Effects Of Endplate Chondrogenic Exosomes (Epcs-Exos) On Intervertebral Disc Degeneration

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Research Article

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Abstract

Study Design.In vitro laboratory studies.

Objectives.To explore the effect of chondrocell-derived exosomes on intervertebral disc degeneration, and provide a new idea for IDD treatment.

Summary of Background Data.The research of exosomes and their regenerative effects have been verified in the research of repair in other tissues and organs at our country and abroad, including osteoarthritis, cardiovascular disease, lung injury, fracture healing, central nervous system disease, cancer and other diseases treatment^[1]. The aim of this study was to investigate whether exosomes derived from endplate cells could inhibit the degeneration of endplate and play an important role in the prevention and treatment of disc degeneration.

Methods.EPCs-Exos derived from endplate chondrocytes were prepared and identified by Nanoparticle tracking analyzer, transmission electron microscope, and immunoblotting to detect surface markers, it was confirmed by immunofluorescence assay that it could be ingested by nucleus pulposus cells. The degeneration of cartilage endplates was evaluated by detecting the contents of Aggrecan, COL 1A, COL 2A and RNA by RT-PCR and WB.

Results.EPCs-Exos can be ingested by nucleus pulposus cells and up-regulate the expression of Aggrecan, COL 2A and their mRNA, down-regulate the expression of COL 1A and their mRNA.

Conclusion.EPCS-Exo can further inhibit the degeneration of intervertebral disc by acting on nucleus pulposus cells and thus the degeneration of cartilage endplate.

1. Introduction

Intervertebral Disc Degeneration (IVDD) is one of the main causes of low back pain (LBP)^[2]. According to research, 80% of people have suffered from LBP in their life^[3], which caused great damage to society and individuals. Exosomes, as nano-sized vesicles, were first proposed as a waste product of the cell's metabolism and as a way for the cell to dispose of excess components^[4]. As research progresses, it has been found that exosomes can carry many substances, including proteins, lipids, metabolites, Mrna, Mirnas, and many non-coding rnas (lncRNA)^[5], which are found in almost all mammals, important for cell-to-cell communication. Exosomes can alter the behavior of nearby or distant target cells by paracrine or endocrine release from the mother cell, so exosomes can be used as a vaccine, drug delivery vectors or biomarkers are appropriately modified to deliver therapeutic interventions to target cells^[5]. In this study, we studied systematically the effects of Chondrocytes secreting exosomes on intervertebral disc degeneration.

2. Materials And Methods

2.1 Ethics The protocol was approved by the Animal Research Committee of Yijishan Hospital, Wannan Medical College.

2.2 Culture and treatment of rat chondrocytes Six 1-week-old female SD rats, were used to isolate chondrocytes. The endplate structure of the Lumbar intervertebral disc of 1 week old SD rats was first isolated, and the normal chondrocytes were digested by 0.1% type II collagenase. Then 10 cm² cells were inoculated and 5106 cells were seeded. 80-90% of the cells were covered with Petri dishes. The supernatant of the cells was stored at -80 °C and the cells were subcultured in 210 cm² petri dishes until 80-90% of the cells were covered with petri dishes, the supernatant was collected and stored at -80 °C. Observe the cell morphology every day, and take photos to record, when the cell morphology change growth rate slowed down obviously, at this time 48-72 hours to collect a cell supernatant preserved at -80 °C, add DF12 culture medium to continue to culture, collect 90 ML supernatant before and after degeneration.

2.3 Exosome extraction

- 1 The particles which are larger than 0.8µm were removed from the cell culture supernatant (with 0.45 µm filter)
- 2 The purified supernatant was mixed with Buffer XBP (1:1) and combined on an exoEasy membrane affinity centrifuge column, and centrifuged for 500 G and 1 Min
- 3 The combined exosomes were rinsed with Buffer XWP and centrifuged for 4000g for 5 min
- 4 Replace the centrifuge tube and then use 500µl Buffer XE for three separate elution, preservation -80 °C

