Differentiation of mesenchymal stem cells into chondrocytes as a future therapy for skeletal diseases

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Objective
The aim of this study was to assess the ability of mesenchymal stem cells (MSCs) to differentiate into chondrocytes *in vitro*.

Background
MSCs with their ability of self-renewal and multilineage differentiation can differentiate into several types of cells including chondrocytes. It is reported that adult cartilage lacks the ability to repair itself, making articular cartilage a very attractive target for regenerative medicine therapy.

Materials and methods
This study was conducted at Clinical Pathology, Gynecology and Obstetrics Departments, Menoufia University Hospital, from December 2016 to May 2018, and included a collection of 30 umbilical cord samples under complete aseptic conditions, after obtaining Menoufia ethical committee approval. Samples were processed with the explant method. MSCs were separated using plastic adherence. MSCs were subcultured in a petri dish 35 mm^2 then treated with chondrocytes' differentiation media.

Results
Identification of isolated MSCs was carried out by morphology and flow cytometry. MSCs were positive to flow cytometric markers CD44 and CD73 and negative for CD34 with a highly statistically significant difference *P* value of less than 0.001 in comparison with differentiated chondrogenic cells. MSCs were able to differentiate into chondrocytes after adding of chondrogenic differentiation media. Chondrocytes could be identified by morphology and immunocytochemistry method. Chondrogenic cells showed negative flow cytometric markers CD44 and CD73.

Conclusion
This study showed the ability of MSCs to differentiate into chondrocytes after the adding of chondrogenic differentiation media *in vitro* as a future therapy for skeletal diseases.

Keywords:
chondrocytes, immunocytochemistry, mesenchymal stem cells, umbilical cord

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Introduction

Stem cells are biological cells present in almost all multicellular organisms with the great ability of self- renewal and multipotency [1]. Adult mesenchymal stem cells (MSCs) can be isolated from several sources including the bone marrow, umbilical cord, adipose tissue, placenta, amniotic fluid, dental pulp, breast milk, and synovium [2].

Adult MSCs have the ability to differentiate into cells of mesodermal (osteocytes, chondrocytes, and adipocytes), ectodermal (neurons) and endodermal origins [2].

The chondrocyte is the main constituent cell of the cartilage with two major functions with respect to the cell's location. The chondrocytes that occupy supporting structures such as the articular cartilage have the function of synthesis and maintenance of the extracellular matrix that is able to withstand physical deformation and facilitate cartilage function. The other function of chondrocytes is the growth of epiphyseal plates [3].

Joint diseases are very difficult to be treated with traditional therapy lines. This is due to the fact that joints are avascular tissues and their chondrocytes are cytoplasmically isolated from their neighboring cells with no ready access to the vascular system, and the tissue is not innervated. Hence, the importance of developing new therapeutic methods such as stem cell therapy [4].

The aim of this study was to assess the ability of stem cells to differentiate into chondrocytes *in vitro*.

Materials and methods

This study was conducted at Clinical Pathology, Gynecology and Obstetrics Departments, Menoufia University Hospital, from December 2016 to May 2018, and involved the collection of 30 umbilical cord samples obtained from healthy full-term pregnant women during their cesarean section delivery under complete aseptic conditions after obtaining Menoufia ethical committee approval. The incusion criteria of the chosen pregnant women were as follows: age ranging from 18 to 40 years; no history of hepatitis, infectious diseases, DM and severe hypertension; and no obstetric problems such as preeclampsia and abruptio placenta. Cords were collected in sterile 0.9% NaCl solution and were transported immediately to the Clinical Pathology Laboratory and kept at room temperature. Cord blood samples used for serum preparation was collected before the expulsion of the placenta and were left at room temperature for clotting; thereafter, they were centrifuged for 15 min at a speed of 1492*g*; the sera were then incubated at 56°C for 20 min for heat inactivation then aliquoted and frozen for future use. Under complete aseptic conditions, the umbilical cord was disinfected by 75% ethanol for 30 s. The umbilical cord was divided into small segments (about 6 cm each) and each segment was opened longitudinally. The umbilical cord vessels were dissected and removed and then the cord tissues were washed with saline. Wharton's jelly was cut into small pieces of about 1.5–2.5 mm. Two tissue culture plastic flasks 25 cm^2 were used for culture. The media were prepared by adding 5 ml of the fresh complete nutrient medium, which was constituted of Dulbecco's Modified Eagle Medium (DMEM)+l-glutamine (50 μg)+cord blood serum (5 ml)+penicillin (500 μg)+Fungizone (250 μg). For proper adherence of the cells, the flasks were incubated in a horizontal position in an incubator with saturated humidity containing 5% CO_2 at 37°C [5]. At day 7, the tissue was removed by changing the medium. The adherent cells (MSCs) were kept in culture and were fed with fresh complete nutrient medium on the 11th day. These cells were kept until the outgrowth of fibroblast-like cells. The flasks were examined by the inverted microscope (100–200×) after removal of the tissue for assessment of the cell morphology (viable cells were round, bright and refractile). The flasks were examined also on the 14th day, to ensure 60–70% confluence, normal cell morphology, and absence of bacterial contamination. Thereafter, these cells were harvested by trypsinization [6].

