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Mesenchymal stem cell-derived exosomes are beneficial to suppressing inflammation and promoting autophagy in intervertebral disc degeneration

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Background: *Intervertebral disc degenerative diseases is one the main causes of lumbago, and its main pathological mechanism is intervertebral disc degeneration (IDD). As shown in previous reports, mesenchymal stem cell (MSC)-exosomes can slow down or even reverse degenerated nucleus pulposus (NP) cells in IDD. Thus, we attempted to clarify the specific role of MSC-exosomes underlying IDD progression.*

Materials and methods: *In the present study, the harvested particles were identified as MSC-exosomes. MSC-exosomes facilitated activation of autophagy pathway in AGE-treated NP cells. MSC-exosomes repressed inflammatory response in AGE-treated NP cells. Autophagy pathway activation enhanced inflammatory response in AGE-stimulated NP cells.*

Results: *Mesenchymal stem cell-exosomes facilitated autophagy pathway activation and repressed inflammation in IDD rats. Autophagy inhibition exerted a protective role against inflammatory response in IDD rats.*

Conclusions: *In conclusion, MSC-exosomes represses inflammation via activating autophagy pathway, which provides a potential novel insight for seeking therapeutic plans of IDD.* (Folia Morphol 2024; 83, 1: 102–112)

Keywords: intervertebral disc degeneration, mesenchymal stem cell, exosomes, autophagy, inflammation

INTRODUCTION

Lumbago is the most common spinal surgery symptom with high morbidity and disability rate, seriously affecting human health and quality of life and also bringing a heavy burden to individuals, families and the whole society [2]. One of the main causes of lumbago is intervertebral disc degenerative diseases, of which main pathological mechanism is intervertebral disc degeneration (IDD). Since most patients with early IDD have no obvious symptoms, they also miss the opportunity for early intervention and therapy [21, 34]. Currently, treatment methods for diseases caused by IDD include conservative therapy and surgery. Both of them can

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relieve clinical symptoms to a certain extent, but the long-term effect is often unfavourable [33]. Thus, it is of great clinical significance to figure out molecular biology to prevent or even reverse IDD with less damage to patients.

The intervertebral disc (IVD) is the largest avascular structure in the human body [9]. The changes in the structure of IVD are caused by a combination of factors such as cellular aging, nutritional disorders, stress factors, inflammatory mediators, reduction of nucleus pulposus cells, abnormal metabolism of extracellular matrix (ECM), etc. [10]. To address IDD and related degenerative diseases, multiple studies have focused on maintaining the amount of nucleus pulposus (NP) cells [11, 25]. Thus, bone marrow mesenchymal stem cells (BMSCs), which have the advantages of easy access, multi-directional differentiation, low immunogenicity, and mature acquisition and identification, have been widely researched [14]. The inflammatory response mediated by pro-inflammatory cytokines can directly cause the loss of water in IVD and exacerbate IDD process [31]; mesenchymal stem cells (MSCs) are capable of migrating to damaged tissues and releasing cytokines, inflammatory regulators, extracellular matrix components, and antimicrobial proteins in this area to create a suitable microenvironment for tissue repair [23]. With the deepening of research, it has been demonstrated that biological information can be transmitted between non-contact cells through paracrine or in the form of exosomes [35]. MSC-exosomes can upregulate autophagy marker proteins LC3 and Beclin-1 and facilitate autophagosome formation [40]. Rat MSCs can produce positive trophic effects on degenerated NP cells by secreting exosomes, and NP degeneration degree can still be improved after non-contact co-culture of BMSCs and NP cells [28]. Moreover, MSC-exosomes are capable of attenuating NP cell apoptosis induced by proinflammatory cytokines [17]. These reports indicate that MSC-exosomes can slow down or even reverse degenerated NP cells in IDD.

Thus, we hypothesized that MSC-exosomes delayed or even reversed IDD via autophagy activation and inflammation inhibition. Herein, we clarified potential role of MSC-exosomes in IDD by establishing animal models and AGE-treated cell models, which may provide a novel insight for targeted therapy of IDD.

MATERIALS AND METHODS

Isolation and identification of MSCs

Human bone marrow specimens were collected from healthy donors. The institutional review board of our hospital approved this research with informed consent signed by patients. MSCs from bone marrow were separated using density gradient centrifugation and adherence to tissue culture plastic. MSCs amplification was performed in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Invitrogen). MSCs were photographed through microscopy (Olympus, USA). For detecting cell surface markers, MSCs were characterized by CD73, CD90 and CD105 positive expression as well as CD34 and HLA-DR negative expression through flow cytometry (BD Biosciences, USA) under manufacturer's guidance. The surface antigens were provided by BD Biosciences.

Isolation and identification of exosomes

Mesenchymal stem cells were cultured in DMEM deprived of FBS for 2 days. Then the collected culture media were centrifuged at 500 g for 10 min, then 2000 g for 30 min to eliminate dead cells and debris, and finally 10000 g for 1 h to eliminate large vesicles. The supernatant containing cell-free culture media was transferred to a new tube without disturbing the pellet and the Total Exosome Isolation reagent (Invitrogen) was added, according to manufacturer's guidelines. After collection, morphology of isolated exosomes was observed through transmission electron microscopy (TEM) (Philips, NLD). The particles were characterized by exosomal markers including CD63, TSG101 and Alix via western blotting.

A cellular model of IDD

Human NP cells (cat no. CP-H097) provided by Procell (Wuhan, China) were cultured in DMEM containing 15% FBS and 1% penicillin-streptomycin. The culture medium was replaced twice every week and NP cells in the second or third passage were harvested for further use. NP cells under AGEs (200 μg/mL) stimulation were co-cultured with MSC-exosomes (cat no. CP-H166) at 10, 50, or 100 μg/mL for 24 h. The untreated NP cells were taken as negative controls. For rescue assays, AGE-stimulated NP cells co-cultured with MSC-exosomes were treated with 5 mM of 3-MA (Sigma-Aldrich), a specific autophagy inhibitor, for 24 h.

