MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2020; 26: e922928
DOI: 10.12659/MSM.922928

Received: 2020.01.17
Accepted: 2020.03.05
Available online: 2020.03.27
Published: 2020.05.21

Exosomes Derived from Bone Marrow Mesenchymal Stem Cells Prevent Acidic pH-Induced Damage in Human Nucleus Pulposus Cells

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: Departmental sources

Background: The exosomes (Exo) derived from mesenchymal stem cells (MSCs) are capable of attenuating the apoptosis of nucleus pulposus cells (NPCs) elicited by proinflammatory cytokines. However, it remains unknown whether MSC-derived Exo also exert a protective effect on NPCs in the pathological acid environment.

Material/Methods: NPCs were divided into 3 groups: Group A, pH 7.1–7.3; Group B, pH 6.5–6.7 and Group C, pH 5.9–6.1. The NPCs were cultured in the above-defined acidic medium, and 3 different amounts of Exo were added into the media. Finally, the expression of the caspase-3, aggrecan, collagen II, and MMP-13 was analyzed and compared among the different groups.

Results: Compared with cells cultured at pH 7.1–7.3 (Group A), proliferation activity of NPCs cultured at pH 5.9–6.7 (Group B and C) decreased significantly. Collagen II and aggrecan expression was also obviously reduced with the decrease of cell proliferation.

Conversely, the expression of caspase-3 and MMP-13 significantly increased. Further experiments showed that proliferation activity was significantly attenuated in NPCs cultured at pH 5.9–6.1 without Exo treatment (Group E) compared with those cultured at pH 7.1–7.3 without Exo treatment (Group D).

Conclusions: In the pathological acid environment, MSC-derived Exo promotes the expression of chondrocyte extracellular matrix, collagen II, and aggrecan, and reduces matrix degradation by downregulating matrix-degrading enzymes, protecting NPCs from acidic pH-induced apoptosis. This study reveals a promising strategy for treatment of IVD degeneration.

MeSH Keywords: Apoptosis • Exosomes • Intervertebral Disc Degeneration • Mesenchymal Stromal Cells

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/922928

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Background

Approximately 80% of adults experience low back pain (LBP) during a certain period of their life [1,2]. In the past, most studies of low back pain were focused on abnormal mechanical load and inflammatory cytokines. In recent years, the role of an acidic environment in intervertebral disc degeneration (IVDD) has increasingly received attention [3–5]. Exploring the role of an acidic environment in IVDD and how to ameliorate IVDD have become important research topics. A previous study showed that accumulation of lactic acid is responsible for decreased permeability of cartilage endplate (CEP), which in the long term can cause abnormal pH levels within the IVD [6]. Acidic pH not only has an adverse effect on the proliferation activity of nucleus pulposus cells (NPCs), but also upsets the metabolic balance of the extracellular matrix [7]. Stem cell transplantation has recently become a useful therapeutic strategy for IVDD because stem cells can differentiate into NPCs and reconstruct the functional disc structure [8–10]. Previous studies showed that acidic pH conditions can reduce the biosynthesis and proliferation of MSCs. Therefore, pH is an important factor limiting the use of MSCs for disc repair since the pH in a severely degenerated disc can drop to as low as pH 5.7, which severely affects gene expression, proliferation, and viability of MSCs [11–14]. Thus, the use of MSC transplantation in disc repair is theoretically limited.

In the past few years, there is growing evidence that MSCs can release some complex extracellular vesicles, called Exo, which provides a new therapeutic strategy for treatment of IVDD [15]. Exo is a membranous nano-sized vesicle, with a diameter about 30–120 nm. It contains complex cargoes, such as proteins, nucleic acids, lipids, and abundant miRNAs, and can carry the therapeutic cargo to the target cells [16]. It has also been proved that bone marrow mesenchymal cells-derived Exo (BMSC-Exo) can inhibit NPC apoptosis and extracellular matrix degradation induced by inflammatory factors [17,18], and has achieved good results in the treatment of many diseases, including cardiovascular disorders, traumatic osteonecrosis of the femoral head, traumatic spinal cord injury, and type I diabetes [19–21] Better than MSCs, Exo can still keep the bioactivity in an acidic environment [22]. Although it is known that BMSC-Exo can ameliorate the apoptosis of NPCs induced by inflammatory factors, it remains to be clarified whether BMSC-Exo can decrease NPC apoptosis and increase extracellular matrix biosynthesis in an abnormal acidic environment. In this study, we explored the protective effect of BMSC-derived Exo on NPCs in acidic pH.