Trypsinization

The medium was aseptically removed. A volume of 2–5 ml of trypsin EDTA was added. The cells were examined microscopically for detachment every 2–3 min. The time required for complete detachment may vary from 5 to 15 min [7].

Neutralization

After complete detachment, DMEM containing 1% serum was added. The cells were transferred to a centrifuge 15 ml falcon tube and centrifuged at 312*g* for 10 min. The supernatant was removed, and the cells were resuspended in complete media as a single cell suspension.

Cells were examined under the microscope, counted and the viability was assessed using the trypan blue dye. The cells were used for MSC identification by flow cytometry and used for chondrogenic differentiation.

Identification of mesenchymal stem cells by flow cytometry

At day 14, the harvested MSCs were identified by flow cytometric analysis of surface markers CD44, CD34, and CD73 using Becton Dickinson FACS Calibur (Biosciences, San Jose, California, USA).

Chondrogenic differentiation

Human Mesenchymal Stem Cell Functional Identification Kit was used for differentiation and identification of chondrocytes and was supplied by R&D Systems (Minneapolis, Minnesota, USA. A total of 2.5 × 10^5 cells were transferred into their existing culture media to a 15 ml conical tube. The cells were centrifuged at 200*g* for 5 min at room temperature. The media was removed, and the cells were resuspended with 1.0 ml of DMEM. The cells were centrifuged again at 200*g* for 5 min. Thereafter, the medium was aspirated and discarded. The cells were resuspended in 0.5 ml of chondrogenic differentiation medium consisting of dexamethasone, ascorbate-phosphate, proline, pyruvate, and recombinant transforming growth factor-β3 (TGF-β3) and were centrifuged at 200*g* for 5 min at room temperature. The medium was replaced with 0.5 ml of fresh chondrogenic differentiation medium every 2–3 days. After 14–21 days, the chondrocyte pellet could be fixed and prepared for frozen sectioning. The chondrogenic pellet can be harvested and cryosection of the chondrogenic pellet can be carried out [8].

Evaluation of chondrogenic differentiation

Postinduction morphological changes by microscopic examination.

Identification of chondrocytes by immunocytochemistry (IHC): the pellet was washed twice with 1 ml of PBS. The pellet was fixed with

0.3 ml of 4% paraformaldehyde in 1 ml PBS for 20 min at room temperature. The pellet was washed again twice with 1 ml of PBS for 5 min. It was then frozen and sectioned. The sections were cut at a nominal thickness of 5–10 μm. Thereafter, the pellet was permeabilized and blocked with 0.15 ml of 0.3% Triton X-100, 1% BSA in PBS at room temperature for 45 min. After blocking, the sections were incubated with the anti-Human Aggrecan antibody working solution overnight at 2–8°C. The sections were washed three times with PBS containing 1% BSA for 5 min The sections were incubated with the secondary antibody working solution in the dark for 60 min at room temperature. The sections were washed three times with PBS containing 1% BSA for 5 min. They were then washed once with distilled water. The cells were stained using the anti-Mouse IgG Secondary Antibodies (red) (R&D Systems), and the nuclei were counterstained with DAPI (blue) (R&D Systems). The sections were covered with a glass coverslip for microscopic observation [8].

Statistical analysis

The results were statistically analyzed by SPSS version 20 (SPSS Inc., Chicago, Illinois, USA). Data were expressed in two phases: descriptive statistics were used for qualitative data, mean, range (minimum and maximum), median and SD for quantitative data. The second was analytic statistics wherein paired samples *t*-test was used for comparison of two dependent quantitative variables normally distributed. *P* value less than or equal to 0.05 was considered a significant difference.

