring the trend observed in cell viability.

3.5. Chondrogenic differentiation

MSCs were induced for chondrogenic differentiation using a defined chondrogenic media using a 3D pellet culture method. Under high centrifugal force, MSCs were settled down and formed a compact spherical pellet. After 28 days of incubation, pellets were transformed into the solid rigid chondrogenic construct, as depicted in Fig. 5. Surprisingly, the human chondrogenic construct was found to have significantly larger dimensions than the rat's, indicating their differentiation potential.

3.6. Histological analysis

Toluidine blue specifically binds to the sulfate groups of proteoglycans and glycosaminoglycans within the cartilage matrix. This interaction induced a metachromatic shift, changing the dye's color from blue to a distinct purple. The results obtained from toluidine blue staining revealed the presence of purple-stained cells in both tissue sections, as illustrated in Fig. 6, confirming successful chondrogenic differentiation.

3.7. Gene expression analysis

Reverse transcriptase PCR analysis through gel electrophoresis revealed the expression of the housekeeping gene GAPDH in both differentiated and non-differentiated cells. In contrast, the cartilage-specific genes COL2A1 and ACAN showed expression exclusively in chondrogenic differentiated cells and were

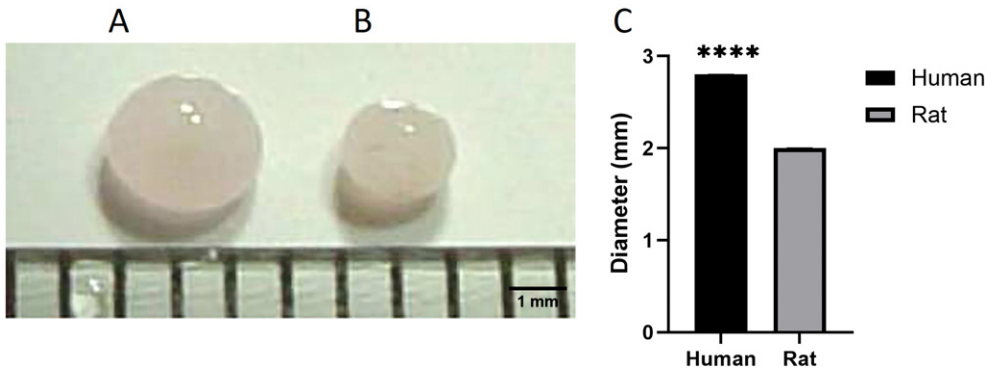

Fig. 5. Chondrogenic differentiation potential. (A) The human chondrogenic construct was significantly larger than (B) the rat construct. (C) The graph showed a statistical difference between the size of both constructs, with a p-value of < 0.0001.

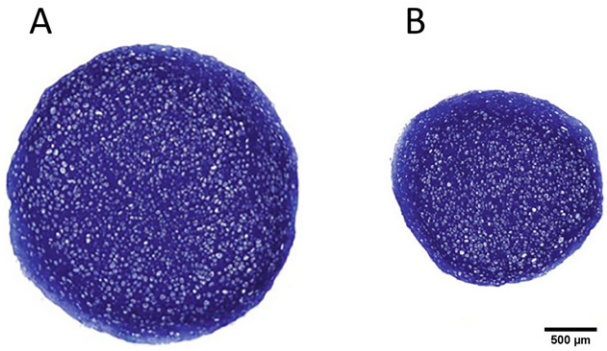

Fig. 6. Histological analysis: Toluidine blue staining confirmed the chondrogenic differentiation in both (A) human and (B) rat-derived constructs.

absent in non-differentiated MSCs (as depicted in Fig. 7). Consequently, the gene expression analysis confirmed chondrogenic differentiation in both chondrocytes.

4. Discussion

AD-MSCs have garnered significant attention as a promising source of stem cells for various regenerative therapeutic applications. Harvesting adipose tissue and isolating MSCs from it is relatively straightforward and more practical than harvesting from other sources. The multipotency and self-renewal capability of these cells make them ideal candidates for cell-based tissue engineering. Consequently, researchers are keenly interested in understanding the mechanisms of differentiation, proliferation, and heterogeneity of these cells [30].