An animal model of IDD

The Animal Experimentation Committee of our hospital approved the animal experiments. A total of 24 three-month-old Sprague-Dawley rats were utilized in our research. After all rats were anaesthetized with 2% (w/v) pentobarbital (40 mg/kg), three IVDs (Co7/8, Co8/9 and Co9/10) in each rat were located by palpation on coccygeal vertebrae and validated using trial radiography. Moreover, three IVDs (Co7/8, Co8/9 and Co9/10) respectively received intradiscal injection of 2 μL of phosphate buffer saline (PBS), AGEs (200 μg/mL) or a mixture of AGEs (200 μg/mL) and exosomes (100 μg/mL) through a 33-gauge needle (Hamilton), termed Sham, Model or Model+exosomes group, with 6 rats in each group. The injections were performed every 2 weeks for 2 months. For rescue assays, IVDs in Model+exosomes group received intraperitoneal injection of 3-MA at 1.5 mg/100 g for 24 h.

MRI examination

Magnetic resonance imaging (MRI) was performed on all rats using a 7.0 T animal specific MRI system (Bruker Pharmascan, Germany). Pfirrmann classification was used to assess the degree of IVD degeneration [26]. The average score of punctured IVDs were calculated as degeneration grading of rats.

Histological analysis

After MRI examination, all rats were sacrificed through intraperitoneal administration with overdose pentobarbital sodium. The specimens were decalcified and fixed in formaldehyde, followed by dehydration, and were finally embedded in paraffin. The slides of each disc were stained with haematoxylin-eosin and observed through microscopy.

Immunofluorescence

Nucleus pulposus cells or tissues attached to slides were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. After washed using PBS, slides were blocked with 2% bovine serum albumin (BSA) in PBS at 37°C for 2 h, followed by incubation with primary antibody against LC3B (ab48394, Abcam, Shanghai, China). After washed twice, slides underwent subsequent treatment with secondary goat anti-rabbit antibody at 37°C for 2 h. Nuclei were co-stained by DAPI (Beyotime) at 0.1 g/mL for 5 min. Images were obtained through microscopy.

Western blot

Total proteins were isolated from NP cells or mouse tissues through a protein extraction kit (Beyotime) according to manufacturer's guidelines. The primary antibodies including CD63, TSG101, Alix, LC3B, Beclin-1, ATG5, interleukin (IL)-6, IL-1β, tumour necrosis factor alpha (TNF-α) and β-actin as well as horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Abcam. Protein bands visualization and detection through enhanced chemiluminescence.

Enzyme linked immunosorbent assay (ELISA)

The concentrations of proinflammatory cytokines were tested with corresponding ELISA kits (IL-6, IL-1β and TNF-α; ThermoFisher) under manufacturer's guidance.

Ethics approval and consent to participate

Animal procedures were approved by the First Affiliated Hospital of Hebei North University and this study was approved by the Ethics Committee of the First Affiliated Hospital of Hebei North University.

Statistical analysis

Data were expressed as the mean ± standard deviation of three independent assays. Statistical analysis was conducted using GraphPad Prism 7 software. Comparisons between two groups were assessed with Student's t-test and comparisons among multiple groups were assessed with one-way ANOVA followed by Tukey's post hoc test. A statistical significance was presented upon $p < 0.05$.

RESULTS

Identification of MSC and its derived exosomes

Mesenchymal stem cells, which have the advantages of easy access, multi-directional differentiation, low immunogenicity, and mature acquisition and identification, have been widely used in IDD-related reports [14]. Herein, human MSCs were harvested from bone marrow aspirates of donors. We observed characteristics of MSCs through microscopy. As a result, it appeared like a spindle with a 60–70% confluence (Fig. 1A). Flow cytometry demonstrated CD90, CD73 and CD105 positive expression as well as HLA-DR and CD34 negative expression in MSCs (Fig. 1B). Then, MSC-exosomes were isolated and purified from MSC culture medium and particle morphology was confirmed under TEM (Fig. 1C). Additionally,