2.4 Exocrine marker detected by WB

- 1) Each 50uL of exosome was added 100ul RIPA (Pik Yuntian P0013B)(containing 1 mm PMSF (Pik Yuntian ST506) ;
- 2) Cells were blown out and cracked on ice for 30 minutes, then mixed up upside down every five minutes;
- 3) The supernatant was centrifuged at 4 °C for 12000 RPM to collect the supernatant, as Post-cleavage protein solution
- 4) Protein concentration was detected by Solarbio PC0020. The protein concentration of the sample was adjusted to the optimum range, adding denaturation 5 loading, boiling water for 5 ~ 10 minutes, placing on ice for 5 ~ 10 minutes, storing at -20 °C, waiting for the follow-up experiment.
- 5) SDS-PAGE Gel (10% separation GEL) was prepared according to the size of the protein;
- 6) SDS-PAGE electrophoresis was carried out on the prepared target protein, the sample size was about 20ug, 80V, 30Min; 120V, 70Min; the Gel was removed from the Gel Sheet;
- 7) The SDS-PAGE Gel was transferred wet-printed in ice bath, constant current 300MA, 30min. Sentence is too long, please supply a shorter sentence.
- 8) Sealing: PVDF membrane after transfer printing is cleaned once with TBST; then it is immersed in the sealing solution and gently sealed for 2 hours at room temperature, then TBST is rinsed once;
- 9) Anti: According to different antibodies, the appropriate proportion of antibodies was diluted with PBS, antibody diluent or blocking solution, and anti-HSP70, anti-Alix, anti-Flotillin-1 and anti-Annexin were respectively diluted by 1: Diluted 1000, set in refrigerator at 4°C overnight, then wash TBST 3 times, every 10min, wash once;
- 10) Second antibody: The second antibody was Solarbio anti-rabbit IgG-HRP and Solarbio anti-rat IgG-HRP
- 11) Rendering: The SOLARBIO PE0010 is applied to the PVDF film for exposure photography.

2.5 Culture and treatment of nucleus pulposus cells After washing with PBS, the nucleus pulposus was cut into small pieces and digested with 0.5% type II collagenase (Roche Diagnosis) for 2 hours. Cells were obtained and maintained in F12 basic medium containing 10% fetal bovine serum and 100u/mL penicillin-streptomycin in a humidified incubator containing 5% CO₂.

2.6 Nucleus pulposus cell experiment The bone marrow nucleus cell culture medium was DMEM-F12, the cells were laid on the slide of 6-well plate, (1) cells were laid on the slide of 6-well plate with 3-well plate, then 1106 cells were added with 20ug/mL exocrine body (before degeneration, after degeneration and normal cell

group) , and the cells were harvested 48 hours later, do QPCR and WB experiments respectively.

2.7 Total RNA extraction

- (1) Trypsin digesting cells were centrifugally collected into a RNase-free 1.5 ml centrifuge tube;
- (2) 500 μ l lysis was added and repeatedly blown and left at room temperature for 5-10 Min;
- (3) 0.2 ml chloroform was added and left to oscillate violently for 30 SEC at room temperature for 3 min.

12000RPM centrifugation at 4 °C for 10 Min;

- (4) Transfer the upper water phase to a clean centrifuge tube, add 1/2 volume anhydrous ethanol, and mix;
- (5) Put the adsorption column into the Collection Tube, add all the solution and the translucent fibrous suspension into the adsorption column with a Pipette, leave it for 2 minutes, centrifuge at 12000rpm for 3 minutes, and dump the waste liquid in the collection tube;
- (6) Put the adsorption column back into the Collection Tube, add 500 μ l RPE Solution, leave for 2 min, 10,000 RPM centrifuge 30 SEC, empty the waste Solution from the collection tube;
- (7) Repeat step 6 once;
- (8) Return the adsorption column to the Collection Tube, centrifuge for 2 min at 10,000 RPM;
- (9) Place the adsorption column in a clean 1.5 mL centrifuge tube, 30 μ l DEPC-TREATED DDH₂O was added at the center of the adsorption membrane, and the RNA was centrifuged at 12,000 RPM for 2 min. The RNA solution was stored at -70 °C or used in the follow-up experiments.

* Total RNA extraction Kit (bio-engineering, B511321).