Results

A total of 30 umbilical cord samples were collected; five of them were excluded because of contamination, and the other 25 cases underwent chondrogenic differentiation. MSCs isolated from Wharton's jelly of the umbilical cord exhibit a characteristic spindle-like, fibroblastic morphology (Fig. 1), and, by flow cytometry, the cells show positive CD44 (r = 60.0–83.0%) with a mean value of 70.24 ± 5.85, and CD73 (r = 64.0–90.0%) with a mean value of 78.16 ± 6.28 and negative CD34 (r = 0.1–1.9%) with a mean value of 0.93 ± 0.50 (Table 1). By contrast, cells induced with the chondrogenic medium began to lose the typical morphology at day 3 and compacted to form a few monolayered aggregates and small multilayered aggregates at days 7 and 14, respectively. Notably, a number of monolayered aggregates and large multilayered aggregates were visible at days 7 and 14 (Fig. 2). After differentiation, chondrogenic cells showed negative CD44 and CD73, and, by comparison of the expression of CD44 and CD73 before and after differentiation, MSCs showed a highly significant difference (P < 0.001) (Table 2). By IHC, the chondrogenic cells were stained red and the nuclei were counterstained blue (Fig. 3).

Discussion

Articular cartilage is an avascular, aneural and alymphatic tissue with a very low regeneration potential because of its limited capacity for self-repair. MSCs are considered the preferred choice for cell-based therapies. MSCs are characterized by their enormous ability of self-renewal and their multipotency [9].

Table 1 Descriptive analysis of the studied cases according to mesenchymal stem cell markers (*n*=25)

Flow marker expression %	Minimum-maximum	Mean±SD	Median
CD73%	64.0-90.0	78.16±6.28	78.0
CD44%	60.0-83.0	70.24±5.85	71.0
CD34%	0.1-1.9	0.93±0.50	0.9

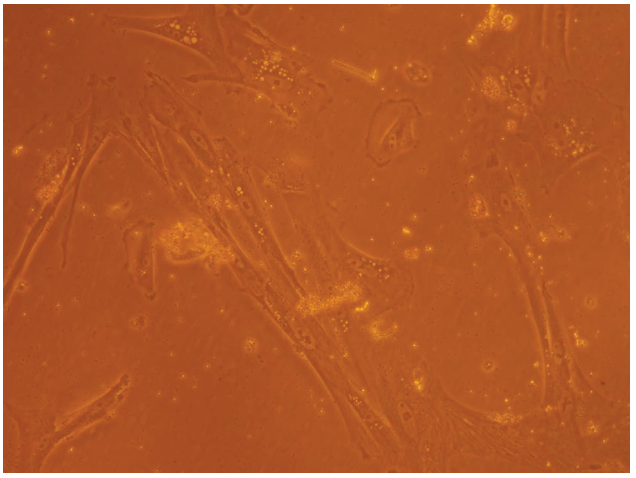
CD, cluster of differentiation.

Table 2 Comparison between CD73 expression and CD44 expression before and after differentiation into chondrocytes (*n*=25)

Variables	Before differentiation	After differentiation	*t*	*P*
CD73%				
Minimum-maximum	64.0-90.0	1.0-15.0		
Mean±SD	78.16±6.28	8.20±3.73	46.198	<0.001*
Median	78.0	8.0		
CD44%				
Minimum-maximum	60.0-83.0	1.0-10.0		
Mean±SD	70.24±5.85	4.65±2.29	50.436	<0.001*
Median	71.0	4.5		

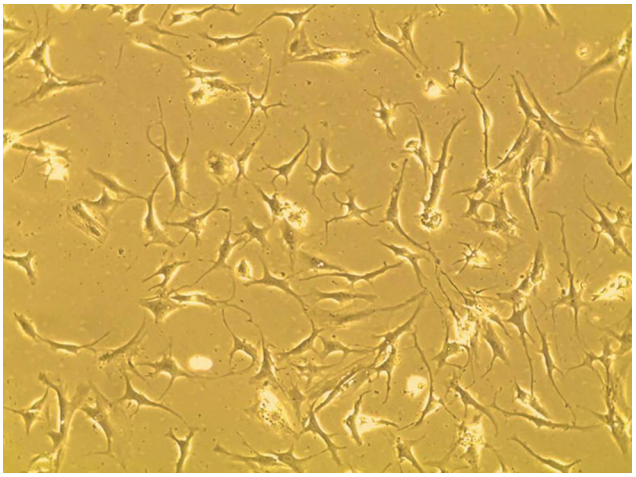
t and *P* values for paired samples *t*-test for comparing between before and after differentiation. *$P \leq 0.05$, statistically significant.