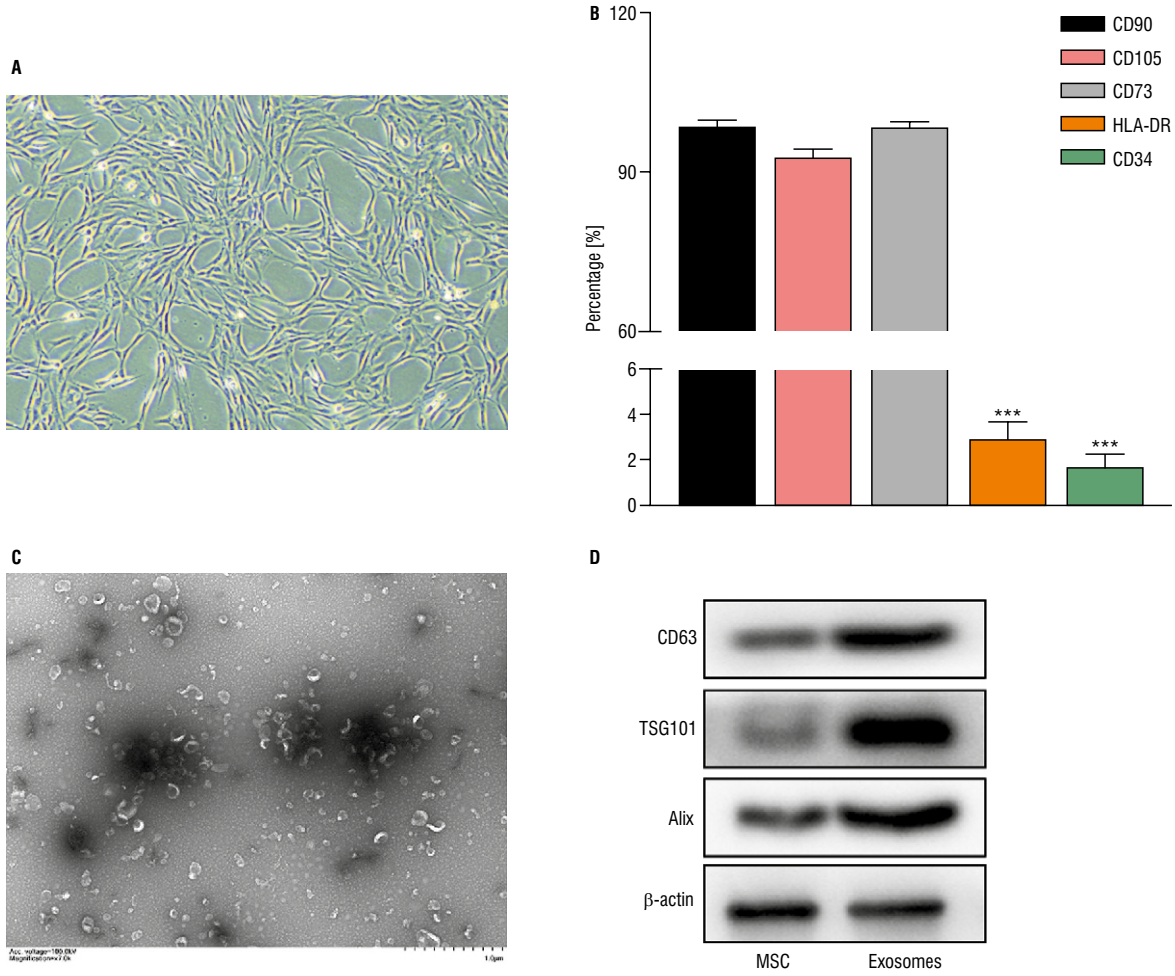

Figure 1. Characterization of mesenchymal stem cell (MSC) and its derived exosomes were identified; **A.** Characteristics of MSCs through microscopy; **B.** Characteristics of MSCs through flow cytometry; **C.** Characteristics of MSC-exosomes through transmission electron microscopy; **D.** Characteristics of MSC-exosomes through western blot; ***$p < 0.001$.

western blotting depicted that the level of exosomal marker proteins (CD63, TSG101 and Alix) presented a remarkable elevation in exosomes relative to MSCs (Fig. 1D). Collectively, these harvested particles were identified as MSC-exosomes.

MSC-exosomes facilitate autophagy in AGE-stimulated NP cells

Previously, AGEs accumulation exerted a pathogenic role in IDD [22]. Thus, we mimicked IDD characteristics in vitro through establishing a cellular model in NP cells under AGE treatment, termed AGEs group, and non-treated NP cells were taken as Control group. To clarify MSC-exosomes role in AGE-stimulated NP cells, AGEs group received additional exosomes treatment at different doses, termed Model+exosomes-10/50/100 group. Previously, MSC-exosomes upregulated autophagy marker proteins LC3 and Beclin-1 and facilitated autophagosome formation [40]. We attempted to clarify impact of MSC-exosomes on autophagy pathway in NP cells. Detecting autophagosomal marker LC3 through immunofluorescence is a reliable means of monitoring autophagy [32]. Thus, we measured fluorescent intensity of LC3 using immunofluorescence. As a result, LC3-labeled NP cells presented depletion in AGEs group and showed a dose-dependent elevation under co-treatment with MSC-exosomes (Fig. 2A). Additionally, western blotting demonstrated the downregulation of LC3B-II, Beclin-1 and ATG5 in AGEs group and this effect was reversed by MSC-exosomes along with increase of concentration of MSC-exosomes (Fig. 2B). Collectively, MSC-exosomes facilitate activation of autophagy pathway in AGE-treated NP cells.

