The regenerative effect of human umbilical cord blood mesenchymal stem cells in a rabbit model of osteoarthritis

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Introduction
Osteoarthritis (OA) is a degenerative process characterized by cartilage destruction and joint stiffness. OA associated with focal articular cartilage loss can be associated with subchondral and periarticular bone changes such as cyst, sclerosis, and osteophyte [1–3]. It comprises specialized cells such as chondrocytes embedded in highly hydrated and organized extracellular matrix consisting of collagen fibers and type I proteoglycans [2].

The current treatments of articular injury aim to decrease inflammation and pain, but fail to curb the progress of OA. The potential of cell-based strategies to provide a biological replacement for damaged cartilage is of great interest in younger OA patients [3].

Strategies using mesenchymal stem cells (MSCs) are emerging tools for cartilage repair; these cells can differentiate into different forms of connective tissues such as cartilage, bone, fat, tendon, ligament, and bone marrow [4]. In addition, MSCs secrete wide forms of bioactive small molecules that could inhibit scar formation, suppress apoptosis, and enhance the activity of resident progenitor cells. This remarkable ‘trophic activity’ can inhibit lymphocyte proliferation and modulate the function of major immune cells [5].

Umbilical cord (UC) blood cells are less immunogenic than other sources of stem cells, and there are no ethical concerns surrounding their use. These cells are isolated from young donors. Human umbilical cord blood mesenchymal stem cells (HUCB-MSCs) appear to be an ideal candidate for cartilage regeneration.

Background
Osteoarthritis (OA) is a degenerative disorder characterized by changes in subchondral and periarticular bone. The limited number of therapeutic choices for articular injury and disease has increased the need for stem cells as a biological replacement for damaged cartilage. Umbilical cord (UC) blood cells are easily available and less immunogenic than other sources of stem cells, and there are no ethical concerns surrounding their use. These cells are isolated from young donors. Human umbilical cord blood mesenchymal stem cells (HUCB-MSCs) appear to be an ideal candidate for cartilage regeneration.

Objective
The aim of the study was to investigate the regenerative effect of HUCB stem cells on degenerated articular cartilage in New Zealand white rabbits experimentally induced with OA.

Materials and methods
This study was performed on 42 New Zealand white rabbits. They were surgically induced with OA in the left knees by cutting the anterior cruciate ligament. After confirmation of the development of OA histopathologically, we delivered a single dose of HUCB-MSCs directly intra-articularly in the cell-treated groups. Controls were injected with only suspension media. Histopathological tests were performed 8 and 24 weeks after injection.

Results
Histopathologically, 8 weeks after the injection, cell-treated rabbits showed better cartilage quality and lower degree of degeneration, whereas 24 weeks after the injection all parameters in the cell-treated groups were significantly better.

Conclusion
HUCB-MSCs are a promising stem cell source for cartilage tissue formation and can decrease the development of OA in rabbits.

Keywords:
mesenchymal cell osteoarthritis, stem cell, umbilical cord

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transplantations [6]. The human UC is considered a major source of MSCs, and therefore is used in stem cell therapy [7].

The first application of MSCs in cartilage repair was conducted on rabbits, where full-thickness defects were filled with collagen scaffold seeded with MSCs and mechanically loaded. The short-term observation showed regeneration of cartilage and bone [8].

Materials and methods
The present study is the first to use human umbilical cord blood mesenchymal stem cells (HUCB-MSCs) in regenerating experimentally induced OA in New Zealand white rabbits.

The present study was conducted at Medical Experimental Research Center (MERC), Mansoura University, between January 2011 and April 2013. The animal ethics committee approved the study.

Forty-two adult (6 months old) male New Zealand white rabbits weighing 3.5–4 kg were housed in quarantine conditions for 14 days to be monitored for normality and to allow time for acclimatization to the new environment and handling. Normal activity and parameter assessment were defined as normal food and water consumption, normal stool formation, normal behavior, no respiratory distress or nasal discharge, and a temperature between 38 and 40.6°C. The rabbits were placed individually into 4 square foot stainless steel cages and housed in the same room with a temperature of 21–22°C.

The rabbits were skeletally mature. They were given the same tap water and food during the experiment. Kept in separate cages, they were allowed to move freely.

All rabbits underwent surgical induction of OA in their left knees by cutting the anterior cruciate ligament (ACL). They were observed clinically for 12 weeks and assessed by measuring their voluntary motion in comparison with controls.