We used collagenase enzyme to digest adipose tissue, releasing MSCs that began attaching to the plastic surface of the culture flask within three days. These cells can be cultured under standard conditions and easily expanded in-vitro without additional requirements, consistent with previously reported studies [31]. Cell culture passages involve repeatedly subculturing stem cells to sustain their growth. However, this process can introduce mutations due to several factors. Each time a cell divides, there is a chance for

Fig. 7. The expression of cartilage-specific genes COL2A1 and ACAN in chondrocytes double-confirmed the chondrogenic differentiation in both constructs.

errors in DNA replication, which can accumulate over multiple passages, ultimately leading to mutations. Additionally, cells in culture are exposed to various environmental factors, such as ultraviolet light and chemicals, which can induce mutations. Furthermore, cells produce reactive oxygen species during cell growth as a by-product of metabolism, potentially damaging DNA and causing mutagenesis. We extended the cell culture to passage 7. Throughout our experiment, we implemented several strategies to minimize the risk of mutagenesis. Firstly, we maintained consistent culture conditions, including temperature, CO_2 levels, and humidity. Secondly, we conducted the experiment in a highly sterile environment, adhering to stringent protocols to prevent contamination. Lastly, we ensured the cell culture was not exposed to ultraviolet light.

The morphological observation of cells at different passages showed that they maintained their homogeneous fibroblast-like morphology throughout the studied period. Previous studies have confirmed the suitability of these cells for in-vitro cultivation [32,33]. The viability and population doubling time of MSCs were monitored until passage 7. Although these cells continued to proliferate until late passage, the population doubling time also increased.

There are three different systems to differentiate stem cells into chondrocytes [34]. The oldest method used to conduct in the culture flask, where MSCs differentiate into chondrocytes without forming any visible structure. The second method, the micromass system, is performed on culture plates containing small wells, where MSCs are differentiated into spherical chondrogenic constructs. The third method, the 3D culture system, is conducted in a 15 ml conical centrifuge tube to differentiate MSCs into small, visible, spherical solid chondrogenic constructs. In the current study, chondrogenic differentiation was accomplished using the 3D culture system, as it is the most efficient method for inducing chondrogenic differentiation [35].

Histological staining and the expression of marker genes are crucial parameters used to confirm chondrogenic differentiation in previous related studies [36,37]. Two stains are commonly used for histological analysis: safranin-O and toluidine staining. For gene expression analysis, there are specific genes related to cartilage, including COL2A1, COL10A1, COL1A1, SOX9, and ACAN, that only express in chondrocytes [38]. Collagen and aggrecan are significant components of the cartilage extracellular matrix [39]. Type II collagen, also known as cartilage collagen, is the primary collagen synthesized by chondrocytes [40]. In the current study, we used toluidine staining for histological analysis. For gene expression analysis, we investigated the cartilage-specific genes COL2A1 and ACAN, which are

responsible for synthesizing Type II collagen. After histological and molecular analysis, we found that the chondrogenic differentiation potential of human AD-MSCs was significantly higher than that of rat AD-MSCs.

5. Conclusion

We used the same differentiation protocol for human and rat adipose-derived mesenchymal stem cells and successfully achieved chondrogenic differentiation. Based on these findings, the current study suggests that research regarding chondrogenic differentiation of rat AD-MSCs can be effectively translated to humans. This discovery is a significant contribution to the field of regenerative medicine and has the potential to advance our understanding of stem cell-based therapy for joint diseases.

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Ethics approval

The study was approved by the University of Veterinary & Animal Science, Lahore, Pakistan (No. DAS/2049).

Conflict of interest

None of the authors have any conflicts of interest to declare.

Author contributions

F.Y. designed the study, conducted the experiments, and wrote the manuscript. M.K.H. and M.S.C. assisted in the pathology part of the project. S.K. assisted in sample collection and proofread the manuscript. M.B.B. assisted in statistical analysis.

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