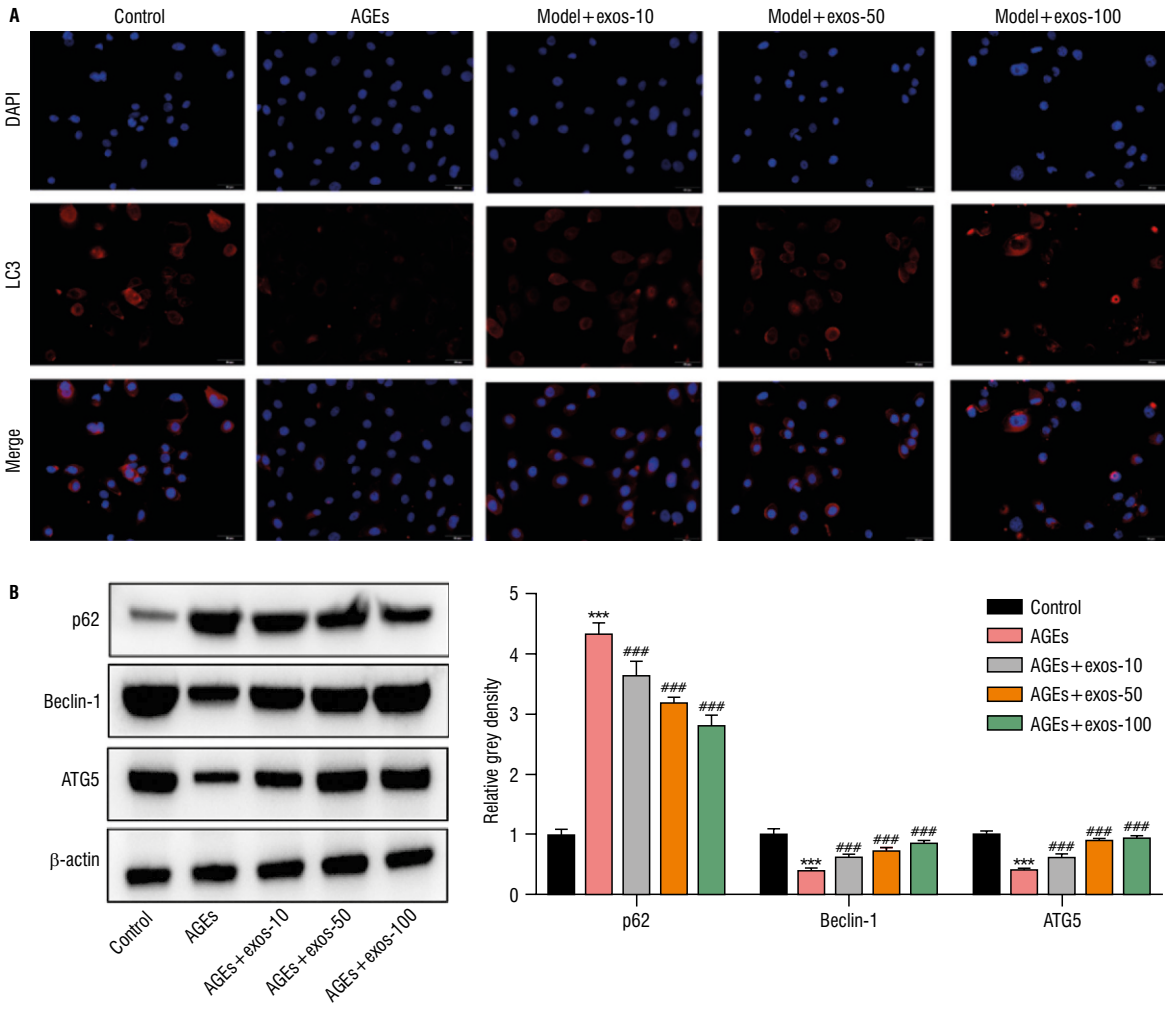

Figure 2. Mesenchymal stem cell-exosomes facilitated autophagy in AGE-stimulated nucleus pulposus (NP) cells; **A.** Immunofluorescence detected LC3 fluorescence in NP cells in Control/AGEs/Model+exos-10/Model+exos-50/Model+exos-100 group; **B.** Western blot detected levels of autophagy-related proteins in NP cells under different conditions; $^{***}p < 0.001$; $^{###}p < 0.001$.

MSC-exosomes suppress inflammation in AGE-stimulated NP cells

Mesenchymal stem cell-exosomes are capable of attenuating NP cell apoptosis induced by proinflammatory cytokines [17]. Thus, we attempted to clarify influence of MSC-exosomes on inflammatory response in NP cells. ELISA demonstrated that contents of proinflammatory cytokines (IL-6, IL-1β and TNF-α) presented elevation in AGEs group and showed a dose-dependent depletion under co-treatment with MSC-exosomes (Fig. 3A). Similarly, western blotting demonstrated the upregulation of IL-6, IL-1β and TNF-α in AGEs group and this effect was reversed by MSC-exosomes along with increase of concentration of MSC-exosomes (Fig. 3B). Collectively, MSC-exosomes repress inflammatory response in AGE-treated NP cells.

Inhibiting autophagy enhances inflammation in AGE-stimulated NP cells

We aimed to clarify whether autophagy pathway exerted a role in inflammatory response in NP cells. Thus, we performed rescue assays using 3-MA, a specific autophagy inhibitor [30], in cellular models under AGEs treatment. Immunofluorescence illustrated that LC3 fluorescence in AG-stimulated NP cells presented elevation under treatment with MSC-exosomes and showed depletion under co-treatment with 3-MA (Fig. 4A). Moreover, ELISA depicted that contents of IL-6, IL-1β and TNF-α in AG-stimulated NP cells presented depletion under treatment with MSC-exosomes and showed elevation under co-treatment with 3-MA (Fig. 4B). Western blotting showed a similar trend to ELISA results (Fig. 4C). Collectively, autophagy pathway activation enhances inflammatory response in AGE-stimulated NP cells.

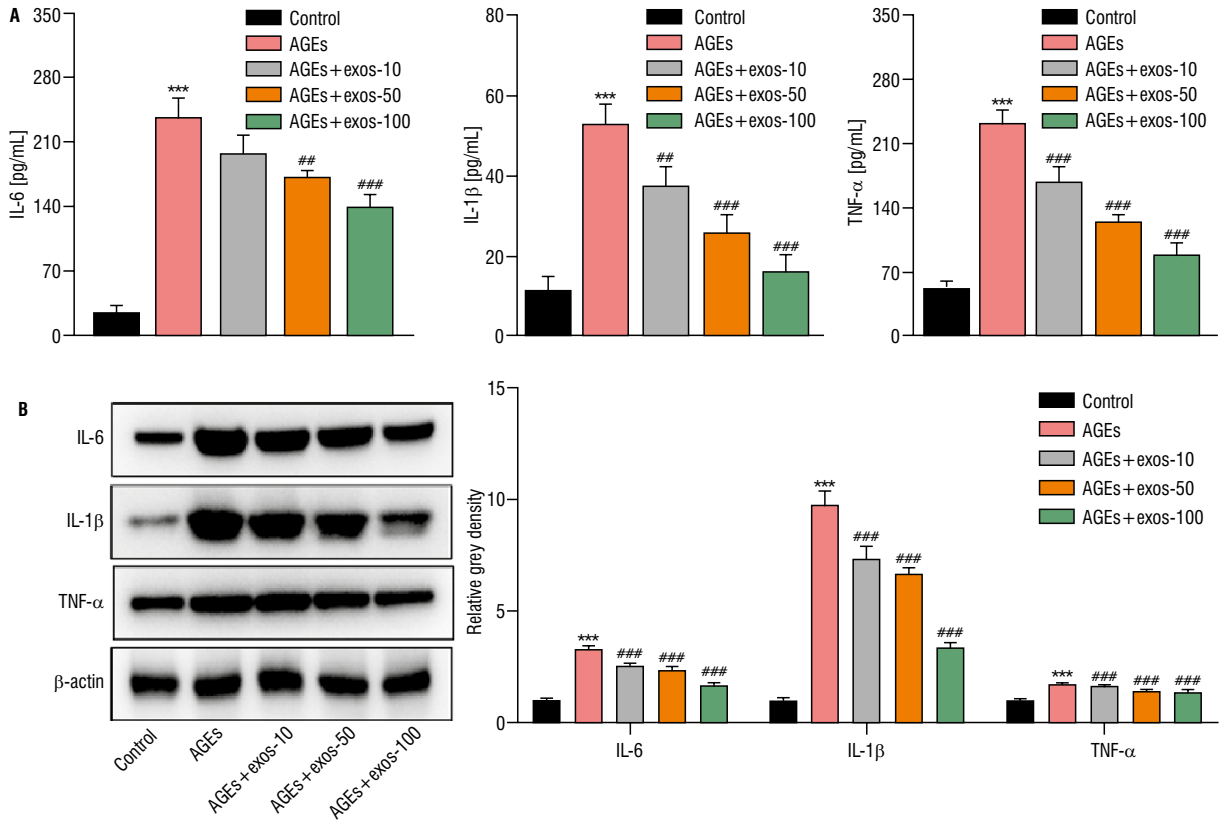

Figure 3. Mesenchymal stem cell-exosomes suppressed inflammation in AGE-stimulated nucleus pulposus (NP) cells; **A.** ELISA detected levels of proinflammatory cytokines in NP cells in Control/AGEs/Model+exos-10/Model+exos-50/Model+exos-100 group; **B.** Western blot detected levels of inflammation-related proteins in NP cells under different conditions; IL — interleukin; TNF-α — tumour necrosis factor alpha; ***$p < 0.001$; ###$p < 0.001$; ##$p < 0.05$.

MSC-exosomes induce autophagy and repress inflammation in a rat model of IDD

We attempted to further elucidate role of MSC-exosomes underlying IDD progression. Thus, we mimicked IDD characteristics in vivo through establishing IDD rat models. All rats were divided into Sham, Model or Model+exosomes group in a random manner. Three independent discs of each rat received intradiscal injection with PBS, AGEs, or AGEs+MSC-exosomes, respectively. We evaluated IDD degree through Pfirrmann MRI grading system [12]. As a result, Pfirrmann scores presented elevation in Model group and showed depletion under co-treatment with MSC-exosomes (Fig. 5A). Histology by haematoxylin-eosin staining indicated that MSC-exosomes reversed AGE-triggered IVD degradation during IDD (Fig. 5B). Moreover, immunofluorescence demonstrated that AGE-triggered depletion in LC3 fluorescence was rescued by MSC-exosomes (Fig. 5C). Western blotting also depicted that AGE-triggered depletion in LC3B-II, Beclin-1 and ATG5 protein abundances was rescued by MSC-exosomes (Fig. 5D), suggesting that autophagy pathway is suppressed in IDD rats while activated by MSC-exosomes. Furthermore, AGE-triggered increase in IL-6, IL-1β and TNF-α concentrations was reversed by MSC-exosomes (Fig. 5E). Western blotting showed a similar trend to ELISA results (Fig. 5F). Collectively, MSC-exosomes facilitates autophagy pathway activation and repress inflammation in IDD rats.

Autophagy inhibition exerts a protective role against inflammation in a rat model of IDD

We aimed to clarify whether autophagy pathway exerted a role in inflammatory response in IDD progression. Thus, we performed rescue assays using 3-MA in IDD rat models. MSC-exosomes-triggered depletion in Pfirrmann scores in IDD rats was rescued by 3-MA treatment (Fig. 6A). Histology by haematoxylin-eosin staining indicated that 3-MA countervailed the inhibitory impact of MSC-exosomes on IVD degradation during IDD (Fig. 6B). Moreover, immunofluorescence demonstrated that MSC-exosomes-triggered elevation in LC3 fluorescence was rescued by 3-MA (Fig. 6C). Furthermore, MSC-exosomes-triggered de-