2.8 Synthesis of cDNA

The cDNA synthesis kit of Quanjin Company was used to synthesize cDNA, and the reverse transcription system and conditions were shown in Table 1.

2.9 Real-time PCR Amplification

- 1 Primer synthesis The qPCR primers were synthesized by Shanghai Sheng Gong, and the primer sequences were shown in Table 2.
- 2 Real time-PCR Reaction system The reaction solution was prepared according to the Real Time -PCR reaction system. DDH₂O, Sybr green qPCR Master Mix, Forward primer, Reverse primer, and cDNA templates were added into the PCR reaction tubes and thoroughly mixed. The reaction system was formulated according to Table 3.

* SYBR Green PCR ELISA (Quanshi AQ131)

- 3 PCR Amplification Procedure • PCR Amplification Procedure Set as shown in Table 4.

2.10 The expression of related genes was analyzed by WB

The gene expression of exosomes and nucleus pulposus cells cocultured in normal cell groups and before and after degeneration was detected by the same procedure as that of exosomes markers.

2.11 Immunofluorescence

• The culture medium of bone marrow nuclear cells was DMEM-F12, cells were spread on a 6-well plate and 5×10⁵ cells were inoculated. The next day, lentivirus pHSI-GFP was added for infection. After 2 days, siRNA CY3-NC was transfected and washed for 3 times with 24h PBS. The anti-fluorescence quenching agent containing DAPI was used to seal the film and the images were collected under a fluorescence microscope.

2.12 Statistic analysis

• Numerical data are expressed as mean $\pm$ standard deviation (SD). All statistical analyses in this study were performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Student t tests were performed to determine the significance between the two groups. P value less than 0.05 is considered significant.

3. Results

3.1 Increased expression of chondrogenic exosomes in the endplate after intervertebral disc degeneration

We first analyzed the histological characteristics of endplate chondrocytes before and after intervertebral disc degeneration. As shown in Fig. 1A, the number of endplate chondrocytes is lower after disc

degeneration than before degeneration. According to the characteristics of exosomes, the morphology and phenotype of exosomes were identified and isolated, and the content of exosomes with a diameter of 100nm was significantly increased after degeneration (Fig. 1B). After intervertebral disc degeneration, the content of chondrogenic exosomes in the endplate increased significantly, with the concentration of about 60mg/ml before degeneration and about 80mg/ml after degeneration (Figure 1C, D). Finally, we detected exosome markers by Western blotting and found that exosome markers (Alix, HSP70, Flotillin, Annexin V) could be detected in the particles (Figure 1E).

3.2 Endplate chondrocyte-derived exosomes are absorbed by nucleus pulposus cells and inhibit disc degeneration.

When chondrocyte derived exosomes were co-cultured with nucleus pulposus cells, it was found that DIL-labeled normal and differentiated exosomes showed red fluorescence in the cytoplasm of nucleus pulposus cells (Figure 2A), indicating that exosomes had been taken up by nucleus pulposus cells. After co-culture of Normal exosomes and Differentiated exosomes with nucleus pulposus cells, the concentrations of Normal exosomes and Differentiated exosomes and COL 2A were higher than those in the control group, while the concentrations of COL 1A were lower than those in the control group, while the results were opposite in the Differentiated group (Figure 2B, C).

3.3 Comparison of RNA concentration and purity after co-culture of Bef-Differentiated-Normal exosomes and nucleus pulposus cells

RNA concentration and purity as shown in Table 5

After the nucleus pulpotheca cells were co-cultured with 20ug/mL exosomes before and after degeneration, RT-PCR detection showed that the RNA concentration in the pre-degenerated exosome group was higher than that in the post-degenerated exosome group.

4. Discussion

The main cause of IDD is usually attributed to the reduction of NP cells, but current treatment strategies (surgery and conservative therapy) can not replenish NP cells or change the pathological state^[6], and lead to a series of more serious complications, bring more pain to the patient. Therefore, more and more scholars focus on the research direction of biological therapy, and achieved great results. Experimental studies have provided many positive evidences for chondrocytes to be used in the treatment of intervertebral disc degeneration. ADKISSON^[7] et Al. demonstrated that chondrocytes can form cartilage matrix in vitro with normal biochemical and morphological matrix, which can be demonstrated in the repair of disc regeneration and degeneration. Fabrice^[8] et Al. injected human immature chondrocytes into the intervertebral disc of rats, and found that the transplanted chondrocytes had no adverse effects on the nerve tissue, and had therapeutic potential to repair the intervertebral disc. Vedicherla^[9] et Al. explored the possibility of autologous chondrocyte transplantation for the treatment of disc degeneration by comparing the nasal cartilage with the articular cartilage, and showed that in the presence of 5% oxygen

and the absence of *tgf*-, nasal cartilage has the ability to produce more nucleus pulposus and extracellular Matrix. This study demonstrates that autologous chondrocyte transplantation can be a promising and effective way to repair disc degeneration in vitro. Although autologous cell transplantation is effective in reducing immune risks, it is cumbersome, time-consuming and requires additional surgery, so both autologous and allogeneic chondrocyte transplants have their limitations ^[10].

Exosomes have many advantages over cell therapy in the treatment of disc degeneration. First exosomes, as vehicles, have the ability to evade the host immune system ^[12]. Exosomes also have a high tissue affinity and targeting ability, so they can act accurately on tissue organs or cells ^[13]. Furthermore, exosomes are simple to manipulate and can be extracted from either cell cultures or patient serum or plasma and modified to transfer back into the same patient ^[13]. Exosomes, because of their polymer nanostructures, are highly stable, have long half-lives and can travel long distances in Vivo under physiological or pathological conditions ^[16].

Exosomes are small, single-membrane secretory Organelles with a diameter of about 30 to 200 nm^[17]. According to the present study, the lowest therapeutic dose of exosomes is 10-100 Ug ^[18], therefore, 20ug was chosen as the experimental dosage of exosomes. Until now, six classes of exosome separation strategies have been reported, including ultra-speed centrifugation, ultrafiltration, immunoaffinity capture, charge neutralization-based polymer precipitation, size-exclusion chromatograph, and microfluidic techniques, with unique sets of advantages and disadvantages for each technique. With the capacity to generate centrifugal forces as high as 1,000,000 ×g (100,000-150,000 ×g is commonly used for exosome separation), ultracentrifugation is an optimal process for separating small particles including bacteria, viruses, and cellular organelles^[19]. In this study, the end plate Chondrocyte exosomes were extracted by this method. International Society for Extracellular Vesicles (ISEV) proposed that two types of proteins (like Transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes, Cytosolic proteins recovered in EVs) need to be evaluated to determine whether the extracted components are exosomes and exosomes derived from biological fluids can be assessed for their purity by determining the presence or absence of certain non-EV structural protein components co-separated with EV^[20]. In this study, the exosomes were identified by Nanoparticle tracking analyzer, transmission electron microscope and immunoblotting to detect the surface markers, it was confirmed by immunofluorescence assay that it could be ingested by nucleus pulposus cells. In order to study the effect of endplate Chondrocyte exosomes on nucleus pulposus cells, we co-cultured blank group, normal exosomes and retrogressive exosomes with nucleus pulposus cells, respectively, using *Gaddh* as a control, *agrecan*, *COL 1A*, *COL 2A* were analyzed by Western blotting. The *AGRECAN*, *COL 2A* were found to be higher in normal exosomes and nucleus pulposus cells than in blank and degenerated cells, the content of *COL 1A* in normal exosome group was lower than that in blank group and degenerate group. At the same time, the RNA concentration of normal exosome group and nucleus pulposus group was higher than that of degeneration group by RT-PCR. Experiments showed that normal exosomes secreted by endplate chondrocytes could up-regulate the expression levels of *Aggrecan*, *Col 2A* and their mRNA in nucleus pulposus cells and down-regulate the expression levels of *Col 1A* and their mRNA. After co-

culture of the degraded exosomes with nucleus pulposus cells, The expression levels of Aggrecan, Col 2A and their mRNA were decreased, while the expression levels of Col 1A and their mRNA were increased, which were completely contrary to the results of normal exosomes. These results suggest that chondrocell-derived exosomes can enhance the proliferation activity of nucleus pulposus cells and thus play a role in the prevention and treatment of intervertebral disc degeneration.

Exosomes, as small single-membrane secreting organelles, are composed of various forms of proteins, lipids and RNA. We only studied exosomes secreted by chondrocytes as a whole to explore their influence on intervertebral disc degeneration, but did not study their specific molecular components. We believe that further study of specific components of chondrogenic exosomes, including interstitial RNA, lipids, or proteins, may influence intervertebral disc degeneration through specific pathways. In addition, this study only extracted specific exosomes and carried out in vitro, without further in vivo experiments. Further studies are expected to demonstrate the potential of chondrogenic exosomes to treat disc degeneration.

To sum up, EPCS-Exo can further inhibit the degeneration of intervertebral disc by acting on nucleus pulposus cells and thus the degeneration of cartilage endplate. These studies reveal the effect of EPCS-Exo on intervertebral disc degeneration and provide a new idea for IDD treatment.

Abbreviations

EPCS-Exos: Endplate Chondrogenic Exosomes

IDD: Intervertebral Disc Degeneration

Declarations

Funding

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Author information

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Contributions

All authors contributed to the study conception and design. Qiu-wei Li and Hong Wang performed all the experiments, collected, and analyzed the data. Qiu-wei Li wrote the manuscript. Hong Wang and Xue-wu Chen supervised the study. The authors read and approved the final article.

Ethics declarations

Ethics approval and consent to participate

All experimental procedures and use of research animals in this study were approved by the Nantong [University](#) of Medical Sciences ethics committee (SYXK(SU)2015-0016).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Reverse transcriptional responses

component	volume
RNA	7μL
Random Primer	1μL
2 ×ES Reaction Mix	10μL
<i>EasyScript</i> [®] RT/RI Enzyme Mix	1μL
gDNA Remover	1μL
They were incubated at 25°C for 10 min and at 42°C for 15 min	
Inactivated at 85°C for 5s and stored at -20°C	

Table 2. Primer sequence

Primer	Sequence[5'-3']
GAPDH-F	GAAGGTGAAGGTCGGAGTCAA
GAPDH-R	CTGGAAGATGGTGATGGGATTT
-F	
-R	

Table 3 qPCR amplification system

component	volume
qPCR Mix	10μL
Forward Primer 10μM	0.5μL
Reverse Primer 10μM	0.5μL
cDNA	2μL
Water, nuclease-free	7μL
Total volume	20 μL

Table 4 qPCR Amplification Procedure

Step	Temperature, °C	Time	Number of cycles
Predegeneration	94	30s	1
denaturing	94	5 s	40
annealing	61	35 s	
melting curve	97	10 s	1
	65	60s	
	97	1s	

Table 5 RNA concentration and purity

Samples	Con.(ng/μl)	OD _{260/280}
Bef exosomes	37.16	1.99
Differentiated exosomes	31.587	2.01
Normal exosomes	59.387	2.01

Figures

Figure 1

Increased expression of chondrogenic exosomes in the endplate after intervertebral disc degeneration

⊠A⊠Morphological comparison of endplate chondrocytes before and after intervertebral disc degeneration under light microscope.(B) The size distribution of exosomes was about 100nm before and post Differentiated.(C) Density comparison of exosomes before and after degeneration under electron

microscopy.(D) Comparison of exosome concentration before and post Differentiated.(E) WB was used to detect marker proteins before and post exosome Differentiated.

Figure 2

Endplate chondrocyte-derived exosomes are absorbed by nucleus pulposus cells and inhibit disc degeneration.

(A) DIL labeled chondrocyte exosomes show red fluorescence in the cytoplasm of nucleus pulposus cells, scale = 20µm.(B) Western blotting results of Aggrecan, Col 1A and Col 2A.The ratio between the target and beta-actin was used to standardize data for comparison.The value is the mean value $\pm$ SEM of the experiment in triplicate.N = 4;* P < 0.05.COL1 collagen type I, COL2 collagen type 2.(C) Western blot analysis of Aggrecan, Col 1A and Col 2A was performed using GADPH as the sample control.