Twelve weeks after surgery, two rabbits died; their knee joints were excised and evaluated histopathologically. The remaining 40 rabbits were randomly assigned to one of four groups (n = 10/group) after proving OA clinically and histopathologically:

Group 1: Osteoarthritic left knees were injected with human UC stem cells and followed up after 2 months.

Group 2: Osteoarthritic left knees were injected with culture media and followed up after 2 months.

Group 3: Osteoarthritic left knees were injected with human UC stem cells and followed up after 6 months.

Group 4: Osteoarthritic left knees were injected with culture media and followed up after 6 months.

Surgical induction of osteoarthritis
The surgery was performed by a veterinary surgeon under sterile conditions. Trimethoprim-sulfamethoxazole 15 mg/kg, subcutaneous, was administered immediately preoperatively. The rabbits were then premedicated and anesthetized with xylazine 3 mg/kg, intramuscular, glycopyrrolate 0.01 mg/kg, intramuscular, and ketamine 17 mg/kg, intramuscular. The left ACL was exposed by a medial parapatellar skin incision, the patella was subluxated laterally, and the knee was fixed in complete flexion. The ACL was transected, and the incision was sutured in a routine manner. Analgesia was achieved by adding buprenorphine 0.03 mg/kg, subcutaneous, preoperatively and then again 12 h later. After the surgery, the rabbits were housed separately and permitted free cage activity until the termination of the study.

Preparation of mesenchymal stem cells
Isolation of umbilical cord-mesenchymal stem cells
Fresh human UCs were extracted after full-term births (cesarean section or normal vaginal delivery) with informed consent.

The UCs were washed with PBS containing 1% antibiotic–antimycotic solution (Thermo Scientific, Miami, Florida, USA). This step was repeated until the cord became clear. The cords were cut longitudinally and minced into 1–2 mm³ fragments, and incubated with enzyme cocktail of 0.1% collagenase and 0.25% trypsin (Sigma Aldrich, USA) at 37°C with shaking at 100 rpm for 45 min. After digestion, the enzyme activity was neutralized by adding the same volume of Dulbecco’s modified Eagle’s medium (DMEM) that contains 10% fetal bovine serum (Thermo Scientific). To achieve complete cell suspension, the mixture was passed through a 100 µm filter (BD Falcon, USA). Then the sample was centrifuged at 2000 rpm for 5 min at room temperature, followed by the cells being cultured in DMEM with 10% fetal bovine serum and 1% antibiotic–antimycotic solution in a 25 cm² culture flask and maintained in an incubator with humidified atmosphere of 5% CO₂ at 37°C [9].

Cell cultivation
After 1 day, nonadherent cells were washed 2–3 times with PBS and adherent cells further cultured in complete medium, which was changed every 3 days
until the monolayer of adherent cells reached 70–80% confluence. For passage 1, trypsinization for cell splitting was achieved using trypsin–EDTA solution (0.25%; Sigma Aldrich). The number of cells was evaluated with a hemocytometer and cellular viability with the trypan blue exclusion test. Each 250–300×10⁳ cell was inoculated in a 75 cm² culture flask incubated at 37°C and 5% CO₂. Cell cultivation was maintained up to the third passage [9].

**Flow cytometric analysis**

Cells were characterized using cell surface markers by fluorescence-activated cell sorting analyses. The cells were stained with different fluorescently labeled monoclonal antibodies (mAb). In brief, 5×10⁵ cells [in 100 µl PBS/0.5% bovine serum albumin (BSA)/2 mmol/EDTA] were mixed with 10 µl of the fluorescently labeled mAb and incubated in the dark at 2–8°C for 30 min. Washing with PBS containing 2% BSA was done twice, and the pellet was resuspended in PBS and analyzed immediately by flow cytometry.

For isolating of HUCB-MSC’s, we used the mouse anti-human CD90-PCY5 mAb, CD105-PE mAb, CD29-FITC mAb, CD13-FITC mAb, CD34-PE mAb, CD11b-FITC mAb, CD19-PCY5 mAb, CD45-PCY5 mAb, and CD14-FITC (all from eBioscience, Miami, Florida, USA). The fluorescence intensity of the cells was evaluated by Epics XL Flow Cytometry (Coulter, Miami, Florida, USA) [10].

**Colony forming unit-fibroblast assay [10]**

For the colony forming unit-fibroblast assay, about 100 cells were plated per 100-mm tissue culture dish (BD Falcon) in complete culture medium. Cells were incubated for 10–14 days at 37°C in 5% humidified CO₂, washed with PBS, and fixed in 95% ethanol for 5 min. Next, the cells were incubated for 20–30 min at room temperature in 0.5% crystal violet (Sigma Aldrich) in 95% ethanol. The plate was washed twice with distilled H₂O₂, and when it dried the colony forming unit-fibroblast units were counted.