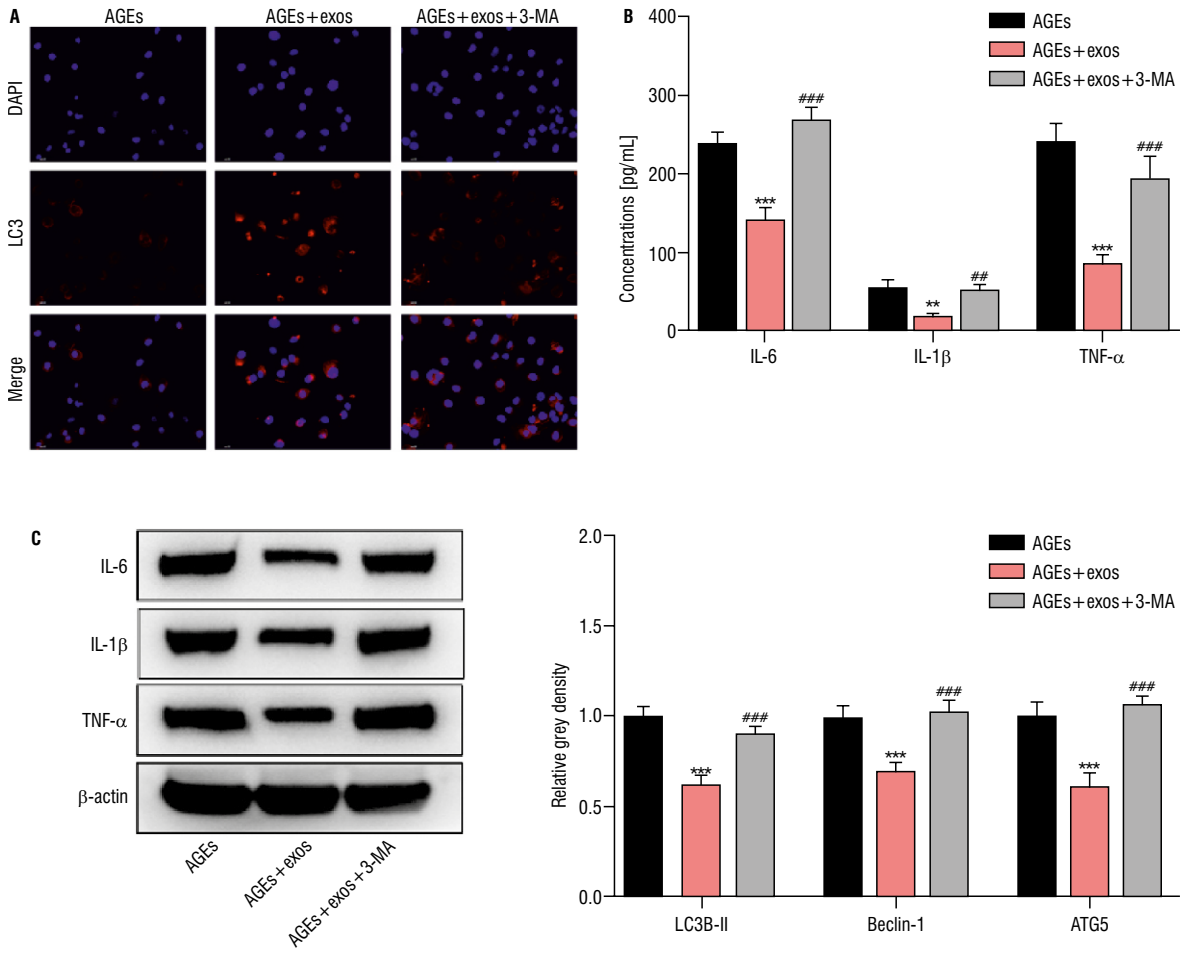

Figure 4. Inhibiting autophagy enhanced inflammation in AGE-stimulated nucleus pulposus (NP) cells; **A.** Immunofluorescence detected LC3 fluorescence in NP cells in AGEs/AGEs+exos/AGEs+exos+3-MA group; **B.** ELISA detected levels of proinflammatory cytokines in NP cells in NP cells under different conditions; **C.** Western blot detected levels of inflammation-related proteins in NP cells under different conditions; IL — interleukin; TNF-α — tumour necrosis factor alpha; ***$p < 0.001$; **$p < 0.05$; ###$p < 0.001$; ##$p < 0.05$.

crease in IL-6, IL-1β and TNF-α concentrations was reversed by MSC-exosomes (Fig. 6D). Western blotting showed a similar trend to ELISA results (Fig. 6E). Collectively, autophagy inhibition exerts a protective role against inflammatory response in IDD rats.

DISCUSSION

At the cellular level, IDD presents pathological characterization by decrease in NP cell amount and a metabolic imbalance of major products of the ECM (proteoglycan and collagen type II) [1, 3, 36]. Thus, finding a source of cells that can replenish degenerated NP cells can effectively improve IDD and simultaneously can achieve the purpose of early prevention and avoid further aggravation of IDD and serious consequences.

Autophagy is a crucial mechanism for cells to self-digest and recycle damaged components, especially under stressful conditions, and is a vital survival mechanism for cells under nutrient deprivation [8, 13]. Autophagy exerts a protective role in IVD cells [24]. Oxidative stress, pH, stress load, inflammatory cytokine stimulation, and hyperglycaemic hypertonic environment in IVD microenvironment can activate the autophagy activity of NP cells, while various extracellular environment stimuli, such as oxidative stress, can reduce ability of cells to proliferate [29, 37, 38]. Our research also validated that IDD degree can be remarkably improved through modulating autophagy level in NP cells. Biological information can be transmitted between cells that are not in contact with each other in the form of paracrine or exosomes, and MSCs can also treat IDD through secreting exosomes [41, 42]. MSC exosomes can alleviate endoplasmic reticulum stress-induced apoptosis by activating AKT and ERK signalling [19]. Herein, exosomes secreted