Figure 1



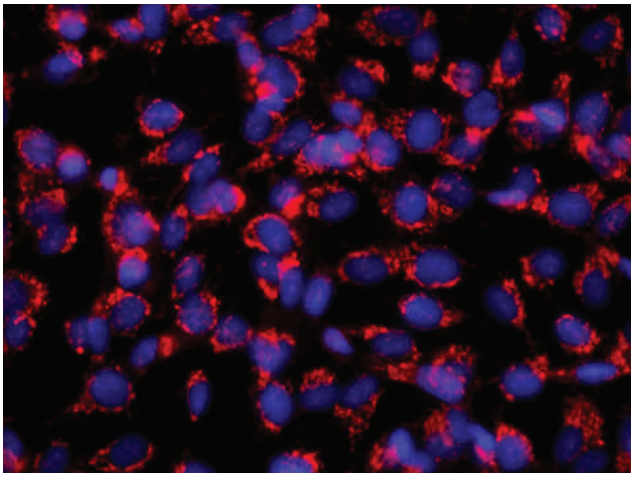
Inverted microscope image ×200 of Wharton's Jelly-derived mesenchymal stem cells.

Figure 2



Inverted microscope image ×200 of chondrogenic cells after adding of chondrogenic differentiation media with fibroblast-like morphology.

Figure 3



Inverted microscope image ×200 of immunocytochemistry detection of chondrogenic cells (the cells were stained red, and the nuclei were counterstained with DAPI; blue).

Human MSCs (hMSCs) are nonhematopoietic with the capacity to differentiate into mesodermal lineages such as adipocytes, osteocytes and chondrocytes, and also ectodermal (neurocytes) and endodermal lineages (hepatocytes) [10]. MSCs are primarily isolated from the bone marrow, and this was studied by many researchers for many years; however, this procedure is invasive and may cause excessive bleeding, infection, and pain. In this study, we chose the umbilical cord as a source for MSCs because of the easy noninvasive collection, no pain and no risk of infections with good sterilization precautions [11]. Moreover, in comparison with marrow-derived MSCs, umbilical-derived MSCs have better and faster proliferative capacity and stronger amplification potential [12]; chondrocytes play a significant role in the formation and maintenance of extracellular matrix with preservation of cartilage functions [13]. In our study, the isolated MSCs showed fibroblast-like morphology. This result agrees with that of Haasters *et al.* [14] who demonstrated an elongated and spindle-shaped morphology of MSCs. By flow cytometric identification, we found that the isolated MSCs showed positive CD44 and CD73 and negative CD34. This finding is in agreement with that of Ullah *et al.* [10] who illustrated that MSCs show cell surface markers like a cluster of differentiation CD29, CD44, CD73, CD90, and CD105 and lack the expression of CD14, CD34, CD45 and HLA (human leukocyte antigen)-DR. After we added the chondrogenic differentiation medium containing recombinant –TGF-β3, the isolated MSCs showed chondrogenic differentiation. TGF-β superfamily is considered as a key mediator of MSC chondrogenesis. Li *et al.* [15] found that TGF-β3 was an effective mediator for inducing chondrogenesis of MSCs and had a more potent chondrogenic potential of a more rapid differentiation than TGF-β1. The same results were approved by Morille *et al.* [16], Ravindran *et al.* [17], and Bian *et al.* [18]. Some authors such as Solchaga *et al.* [19] used TGF-β1 as a chondrogenic differentiation inducer. However, Legendre *et al.* [20], thought that there was no real difference between TGF-β1 and TGF-β3 as chondrogenic differentiation inducers. We used IHC method for identification. The IHC technique consists of two phases: (a) slide preparation (specimen fixation and tissue processing), and this step includes (in order) antigen retrieval, nonspecific site block, endogenous peroxidase block, primary antibody incubation, and the employment of systems of detection, revealing and counterstaining and also slide mounting and storage; (b) interpretation of the obtained expression [21].

The IHC has many advantages including the possiblity to use fresh or frozen tissue samples for IHC; IHC is well established and readily available, The cost of IHC is relatively low, It has a fast turn-around time, and there are no infectious agents involved in the study; the risk to human health is minimal [22].

However, according to Yaziji *et al.* [23] IHC also has the disadvantages of being not standardized worldwide; the equipment needed to perform IHC is costly. Quantifying results is difficult, and IHC is subject to human error. By IHC, the chondrogenic cells were stained red, and the nuclei were counterstained blue. This is the same result approved by Tanthaisong *et al.* [9] and De Matos *et al.* [24].

Conclusion

MSCs are able to differentiate into chondrocytes after adding of chondrogenic differentiation medium *in vitro* as a future therapy for skeletal diseases.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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