**Umbilical cord-mesenchymal stem cells differentiation assay [11]**

**Chondrogenic differentiation**

For chondrogenic differentiation, the pellet culture system described by Sekiya et al. [12] was used. The cells were counted and seeded at a density of 0.25×10⁶ per Eppendorf tube in a chondrogenic differentiation medium, which consisted of high glucose DMEM supplemented with 10 ng/ml TGF-β3, 100 nmol/l dexamethasone, 200 µmol/l ascorbate-2-phosphate, 40 µg/ml proline, 1 mmol/l pyruvate, 1 mg/ml BSA, and 50 mg/ml ITS*3. The medium was replaced every 2–3 days for 21 days.

Sulfated glycosaminoglycan (GAG) appeared on the frozen sections (5-µm-thick) with 1% toluidine blue (Sigma Aldrich). The production of sulfated GAG was measured in an alcian blue binding assay after digestion in 100 µl papain solution. Absorbance was reported at 630 nm.

**Cell culture**

The cultures were observed using an inverted microscope. The attachment of spindle-shaped cells to the tissue culture plastic flask was recorded after 1 day. Primary cultures reached 70–80% confluence in 5 days. After the third passage, cultures were composed of a homogenously fibroblastic cell.

**Immunophenotypic characterization**

The third passage UC-MSCs were analyzed for markers of cell surface; UC-MSCs were negative for CD34, CD11b, CD19, CD45, and CD14, with percentages 0.18, 1.02, 0.06, 0.03, and 0.5, respectively; UC-MSCs were positive for CD90, CD105, CD29, and CD13, with percentages 97.3, 99.8, 99.5, and 76.9, respectively.

**Injection of stem cells**

**Stem cell-treated group**

A single dose of one million cells suspended in 1 ml of medium was delivered to the operated knee by direct aseptic intra-articular injection.

**Control group**

The control group received 1 ml of medium without cells. All rabbits were killed by an intravenous injection of thiopentone.

**Assessment of osteoarthritis**

After killing the animals, the femoral condyle and tibial plateau were resected and fixed in 10% formalin solution. The sections were decalcified in 0.5% EDTA and stored in paraffin. We collected the tissue section from the same anatomical site on the condyle and plateau to avoid bias. All samples were read blindly by one observer [13].
Macroscopic assessment

Hind legs were thawed, knee joints opened, and articular surfaces subjected to photographic documentation.

Macroscopic grading was used to assess cartilage integrity in each of the four compartments of the rabbit femorotibial joint [13].

Histological grading

The grading of OA was carried out following the procedure of Mankin et al. [14].

Statistical analyses

The distribution of data was nonparametric; therefore, we used a nonparametric test (the Mann–Whitney U-test) for comparison between groups. Statistical analyses were performed using SPSS (version 20; SPSS Inc., Chicago, Illinois, USA). Data were presented as median and range. P values less than 0.05 were considered statistically significant.

Results

The results are shown in Tables 1–4 (Figs 1–5).

It was noted that, compared with the control group, the patient group showed great limitation in voluntary movements.

Discussion

This study was conducted on 42 New Zealand white rabbits. All rabbits underwent surgical induction of

Table 1 The weight and macroscopic and histologic grading of the left tibial plateau and femoral condyle in the animal model after 12 weeks

| Characteristics       | First rabbit | Second rabbit |
|-----------------------|--------------|---------------|
| Weight                | 3.20         | 3.75          |
| **Left tibial plateau** |              |               |
| Macroscopic assessment (9) |            |               |
| Left tibial naked eye (N/E) (1–6) | 4  | 5             |
| Histological assessment (13) |            |               |
| Left tibial cartilage (0–5) | 3  | 4             |
| Left tibial chondrocytes (0–3) | 3  | 3             |
| Left tibial safranin (0–5) | 3  | 3             |
| Left tibial tidemark (0/1)  | 0  | 0             |
| **Left femoral condyles** |              |               |
| Macroscopic assessment (9) |            |               |
| Left femoral N/E | 3  | 4             |
| Histological assessment (13) |            |               |
| Left femoral cartilage | 2  | 3             |
| Left femoral chondrocytes | 3  | 3             |
| Left femoral safranin | 2  | 2             |
| Left femoral tidemark | 0  | 0             |
we delivered a single dose of HUCB-MSCs directly intra-articularly into the cell-treated groups. The control groups were injected only with suspension media.