Material and Methods

The human NPCs and BMSCs were brought from Science Cell Research Laboratories. To prove the adverse effect of an acid environment on NPCs and find the appropriate acidic environment that mimics the degenerated IVD, NPCs were cultured in 3 different pH media: Group A (Control, pH 7.1–7.3); Group B (pH 6.5–6.7); and Group C (pH 5.9–6.1). After the expression of caspase-3, MMP-13, aggrecan, and collagen II, as well as cell proliferation, were analyzed, we chose pH values of the medium that satisfied the following 2 conditions: (1) The proliferation of NPCs was obviously decreased, the expression of the above-mentioned proteins was markedly altered. (2) The acid level was the nearest to that in degenerated IVD [7,11–13,23]. NPCs were incubated in acidic medium with 7 different concentrations of BMSCs-Exo – 1, 5, 10, 15, 20, 25, and 30 μg/mL – and cell proliferation was assessed to find the appropriate BMSCs-Exo concentration. Finally, NPCs were cultured in these acidic media supplemented with appropriate concentration of BMSCs-Exo. The expression of caspase-3, MMP-13, aggrecan, and collagen II was detected by Western blot and qRT-PCR.

Cell culture

NPC culture medium is composed by 10 mL of FBS, 5 mL of penicillin/streptomycin solution (P/S), 5 mL of nucleus pulposus cell growth supplement, and 500 mL of basal medium. BMSC medium consists of 5 mL of FBS, 5 mL of P/S, and 500 mL of basal medium. All reagents were mixed to make the complete NPC medium and BMSC medium. Subsequently, NPCs and BMSCs were thawed in a 37°C water bath and then the cells were transferred as quickly and gently as possible to the T75 flask containing 10 mL of complete medium. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced the next day to remove unattached cells and DMSO. Finally, the medium was replaced every 3 days. NPC culture medium was replaced with acidic medium before the culture reached 80% confluence. After 36 h, the NPCs were collected for the following experiments.

Preparation of culture medium with different pH values

Culture media with 3 different pH levels – pH 7.1–7.3, pH 6.5–6.7, and pH 5.9–6.1 – were prepared by adding the appropriate amount of HCl (1 mM) and NaOH (1 mM), and the pH value of the culture medium was measured using a pH meter (MC, PHBJ-260F, Shanghai, China). The culture medium was placed at 37°C in 5% CO_2 for 2 days to reach equilibrium.

Exosome isolation and characterization

BMSCs-Exo isolation was carried out essentially as described previously [18]. Briefly, MSC culture medium was replaced with exosome-free medium before the culture reached 80% confluence. After 48 h, the cultured supernatant was collected for Exo isolation. The medium was centrifuged at 300 g for 10 min at 4°C to remove cells, at 2000 g for 20 min at 4°C to remove

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Table 1. Primer sequences used in qRT-PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
Collagen II	AGAACTGGTGGAGCAGCAAGA	AGCAGGCGTAGGAAGGTCAT
MMP-13	CCCAACCCTAAACATCCAA	AAACAGCTCCGCATCAACC
Caspase-3	TGGAATTGATGCGTGATGTT	GTCGGCATACTGTTTCAGCA
Aggrecan	ACCAGACTGTCAGATACCCC	CATAAAAGACCTCACCCTCC
GAPDH	GACCTGACCTGCCGTCTA	AGGAGTGGGTGTCGCTGT

some substructures, and at 10 000 g for 1 h at 4°C to remove apoptotic bodies and small vesicles. At each step, the supernatant was transferred to a new tube, then the supernatant was filtered through 0.22-μm filter to remove substructures and vesicles larger than 220 nm. Next, BMSC-Exo was separated by ultracentrifugation at 120 000 g for 4 h, and the pellets were rinsed with PBS and resuspended in PBS. The protein levels of the exosome preparations were determined using the BCA Protein Assay kit (Pierce) according to the manufacturer's instructions. The preparations were stored at –80°C or used directly for experimentation.

The number and size distribution of the isolated particles were analyzed using the Nanoparticle Tracking Analyzer PMX110 (Meerbusch, Germany). The particle morphology was observed using transmission electron microscopy (TEM). Five uL of the resuspended samples were added dropwise to 100-mesh grids and incubated at room temperature for 10 min, then the grids were negatively stained with 1% phosphotungstic acid for 1 min, and the remaining liquid was removed by filter paper. Finally, the grids were placed under a TEM operating at 80 Kv. Western blot analysis was performed to detect the expression of Exo markers (TSG101 and CD63).

Morphological observation

The human NPCs were grown in 6-well plates at a density of 1×10^5 cells per well. When the culture reached 80% confluence, cells were treated with the appropriate conditions, such as acidic medium or exosomes. After incubation for 36 h, the cells were observed by inverted phase-contrast microscopy (×10) (Olympus, Tokyo, Japan) and photographed with a digital camera (Nikon, Japan).

Quantitative real-time PCR (qRT-PCR)

The expression of caspase-3, aggrecan, collagen II, and MMP-13 in the different groups was detected by qRT-PCR. The total RNA was isolated from the sample by the RNAiso method and then used as a template to generate cDNA using the TaKaRa PrimeScript RT-PCR Kit. Primer sequences are shown in Table 1, and GAPDH served as the reference gene. The reaction system was 20 μL, containing 10 μL of SybrGreen qPCR Master Mix, 4 μL cDNA, 0.4 μL ROX, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), and 4.6 μL ddH_2O. