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ORIGINAL RESEARCH

Adipose Tissue-Derived Exosome Maintains Metabolic Balance of Extracellular Matrix in Rat Nucleus Pulposus Cells

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Purpose: This study aimed to investigate the protective effect of adipose tissue-derived exosomes (AT-Exo) on rat nucleus pulposus cells (NPCs).

Methods: Ultracentrifugation was used to extract exosomes from rat adipose tissue. Transmission electron microscopy (TEM), Western blot, and nanoparticle tracking analysis (NTA) were used to characterize the exosomes. Tert-butyl hydrogen peroxide (TBHP) was used to induce apoptosis of rat NPCs. Cell viability was determined by CCK-8 assay. AT-Exo was administered to investigate its effect on rat NPCs using Western blot and immunofluorescence staining.

Results: AT-Exo was successfully extracted and characterized by NTA, TEM, and Western blots. Uptake assay showed that AT-Exo can be taken up by the NPCs. TBHP (60 μM) resulted in decreased cell viability and increased apoptosis of NPCs. Interestingly, AT-Exo protected NPCs against TBHP, indicated by increased cell viability, decreased apoptosis, upregulated Aggrecan and type II collagen deposition, and downregulated matrix metalloproteinase 3/13.

Conclusion: In summary, rat adipose tissue-derived exosomes can increase the levels of Aggrecan, type II collagen, and Bcl2, and decrease the levels of matrix metalloproteinase 3/13, cleaved caspase3, and Bax. Therefore, rat adipose tissue-derived exosomes can maintain metabolic balance of extracellular matrix and protect against apoptosis in rat nucleus pulposus cells.

Keywords: exosome, adipose, intervertebral disc degeneration, nucleus pulposus, extracellular matrix

Introduction

Intervertebral disc degeneration, as a common degenerative disease of articular cartilage, causes lower back pain (LBP), lumbar disc herniation, and a series of other degenerative diseases that seriously affect people's quality of life.[1] The main molecular mechanism of disc degeneration is that the catabolism of the extracellular matrix in the disc tissue is stronger than its anabolism, resulting in a decrease in the extracellular matrix, a decrease in the water content of the nucleus pulposus (NP), and degeneration of the NP, which leads to the collapse of the intervertebral space, resulting in symptoms such as LBP.

As an extracellular vesicle with a diameter of 30–150nm, exosomes contain a variety of substances such as DNA, RNA, proteins, and lipids. Recent studies have shown that exosomes secreted by bone marrow mesenchymal stem cells, adipose mesenchymal stem cells, NP mesenchymal stem cells and other types of stem cells are able to treat apoptotic nucleus pulposus cells (NPCs), thus slowing down intervertebral disc degeneration.[2–5] Exosomes obtained by cell culture are time-consuming and have a low yield. The extraction of exosomes from adipose tissue (AT) is simpler and easier to modify. AT in the human body is a type of connective tissue that can perform many functions. Adipose tissue-derived

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exosomes (AT-Exo) contain a variety of adipokines and play an active role in the treatment of metabolic diseases.[6] However, whether AT-Exo can protect NPCs and mitigate intervertebral disc degeneration remains unknown.

Thus, this study was performed to investigate the potential protective effect of AT-Exo on rat NPCs, focused on maintaining metabolic balance of extracellular matrix.

Materials and Methods

Ethics

Sprague-Dawley (SD) rats used in this study were from the Animal Experiment Centre of Hebei Medical University. The use of animals was approved by the Ethics Committee of Hebei Medical University (IACUC-4th Hos Hebmu-2023101). In this study, the use of animals complied with the 3R principles, namely reduction, replacement, and refinement, as well as the Five Freedoms principles. Three SD rats were used to isolate rat NPCs, and another three were used to extract AT-Exo.

Extraction of Rat Adipose Tissue

Four-week-old SD rats (80–120g, male) were sacrificed by carbon dioxide, soaked in iodophor for 5 minutes and immersed in 75% alcohol for 15 minutes. Tissue scissors were used to separate the skin at the groin and the AT was removed and placed in PBS at 4°C. AT was washed 3–4 times in PBS and the hairs and blood were removed. The AT was cut into pieces around 2mm*2mm*2mm using tissue shears and placed in a petri dish with a diameter of 10cm. Around 15mL of serum-free medium was added to this dish and incubated at 37°C for 48 hours.

Isolation and Characterization of AT-Exo

After 48 hours, the supernatant in the petri dish was collected in a centrifuge tube. At 300g, 4 °C, this tube was centrifuged for 10 minutes to remove the precipitate and only keep the supernatant. The supernatant was centrifuged again, at 3000g, 4 °C, for 10 minutes, and only the supernatant was collected. Centrifuge the supernatant at 4 °C, 1×10^4g, for 30 minutes, collect the supernatant and discard the sediment. The supernatant was filtered through a 0.22 μm filter. The filtered supernatant was centrifuged at ultra-high speed at 4 °C, 1.1×10^5g for 70 minutes. Next, the supernatant was discarded, and the sediment was resuspended in PBS and filtered through a 0.22 μm filter. After filtration, the fluid containing exosomes was centrifuged for 70 minutes at 4 °C, 1×10^5g. After centrifugation, discard the supernatant and use 200 μL PBS to resuspend the sediment.

The concentration of exosomes was determined by BCA assay (Solarbio, PC0020, China). The expression of exosome surface markers Alix, Calnexin, CD63, TSG101 and SDCBP was examined in this study. Transmission electron microscopy (TEM) was used to observe the structure of exosomes, and nanoparticle tracking analysis (NTA) was used to analyze the particle size of exosomes.

Cell Isolation, Culture, and Characterization

Four-week-old SD rats (80–120g, male) were sacrificed by carbon dioxide. The tails of rats were cut off with tissue scissors, soaked in iodophor for 5 minutes, and then transferred to 75% ethanol for 15 minutes. After 15 minutes, the rat tails were transferred to a biosafety cabinet. The tails were rinsed and cleaned with PBS solution. The tail skin was incised and removed, and only the spinal columns were collected, kept in PBS for use. Next, the soft tissues were removed and the annulus fibrosus was incised with surgical blade. After the intervertebral space was open, the jelly-like NP tissue was obtained using a fine needle, put into 3 mL 0.25% trypsin, at 37 °C for 30 minutes. Next, centrifuged at 1000 rpm for 5 minutes, the supernatant was discarded. Add 3mL of 0.2% type II collagenase to the pellet, at 37 °C for 4 hours, centrifuge at 1000 rpm for 5 minutes, and discard the supernatant. Resuspend the pellet with DMEM/F12 complete medium containing 10% FBS (Gibco, US), transfer the resuspended solution to a T25 culture flask. This flask stayed in a cell incubator with 5% CO_2 at 37 °C. The culture medium of NPCs was refreshed every 2–3 days. Cells were passaged when reaching 80% confluence. The NPCs of passages 3–4 were used for subsequent experiments.

The expression of NP cell surface markers was detected by flow cytometry. The positive markers CD73, CD90, CD105, and the negative markers CD31 and CD45 were detected in this study.[7] NPCs with 80% confluence were

dissociated with 0.25% trypsin and resuspended with PBS after centrifugation. 1×10^6 single cells were generated. NP cells were labeled with PE-tagged CD73, CD31, CD105 and FITC-tagged CD90, CD45 for 30 min at 4°C. Isotype control was set at the same time.

Exosome Uptake Assay

Mix 5 μL of Evlink555 (Tingo exosomes technology co ltd, EL012100200, China) with 150 μL of exosome stock solution and incubate it in the dark for 30 minutes at room temperature. Transfer the labeled exosomes to a 100kD ultrafiltration tube, at 10^4 g at room temperature, 15 minutes × 4 times, to remove free fluorescent probe and small molecule substances. Resuspend the pellet with 100 μL of PBS. The NPCs were seeded at 3×10^4 cells per well in a 24-well plate. When cell confluence reached 80%, the labeled exosomes were added to the cell culture medium at 50 μg/mL. After 4 hours of cell culture, the cell membrane was stained, the cell culture medium was discarded, and PBS was used to clean the cells three times for 5 minutes each time. 5 μL of Celllink505 (Tingo exosomes technology co ltd, CL012100210, China) probe was diluted to 500 μL with PBS, added to the well plate, and incubated at room temperature in the dark for 30 minutes. After incubation, clean the cells with PBS three times for 5 minutes each time. The cells were fixed with 4% paraformaldehyde for 30 minutes, the cells were washed three times for 5 minutes each time. DAPI was added to stain nuclei for 5 minutes, washed three times for 5 minutes each time. The images were observed and collected under a fluorescence microscope.

CCK-8 Assay

The apoptosis model of NPCs was established by Tert-butyl hydrogen peroxide (TBHP) (Macklin, Shanghai). CCK-8 (MedChemExpress, HY-K0301, US) assay was performed to determine the effects of TBHP and AT-Exo on rat NPCs, according to the manufacturer's manual.

Experimental Grouping

Based on the CCK-8 assay results, rat NPCs were divided into three experimental groups: (1) the control group: the cells were treated with serum-free medium; (2) the TBHP group: after the cells were treated with serum-free medium for 4 hours, TBHP was added and worked for another 4 hours (60 μM); (3) The TBHP + AT-Exo group: 150 μg/mL AT-Exo were pretreated for 4 hours, followed by 60 μM TBHP for another 4 hours of treatment.

Western Blotting

Western blotting was used to detect the protein level of type II collagen, Aggrecan, MMP3, MMP13, cleaved caspase-3, Bax and Bcl2 in each experimental group. After the proteins in the experimental groups were collected, the protein concentration was determined by a BCA assay. After the protein concentration was determined, the protein was mixed with loading buffer, boiled in water bath for 10 minutes. After being aliquoted, the proteins were stored at −80 °C for Western blotting use. For electrophoresis, 90V constant voltage was firstly applied to concentrate the protein. After bromophenol blue entered the separating gel, the voltage changed to 120V to the end of electrophoresis. For membrane transfer, a PVDF membrane was used. The membrane transfer ran using a 400mA constant current for 50 minutes. After membrane transfer, the PVDF membrane was blocked in fast blocking solution (EpiZyme, PS108, China) for 30 minutes at room temperature. The following primary antibodies were used to incubate the PVDF membrane at 4 °C for overnight: rabbit GAPDH (1:1000, Abways, AB0038, US), Aggrecan (1:1000, Affinity Biosciences, DF7561, China), type II collagen (1:1000, Affinity Biosciences, AF0135, China), MMP3 (1:2000, Proteintech, 17873-1-AP, China), MMP13 (1:4000, Proteintech, 18165-1-AP, China), cleaved caspase-3 (1:1000, Cell signaling, 9661s, US), Bax (1:1000, ImmunoWay, YTO455, US), Bcl2 (1:1000, ImmunoWay, YTO470, US). Next, the membrane was washed with TBST three times, 10 minutes each time. Goat anti-rabbit HRP-conjugated secondary antibody (1:5000, Abways, AB0101, US) was used to incubate the membrane for 1 hour at room temperature, at 70 rpm on a shaker. The membrane was then washed for 10 minutes, three times in total. A chemiluminescence imager was used to scan and record the PVDF film soaked in the chemiluminescence chromogenic solution.

Immunofluorescence Staining

Immunofluorescence (IF) was used to detect the level of type II collagen, Aggrecan, and cleaved caspase3. A coverslip was firstly placed into the well of a 24-well plate. Around 10^5 NPCs were seeded in this well, cultured in 1-mL growth medium at 37 °C and 5% CO_2. When reaching 80% confluence, the NPCs were treated using TBHP, AT-Exo or both according to the experimental grouping mentioned above. Next, the coverslip-loaded NPCs were fixed with 4% paraformaldehyde for 15 minutes, washed with PBS three times, 5 minutes each time. Two mL of 0.5% Triton X-100 was used to penetrate the cells for 10 minutes, followed by washing in PBS for three times, 3 minutes each time. Immunostaining blocking solution (Beyotime, P0102, China) was used to block non-specific binding sites of the antibodies for 30 minutes at room temperature. Next, the NPCs were incubated with rabbit cleaved caspase-3 (1:400), Aggrecan (1:400), and type II collagen (1:400) overnight at 4 °C in the dark. After primary antibody incubation, the coverslip was soaked in PBS three times, 5 minutes each time. Next, fluorescent secondary antibody (1:500, Abways, AB0141, US) and Phalloidin (1:1000, Abcam, ab76757, US) were used to incubate the NPCs at room temperature in dark for 1 hour. The coverslip was then soaked in PBS three times, 5 minutes each time. DAPI (1:5000) was then added dropwise to stain cell nuclei, incubated in dark for 5 minutes. Excessive DAPI was washed away with PBS for 5 minutes, three times in total. An absorbent paper was used to clear the residual liquid on the coverslip, which was then sealed with mounting medium containing anti-fluorescence quencher. Imaging was performed under a fluorescence microscope (Leica, Germany, DM2500). The IF images were analyzed using ImageJ software (Java 1.8.0, US).

Reactive Oxygen Species Detection

Reactive oxygen species (ROS) was detected to assess the level of oxidative stress during TBHP-induced apoptosis of rat NPCs. A ROS detection kit (1:1000, Beyotime, S0033s, China) was used. This assay was performed according to the directions for use provided by the manufacturer. The results were observed and imaged using a fluorescence microscope (Leica, Germany, DM2500).

Statistical Analysis

Statistical analysis was conducted with GraphPad Prism 8.0 (GraphPad Software Inc, US). Data were presented as mean ± standard deviation (SD). Data analysis was determined by one-way analysis of variance (ANOVA) followed by Tukey's tests. A p-value of less than 0.05 was considered statistically significant.

Results

Extraction and Characterization of Rat AT-Exo Exosomes

Western blot showed that the levels of Alix, SDCBP, CD63, and TSG101 in AT-Exo were higher than those in AT, indicating that exosomes were indeed enriched in exosome samples (Figure 1A). Calnexin, a negative exosome marker, was almost not expressed in exosomes, while it was highly expressed in AT, indicating that exosome samples were not contaminated by other membrane structures. NTA showed that the diameter of AT-Exo was about 100nm, and the concentration was 3.4 ×10^9 particles/mL (Figure 1B). TEM showed that the extracted exosomes had obvious hemi-spherical and saucer-type typical exosome structures, and the diameter of exosomes was between 100nm-200nm (Figure 1C).

Isolation and Characterization of Rat NPCs

The rat NPCs were successfully isolated and cultured. The NPCs cultured in vitro showed a short spindle shape at the initial stage of adhesion, and the cytoplasm raised outward and then gradually extended. As shown in Figure 2A, when cells reached 80% confluence, the NPCs changed to a long spindle shape with the increase of the number of passages, and the growth became slower and the time for confluence was longer than an early stage.

The CD73, CD90, CD105, CD31, and CD45 were used to characterize the primarily cultured NPCs. As shown in Figure 2B, the positive markers CD73, CD90 and CD105 accounted for 88.8%, 80.7%, and 93.5%, respectively, while the negative markers CD31 and CD45 accounted for 0.09% and 0.63%, respectively.

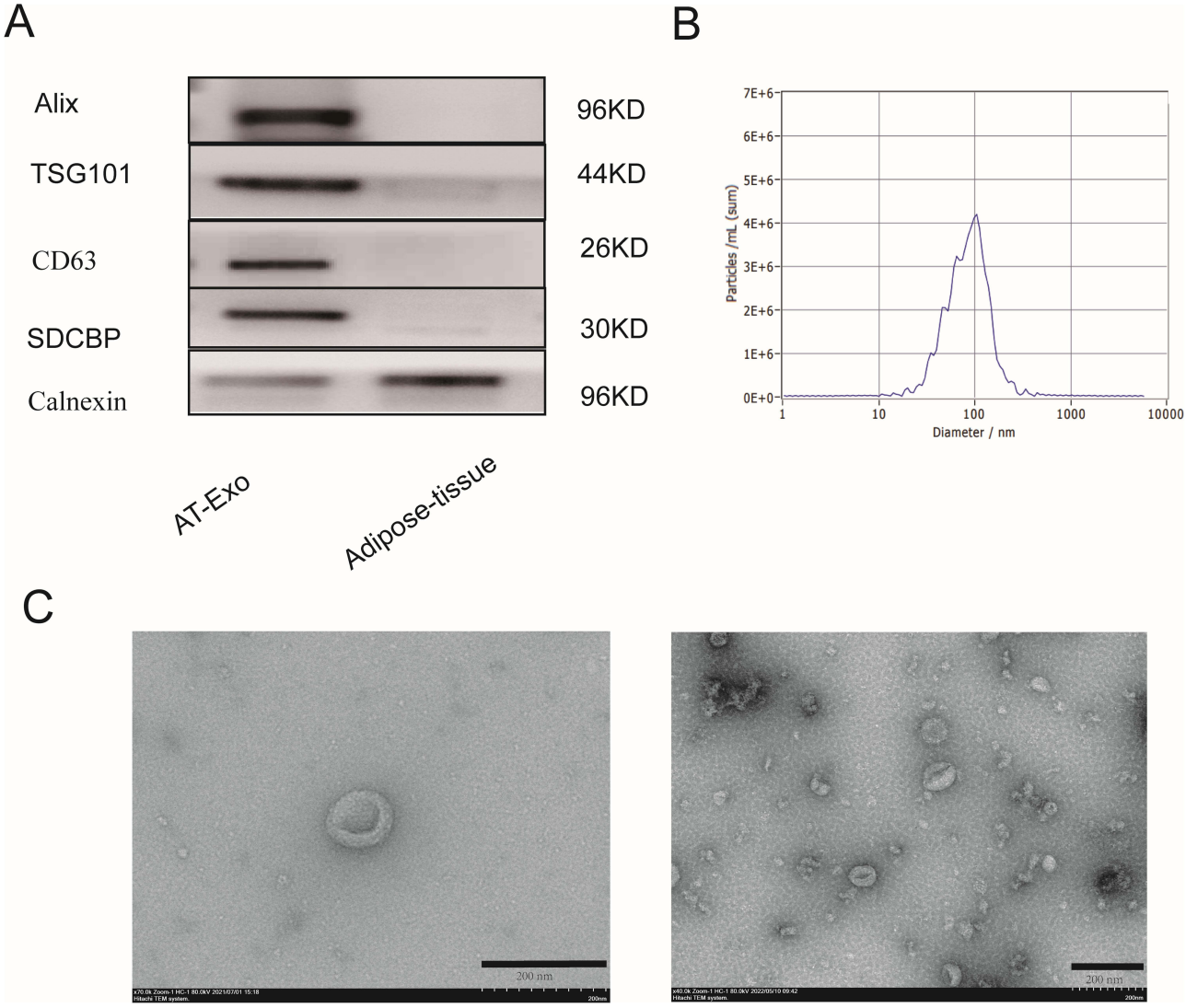

Figure 1 Characterization of adipose tissue-derived exosomes. (**A**) Western blot was used to detect Alix, SDCBP, CD63, TSG101 and Calnexin to identify AT-Exo surface markers. (**B**) Nanoparticle tracking analysis identified AT-Exo with a particle size of 100nm. (**C**) AT-Exo was observed to have a typical exosome structure by transmission electron microscope.
Abbreviations: AT-Exo, adipose tissue-derived exosome.

AT-Exo Can Be Taken Up by NPCs

As shown in Figure 3A, the efficiency of rat NPCs in taking up AT-Exo varied at different time points (2h, 4h, 8h, 12h, 24h). As shown in Figure 3B, the fluorescence staining showed that the cell membrane of NPCs appeared green, while red fluorescence-labeled AT-Exo appeared in the cytoplasm of rat NPCs, indicating that rat NPCs can take up AT-Exo. As shown in Figure 3C, the uptake of AT-Exo by rat NPCs reached a maximum after 4 hours of administration.

CCK-8 Assay Determined the Optimal Concentration and Duration of TBHP and AT-Exo

It was found by CCK-8 assay that TBHP decreased the cell survival in a dose- and time-dependent manner. To achieve a 50% survival rate, the concentration of TBHP was determined to be 60 μM and the time was 4 hours (Figure 4A). The cell survival reached the highest when the exosome concentration was 150 μg/mL at time point of 4 hours (Figure 4B).

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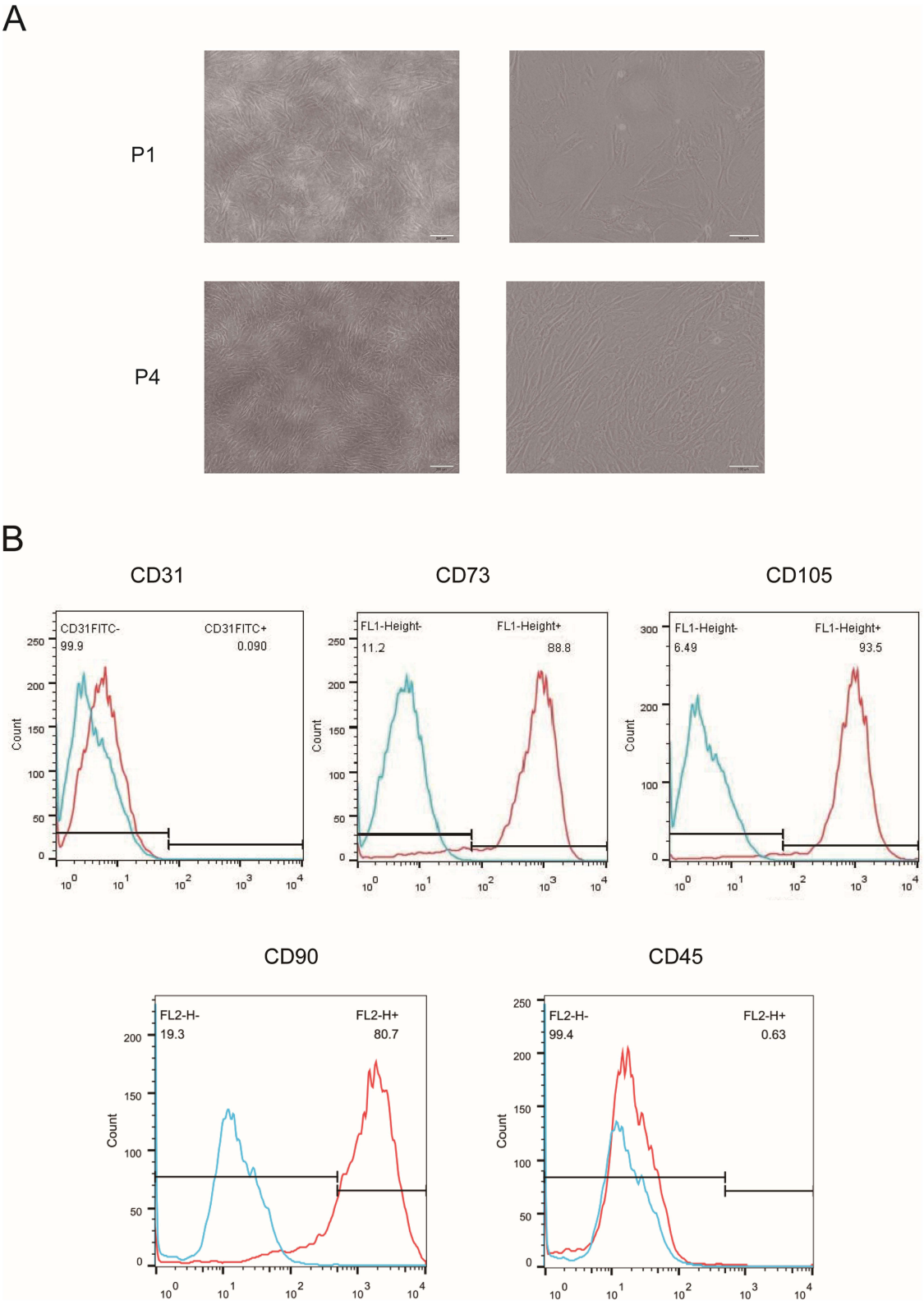

Figure 2 The morphology of nucleus pulposus cells and flow cytometry. (**A**) The morphological changes of NPCs at passage 1 (P1) and passage 4 (P4) were observed under a microscope. The NPCs cultured in vitro showed a short spindle type at the initial stage, and with the increase of passages, the NPCs gradually showed a long spindle shape. The left column scale bar=200μm. The right column scale bar=100μm. (**B**) Flow cytometry was used to detect the surface markers of rat NPCs.
Abbreviation: NPCs, nucleus pulposus cells.

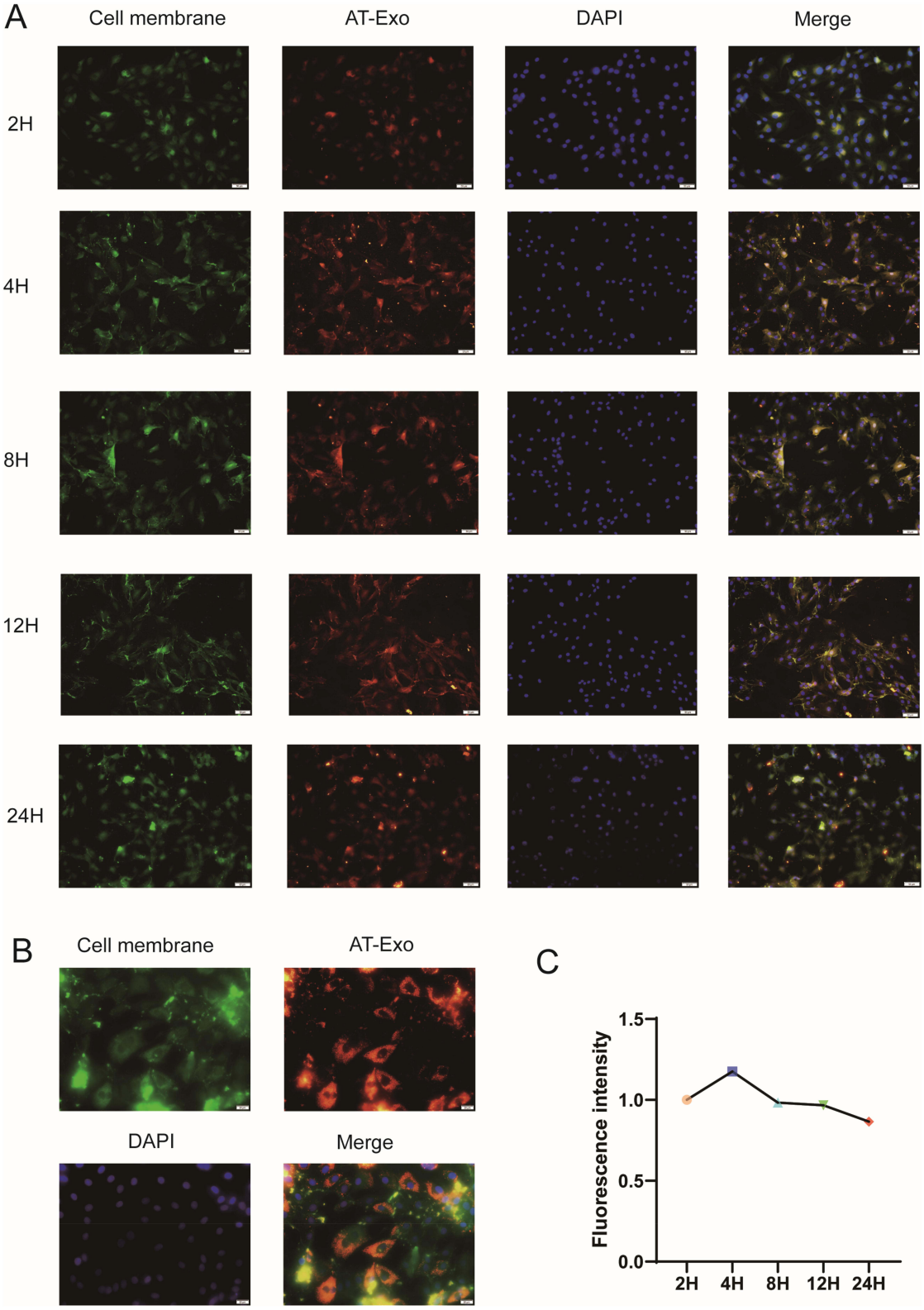

Figure 3 Adipose tissue-derived exosomes can be taken up by nucleus pulposus cells. (**A**) After AT-Exo was co-incubated with NPCs for 2 hours, 4 hours, 8 hours, 12 hours, and 24 hours, AT-Exo can be uptaken by NPCs. Scale bar = 50μm. (**B**) The uptake of AT-Exo by NPCs was observed under magnification. AT-Exo labeled with red fluorescence appeared in the cytoplasm of NPCs. Scale bar = 20μm. (**C**) The uptakes of AT-Exo by NPCs were recorded at different time points.
Abbreviations: AT-Exo, adipose tissue-derived exosome; NPCs, nucleus pulposus cells.

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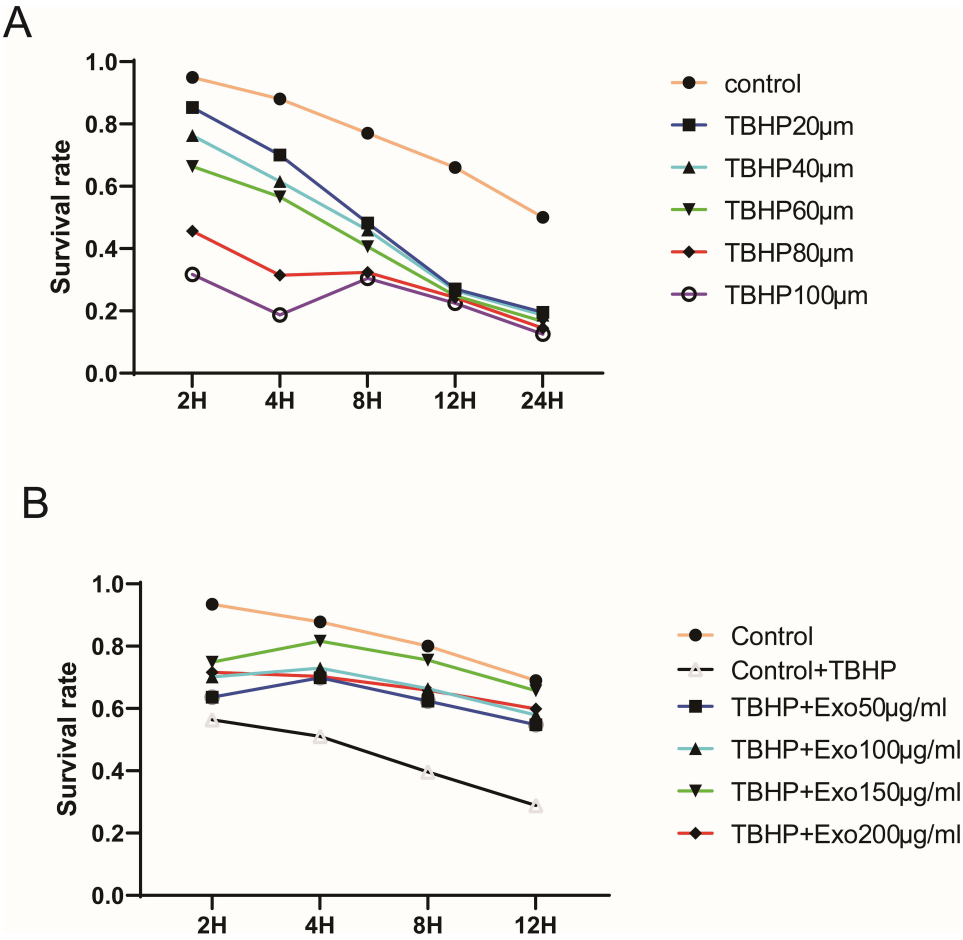

Figure 4 CCK8 was performed to determine the concentration and duration of tert-butyl hydroperoxide and adipose tissue-derived exosome. (**A**) CCK8 was used to determine the concentration and duration of TBHP on NPCs. (**B**) CCK8 assay was used to determine the concentration and duration of AT-Exo acting on TBHP-stimulated NPCs.

Abbreviations: AT-Exo, adipose tissue-derived exosome; NPCs, nucleus pulposus cells; TBHP, tert-butyl hydroperoxide.

Western Blotting

To investigate the effect of AT-Exo on the extracellular matrix of NPCs, Western blotting was used to detect the protein levels of Aggrecan and type II collagen. As shown in Figures 5A and B, the levels of Aggrecan and type II collagen in the TBHP group were significantly lower than those in the Control group ($P < 0.05$). After AT-Exo was administered, the levels of Aggrecan and type II collagen in the TBHP+AT-Exo group were significantly higher than those in the TBHP group. These results indicate that AT-Exo can promote the deposition of extracellular matrix proteins in degenerative NPCs.

Western blotting showed that AT-Exo down-regulated the expression of MMP3 and MMP13 in rat NPCs. As shown in Figure 5C and D, the levels of MMP3 and MMP13 in the TBHP group were significantly higher than that in the Control group ($P < 0.05$). After administration of exosomes, the levels of MMP3 and MMP13 in the TBHP+AT-Exo group were significantly lower than that in the TBHP group ($P < 0.05$). In the TBHP+AT-Exo group, the level of MMP3 was higher than that in the Control group ($P < 0.05$), while the level of MMP13 was lower than that in the Control group without significance. These results suggest that AT-Exo can down-regulate the expression of matrix metalloproteinases in degenerative NPCs, thereby reducing degradation of extracellular matrix proteins.

To investigate the effect of AT-Exo on apoptotic proteins in degenerative NPCs, cleaved caspase3, Bcl2 and Bax were determined by Western blotting. As shown in Figure 5E, Western blotting indicated that the level of cleaved caspase3 in the TBHP group was significantly higher than that in the Control group ($P < 0.05$), while it was significantly lower than the TBHP group ($P < 0.05$) after administration of AT-Exo. It is known that the ratio of Bcl2/Bax determines cell

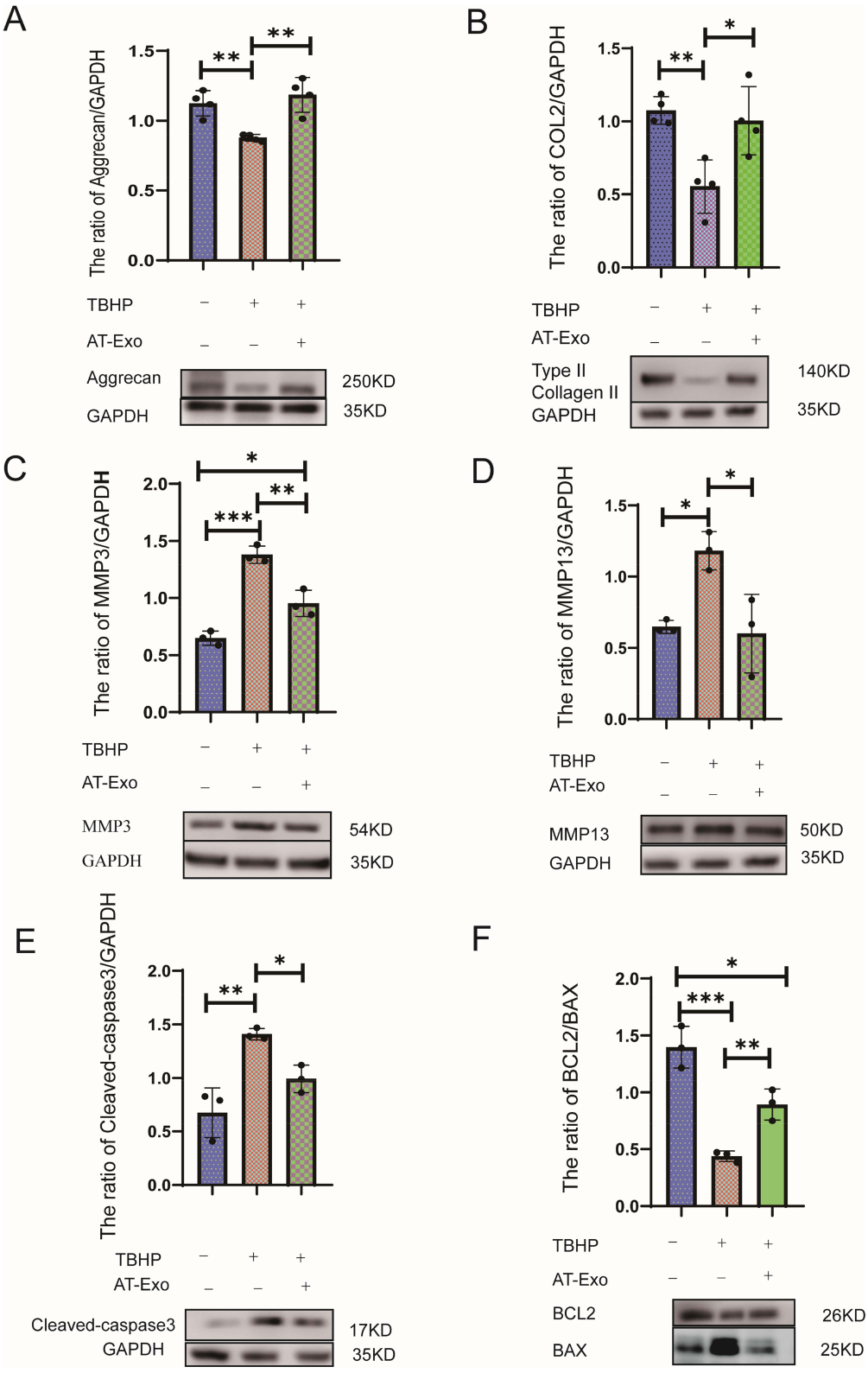

Figure 5 Western blot was conducted to detect the effect of adipose tissue-derived exosomes on nucleus pulposus cells. (**A** and **B**) AT-Exo promoted the levels of Aggrecan and type II collagen in extracellular matrix of TBHP-stimulated NPCs. (**C** and **D**) AT-Exo reduced the levels of MMP3 and MMP13 in TBHP-stimulated NPCs. (**E** and **F**) AT-Exo can down-regulate the levels of cleaved caspase3 and Bax and upregulate the level of Bcl2 in TBHP-stimulated NPCs. *P <0.05, **P <0.01, ***P <0.001.
Abbreviations: AT-Exo, adipose tissue-derived exosome; NPCs, nucleus pulposus cells; TBHP, tert-butyl hydroperoxide.

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apoptosis level. As shown in Figure 5F, the ratio of Bcl2/Bax in NPCs following TBHP administration was significantly lower compared with the Control group ($P < 0.05$), while it was significantly higher compared with the TBHP group after AT-Exo administration ($P < 0.05$). These results suggest that AT-Exo can down-regulate the expression of apoptotic proteins in degenerative NPCs and inhibit the apoptosis of rat NPCs.

Immunofluorescence Staining

As shown in Figure 6A, the fluorescence intensity of Aggrecan in NPCs after being stimulated by TBHP was significantly lower compared to the Control group ($P < 0.05$). After administration of AT-Exo, the fluorescence intensity of Aggrecan (TBHP+AT-Exo group) was significantly increased and higher compared with the TBHP group ($P < 0.05$), although still a bit lower compared with the Control group.

As shown in Figure 6B, the fluorescence intensity of type II collagen in the TBHP group was slightly but significantly lower compared with the Control group ($P < 0.05$). The level of type II collagen in the TBHP+AT-Exo group was significantly higher than that in the TBHP group ($P < 0.05$).

As shown in Figure 6C, the fluorescence intensity of cleaved caspase3 in the NPCs after being stimulated by TBHP was significantly higher compared to the Control group ($P < 0.05$). After administration of AT-Exo, the level of cleaved caspase3 in the TBHP+AT-Exo group significantly decreased ($P < 0.05$), although still slightly higher than that in the Control group ($P < 0.05$).

Reactive Oxygen Species Detection

To detect the intracellular oxidative stress level, ROS detection reagent was used. As shown in Figure 7, after adding TBHP to rat NPCs, the intracellular ROS level significantly increased. After administration of AT-Exo, the intracellular ROS level greatly decreased. These results indicate that AT-Exo can effectively scavenge the ROS induced by TBHP.

Discussion

Intervertebral disc degeneration is the main cause of lower back pain.[1] With increasing age, the water content of the NP tissue in the intervertebral disc gradually decreases, resulting in a decrease in the height and elasticity of the disc, which ultimately leads to lower back pain.[8] The decrease in water content of the NP is mainly caused by abnormal stress in the intervertebral disc, release of inflammatory factors, oxidative stress, and nutritional deficiencies that result in senescence and apoptosis of the NPCs, which in turn forms a vicious circle that accelerates the metabolic disorders of the NPCs, resulting in a decrease in the extracellular matrix and loss of the water-containing ability of the NP tissue, ultimately triggering intervertebral disc degeneration.[9] Studies have shown that biomaterial therapy, growth factor therapy, stem cell therapy, immunotherapy and exosome therapy can protect the damaged NPCs and delay intervertebral disc degeneration.[10–14]

Exosome treatment for disc degeneration as an emerging biological treatment is widely noticed by spine surgeons.[15] Almost all cells in the body are capable of secreting exosomes, which are found in a wide range of natural body fluids such as blood, saliva, urine, and breast milk. Exosomes contain a variety of substances such as DNA, RNA, proteins, and lipids. After being secreted by cells, exosomes are able to reach other tissues through the circulatory pathway, go through the paracrine pathway or act directly on neighboring cells. Exosomes can deliver substances from the exosomes to the cells through various pathways such as phagocytosis or fusion of the membrane structure of the exosomes with the cell membrane, thus influencing the physiological functions of the cells.[16,17]

Recent studies have shown that exosomes secreted by bone marrow mesenchymal stem cells, adipose mesenchymal stem cells, and other types of stem cells can play a positive role in promoting osteogenesis, treating intervertebral disc degeneration, osteoarthritis, and other diseases.[2,3,5,18] Compared with stem cells in treating disc degeneration, exosomes in treating disc degeneration have the advantages of smaller immune rejection and easier preservation. However, the process of extracting exosomes from cells takes longer and is more expensive, and fewer exosomes are extracted.[19] In contrast, AT, as a widespread loose connective tissue, consists of a variety of cells, one-third of which are mature adipocytes developed from pluripotent mesenchymal stem cells, and the rest of which includes cells such as fibroblasts, endothelial cells, macrophages, and preadipocytes.[20] AT is not only a tissue used for energy storage, but also a tissue

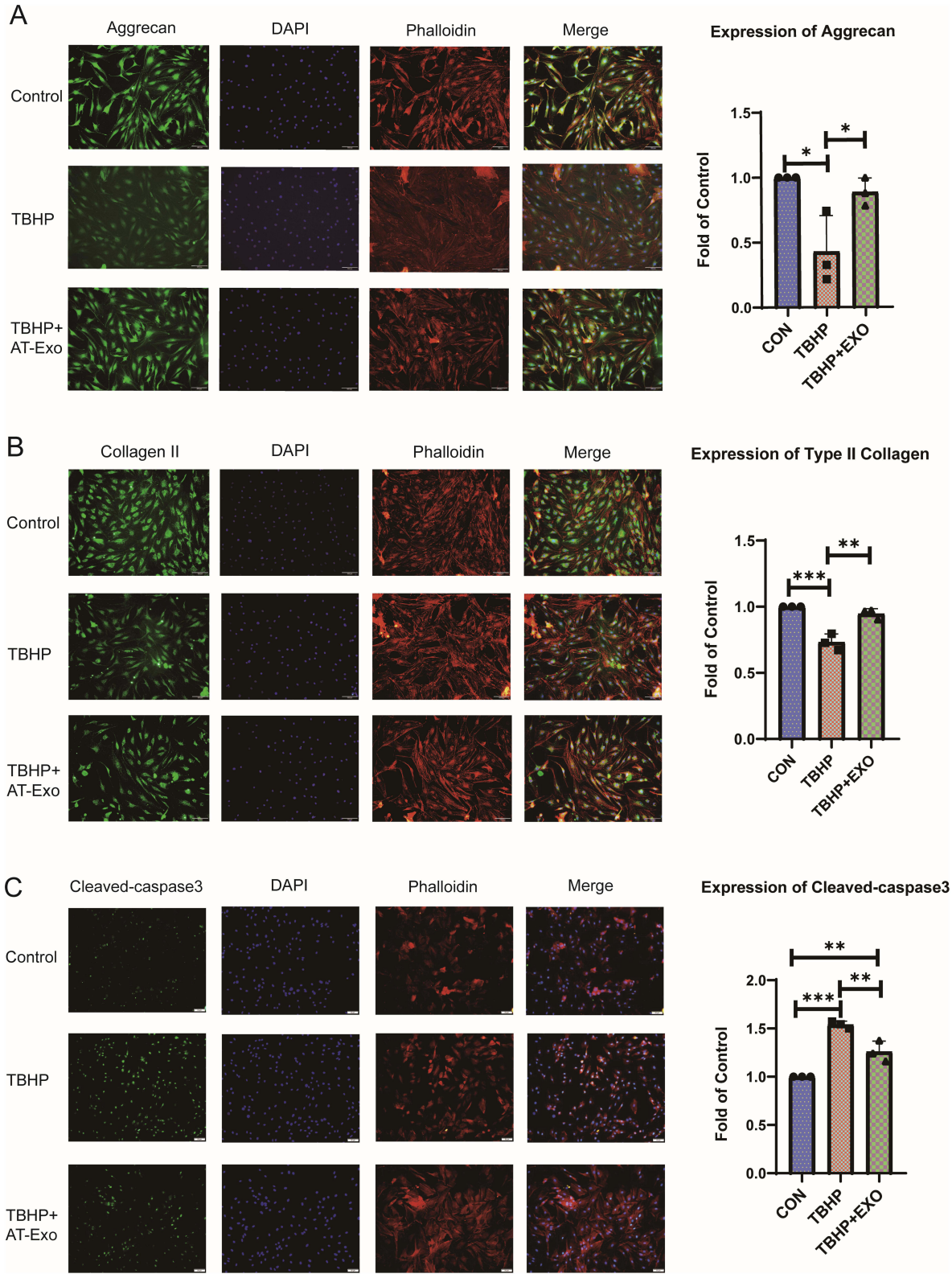

Figure 6 Immunofluorescence staining. (**A**) IF showed that AT-Exo can promote the expression of Aggrecan in NPCs. Scale bar = 100μm. (**B**) IF showed that AT-Exo can promote the expression of type II collagen in NPCs. Scale bar = 100μm. (**C**) IF showed that AT-Exo can downregulate the level of cleaved caspase3 in NPCs. Scale bar = 50μm. *P <0.05, **P <0.01, ***P <0.001.

Abbreviations: IF, immunofluorescence staining; AT-Exo, adipose tissue-derived exosome; NPCs, nucleus pulposus cells; TBHP, tert-butyl hydroperoxide.

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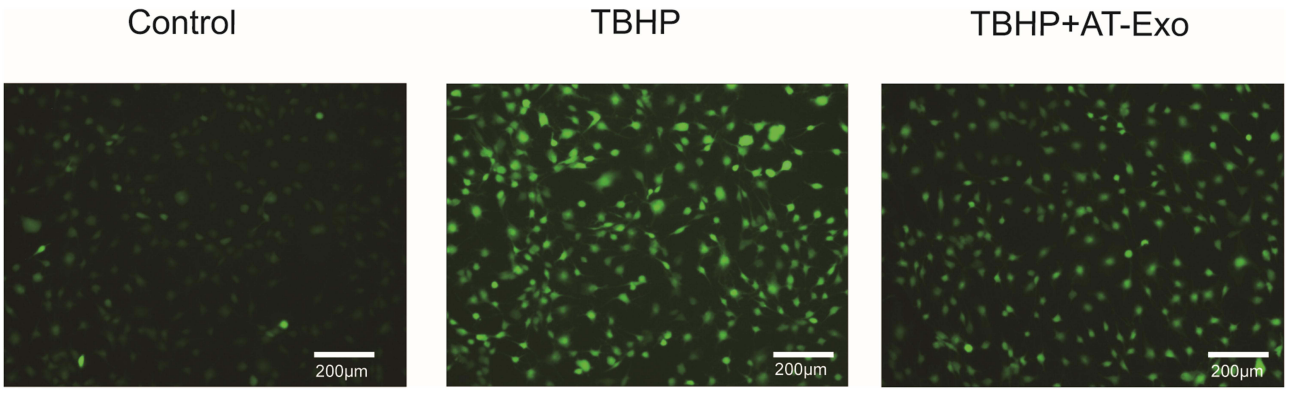

Figure 7 Reactive oxygen species assay was used to detect the intracellular oxidative stress level. After administration of TBHP, the intracellular ROS level substantially increased while it significantly decreased following the addition of AT-Exo.
Abbreviations: AT-Exo, adipose tissue-derived exosome; ROS, reactive oxygen species; TBHP, tert-butyl hydroperoxide.

capable of performing multiple functions. The basis for this multifunctionality of AT lies in the ability of adipocytes to secrete a large number of hormones, growth factors, enzymes, cytokines, complement factors, and matrix proteins, which are collectively known as adipokines or adipocytokines. The exosomes secreted by the various cells in adipose tissue are able to act on surrounding cells or enter the bloodstream to play a role in other tissues, producing different physiological effects. Researchers have isolated exosomes from AT, and the isolated exosomes contain a variety of adipokines, which have played an active role in the treatment of metabolic diseases.[6,21–23] Adipocytes are the most important cell type in adipose tissue. In tissues, there are also non-adipocyte components mainly including preadipocytes, adipose-derived stem cells, endothelial cells, pericytes and various immune cells. In this study, AT-Exo were extracted from adipose tissue and thus, most of AT-Exo were derived from adipocytes. The characterization of AT-Exo using WB, NTA, and TEM showed that the rat AT-Exo extracted in our study conformed to the basic characteristics of exosomes.[17]

Degeneration of the intervertebral disc is mainly manifested by the reduction of extracellular matrix in the NP tissue. The reduction of extracellular matrix in the NP tissue leads to disc degeneration. In the current study, when NPCs were stimulated with TBHP, the contents of type II collagen and Aggrecan in the extracellular matrix decreased. However, when exosomes were administered, the levels of type II collagen and Aggrecan were increased, indicating that AT-Exo can alleviate intervertebral disc degeneration by promoting the expression of extracellular matrix proteins. Matrix metalloproteinase is a collagen hydrolase identified in 1962 that causes degradation of the extracellular matrix.[24] In our study, when TBHP was applied to NPCs, Western blotting showed an increase in the levels of MMP3 and MMP13, which belong to the matrix metalloproteinase family. When exosomes were administered, the levels of MMP3 and MMP13 were significantly decreased, further suggesting that exosomes can inhibit disc degeneration by down-regulating matrix metalloproteinase levels and reducing extracellular matrix degradation. Collectively, AT-Exo promoted the expression of type II collagen and Aggrecan and decreased the expression of MMP3/13, suggesting that AT-Exo can protect NPCs by maintaining the metabolic balance of extracellular matrix.

Bax, Bcl2, and cleaved caspase3, as important genes regulating apoptosis, play important roles in the apoptotic process of NPCs. While Bax and Bcl2 are a pair of regulatory genes that are currently agreed to function independently of each other during apoptosis. Bcl2 can exert anti-apoptotic effects from the surface of organelles such as mitochondria and endoplasmic reticulum.[25] In contrast, Bax promotes apoptosis by disrupting the integrity of the mitochondrial membrane.[26] When TBHP stimulates NPCs, the Bcl2/Bax ratio decreases, at which time the Bax/Bax dimer content rises, initiating apoptosis. When the Bcl2/Bax ratio increased after supplementation of exosomes, the Bax/Bax dimer dissociated from Bcl2 to generate a more stable Bcl2-Bax dimer, which inhibited apoptosis. Caspase-3 is the most critical apoptosis execution protein in the apoptotic process, and promotes apoptosis by affecting DNA replication, transcription, and damage repair. Caspase-3 occupies a central position in apoptosis and is known as the "death execution protein".[27,28] In our study, the level of Caspase-3 in NP cells was significantly decreased following administration of AT-Exo, indicating the anti-apoptotic effect of the exosomes.

Some studies have shown that Bax and Bcl2 can act as the upstream regulatory mechanism of Caspase-3, and Caspase-3 can also act on Bcl2 to regulate apoptosis.[29,30] Thus, the relationship between Bax, Bcl2 and Caspase-3 in the apoptotic process is complex and needs to be further studied. The impact of AT-Exo on the extracellular matrix metabolism of rat NPCs involves multiple molecular mechanisms.[19,31] Previous study suggests that adipose-derived extracellular vesicles contain active STAT3, which can be transported to macrophages to induce polarization of macrophages toward the anti-inflammatory M2 phenotypes through transcriptional activation of arginase-1.[32] Melatonin in the exosomes extracted from adipose-derived stem cells, as a hormone secreted by the pineal gland with anti-inflammatory activity, can promote adipocytes to secrete exosomes containing α-ketoglutarate. After macrophages take up α - ketoglutarate in exosomes, it prompts macrophages to polarize towards the anti-inflammatory M2 phenotype, thus alleviating adipose inflammation in the obese state.[33] The PI3K-AKT-mTOR signaling pathway, as a widely studied signaling pathway, has been proven to play an important role in the process of intervertebral disc degeneration.[34,35] In subsequent research, we will conduct in-depth studies on the mechanism by which adipose-derived exosomes treat intervertebral disc degeneration, such as PI3K-AKT-mTOR signaling pathway. Meanwhile, an in vivo model will be used to further validate the effect of AT-Exo on intervertebral disc degeneration.

Bone marrow mesenchymal stem cell-derived exosomes and other cells-derived exosomes have been shown to mitigate disc degeneration through a variety of mechanisms.[2–5] In contrast, exosomes of adipose origin and exosomes of cellular origin in slowing disc degeneration have not been studied and compared in depth, which would be the main focus of our future research. However, applying AT-Exo to clinical treatment of intervertebral disc degeneration still faces many problems. The yield, cost, and preservation of exosomes all restrict their clinical application. With the development of science and technology, these issues are being gradually resolved. However, new problems have emerged. Whether exosome treatment for intervertebral disc degeneration should be administered intravenously or intradiscal remains to be investigated, although some studies have shown that injecting gel-loaded exosomes into the intervertebral disc can ameliorate intervertebral disc degeneration.[36,37]

There are some limitations in this study. To start with, the protective effect of AT-Exo on disc degeneration still needs to be validated by relevant animal models. Additionally, the specific molecular mechanism of AT-Exo for the treatment of disc degeneration remains to be further studied.

Conclusion

In summary, rat adipose tissue-derived exosomes can increase the levels of Aggrecan, type II collagen and Bcl2, and decrease the levels of MMP3, MMP13, cleaved caspase3, and Bax. Thus, the rat adipose tissue-derived exosomes can maintain metabolic balance of extracellular matrix and protect rat nucleus pulposus cells against apoptosis.

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Disclosure

The authors declare no conflicts of interest in this work.

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