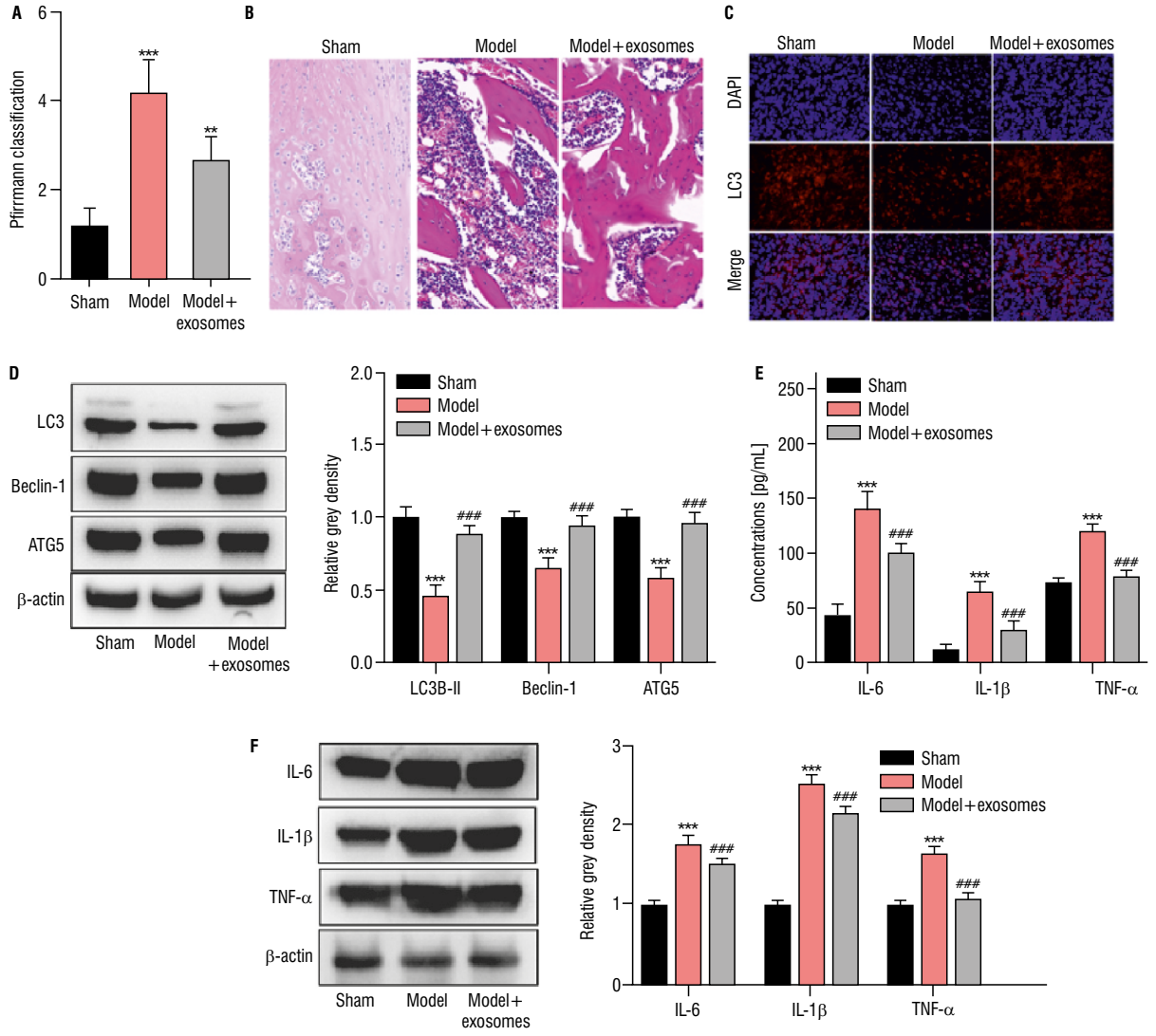

Figure 5. Mesenchymal stem cell-exosomes induced autophagy and repressed inflammation in a rat model of intervertebral disc degeneration; **A.** Pfirrmann magnetic resonance imaging grading system evaluated pathological changes in discs in Sham/Model/Model+exosomes group; **B.** Haematoxylin-eosin staining evaluated pathological changes in discs in each group; **C.** Immunofluorescence detected LC3 fluorescence in discs in each group; **D.** Western blot detected levels of autophagy-related proteins in discs in each group; **E.** ELISA detected levels of proinflammatory cytokines in discs in each group; **F.** Western blot detected levels of inflammation-related proteins in discs in each group; ***$p < 0.001$; **$p < 0.05$; ###$p < 0.001$.

by MSCs were successfully detected through TEM and immunoblot. By establishing a co-culture model, we discovered that in NP cellular model under AGEs stimulation, autophagy-related proteins in AGE-stimulated NP cells presented downregulation, while autophagy-related proteins in AGE-stimulated NP cells with MSC-exosome co-culture presented upregulation along with elevation of MSC-exosomes concentration, fully suggesting that MSCs enhances autophagy level in AGE-stimulated NP cells through MSC-secreted exosomes. Additionally, we established an IDD rat model and obtained similar results to a cellular model.

Inflammatory response mediated by pro-inflammatory cytokines can directly cause loss of water in IVD and exacerbate IDD process [20]. The enhanced "pro-inflammatory" role and insufficient "anti-inflammatory" role, that is, the imbalance of "inflammatory arm", may be a vital reason for aggravated inflammatory response in IDD [39]. Previously, pro-inflammatory cytokine IL-1 presented upregulation and its inhibitor IL-1a presented depletion in IDD [4]. Kritschil et al. [15] illustrated that insulin-like growth factor-1, which exerted an anti-inflammatory role, presented downregulation in IDD. Herein, in AGE-treated NP cellular model, pro-inflammatory

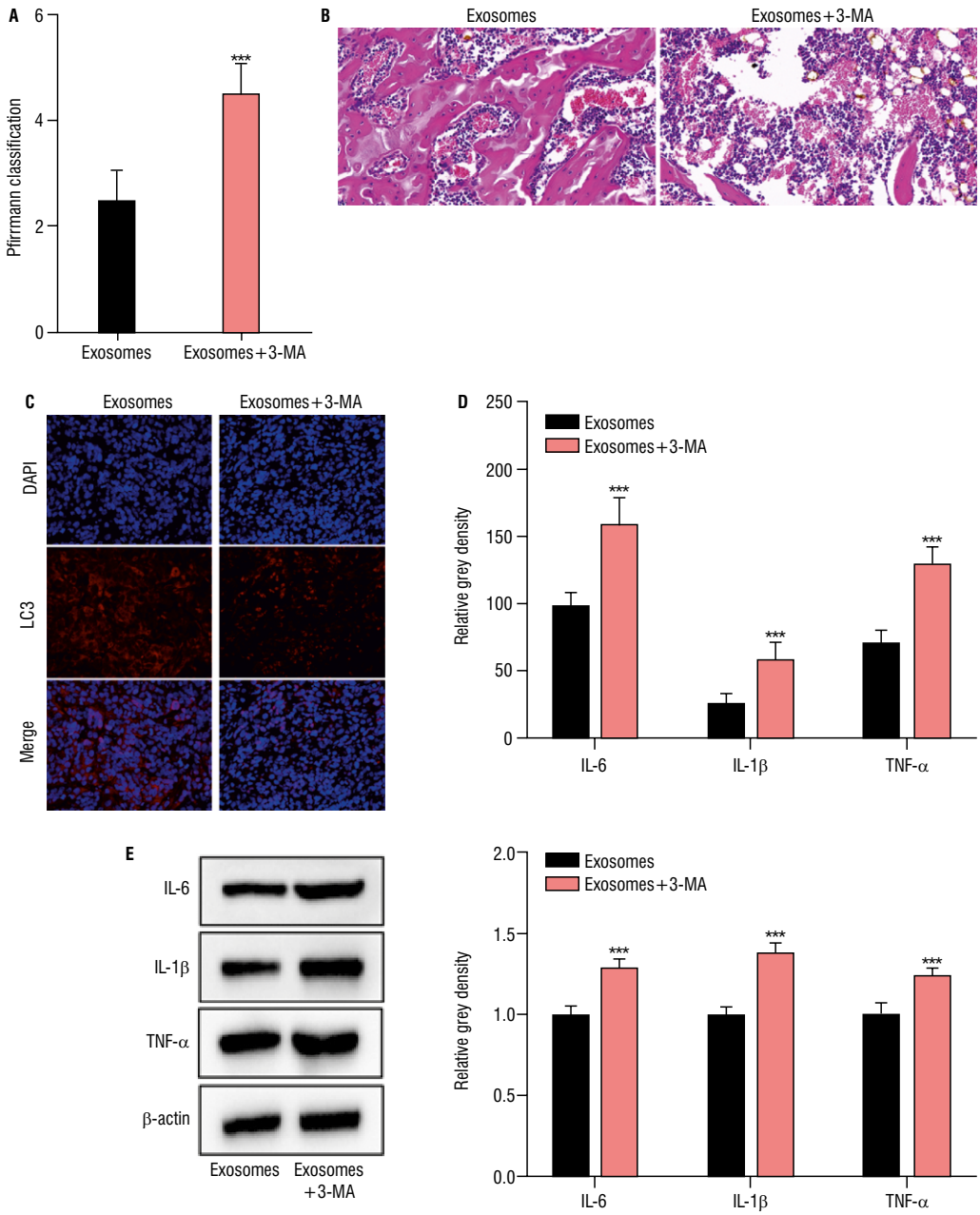

Figure 6. Autophagy inhibition exerted a protective role against inflammation in a rat model of intervertebral disc degeneration; **A.** Pfirrmann magnetic resonance imaging grading system evaluated pathological changes in discs in Exosomes/Exosomes+3-MA group; **B.** Haematoxylin--eosin staining evaluated pathological changes in discs in each group; **C.** Immunofluorescence detected LC3 fluorescence in discs in each group; **D.** ELISA detected levels of proinflammatory cytokines in discs in each group; **E.** Western blot detected levels of inflammation-related proteins in discs in each group; ***$p < 0.001$.

cytokines in AGEs-treated NP cells presented upregulation, while pro-inflammatory cytokines presented downregulation along with elevation of MSC-exosomes concentration, fully suggesting that MSCs repress inflammatory response of NP cells through MSC-secreted exosomes. Additionally, we established an IDD rat model and obtained similar results to a cellular model.

Autophagy is a widespread self-protection mechanism in eukaryotic cells [18]. Recent research has revealed that there is a close connection between autophagy and inflammation [7, 16]. Though inflammatory responses innately protect the body from infection or tissue damage, persistent or excessive inflammatory responses can result in irreversible damage to the body [5]. It has been demonstrated that

autophagy suppresses the inflammatory response possibly through directly repressing inflammatory complexes and indirectly through removing inflammatory stimuli such as damaged organelles or pathogenic microorganisms, thereby protecting cells from excessively persistent inflammation [6, 27]. Herein, in AGE-stimulated NP cellular model co-cultured with MSC-exosomes, 3-MA, an autophagy inhibitor, counteracted promoting influence of MSC exosomes on autophagy activation and reversed inhibitory impact of MSC-exosomes on inflammation, fully suggesting that MSC-exosomes suppresses AGE-induced NP cell inflammation via activating autophagy pathway. These results were also validated in animal models.

CONCLUSIONS

In conclusion, MSC-exosomes can improve degeneration of IDD via activating autophagy pathway to suppress inflammation, providing a potential novel insight for seeking therapeutic plans of IDD. However, due to complex and diverse components in exosomes, it remains elusive which component or cytokine in exosomes and which pathway exerts the main role, which will be addressed one by one in our future research.

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Conflict of interest: None declared.

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