Twenty-four weeks after the stem cell injection, macroscopic and histopathologic grading was significantly better than that performed 8 weeks after the injection, and the scores were significantly higher.

Løken et al. [15] pointed out that only long-term results are of great interest. Different time intervals have been used to study cartilage repair simulating clinical settings.

In the present study 24 weeks was deemed to be an adequately long observation time to keep the rabbits alive to perform reasonable statistical analysis.

In animal models, OA is induced primarily by surgical procedures such as ACL transaction [16], ACL transaction combined with complete medial meniscectomy [17], or chemical adjuvants (using collagenase enzyme type II) [18]. Surgically induced OA is better than chemically induced OA because the biochemical and pathological changes are similar to human OA [19].

Our findings are in accordance with a study by Toghraie et al. [20], who reported that participants injected with MSCs showed good cartilage quality and lower degrees of cartilage degeneration, osteophyte formation, and subchondral sclerosis compared with the control group. They added that direct intra-articular injection of MSCs decreased the progress of advanced OA lesions in a rabbit model.

To the best of our knowledge, this study is the first to evaluate the efficacy of direct intra-articular injection of HUCB stem cells in regenerating rabbit articular cartilage.

In the present study the assessment of cell-treated rabbits was performed twice instead of once (8 and 24 weeks after injection). This allowed better follow-up of the effect of injected cells in regeneration of cartilage at a longer duration.

OA by cutting the ACL in their left knees. After the confirmation of development of OA histopathologically,
In the present study we opted for HUCB stem cells as it is an available and cheap source of stem cells. The UC blood is a source of noncontroversial, ‘embryonic-like’ stem cells that are collected at the time of birth through a simple, safe and painless procedure, and are preserved for future use [7].

HUCB-MSCs have a high chondrogenic differentiation potential that might lead to regeneration of damaged cartilage. HUCB-MSCs also show potent immunosuppression and anti-inflammatory effects [21].

We concluded that HUCB-MSCs are a promising cell source for cartilage tissue engineering and can reduce the development of OA lesions in a rabbit model.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Table 3 Comparison of macroscopic and histological grading scores of the left tibial plateau and femoral condyle in cell-treated and cell-free groups at 2 months (groups 1 and 2) after injection

| Characteristics                        | Median  | Group 1 | Group 2 | U value | Level of significance (P value) |
|----------------------------------------|---------|---------|---------|---------|---------------------------------|
| Left tibial plateau macroscopic assessment |         | (N = 9) | (N = 7) |         |                                 |
| Histological assessment                |         |         |         |         |                                 |
| Left tibial cartilage                  | 3       | 4       | 4       | 0.002   |                                 |
| Left tibial chondrocytes               | 1       | 3       | 2       | 0.001   |                                 |
| Left tibial safranin                   | 1       | 2       | 10      | 0.011   |                                 |
| Left femoral condyles macroscopic assessment |         |         |         |         |                                 |
| Histological assessment                |         |         |         |         |                                 |
| Left femoral cartilage                 | 0       | 2       | 2       | 0.001   |                                 |
| Left femoral chondrocytes              | 1       | 2       | 11.5    | 0.023   |                                 |
| Left femoral safranin                  | 1       | 2       | 7       | 0.03    |                                 |

Table 4 Comparison of macroscopic and histologic grading scores of the left tibial plateau and femoral condyle in cell-treated and cell-free groups at 6 months (groups 3 and 4) after injection

| Characteristics                        | Median  | Group 3 | Group 4 | U value | Level of significance (P value) |
|----------------------------------------|---------|---------|---------|---------|---------------------------------|
| Left tibial plateau macroscopic assessment |         | (N = 7) | (N = 5) |         |                                 |
| Histological assessment                |         |         |         |         |                                 |
| Left tibial cartilage                  | 5       | 6       | 7.5     | 0.076   |                                 |
| Left tibial chondrocytes               | 0       | 4       | 0.00    | 0.004   |                                 |
| Left tibial safranin                   | 0       | 3       | 0.00    | 0.002   |                                 |
| Left femoral condyles macroscopic assessment |         |         |         |         |                                 |
| Histological assessment                |         |         |         |         |                                 |
| Left femoral cartilage                 | 0       | 2       | 1.00    | 0.005   |                                 |
| Left femoral chondrocytes              | 0       | 3       | 1.5     | 0.007   |                                 |
| Left femoral safranin                  | 1       | 2       | 0.00    | 0.001   |                                 |

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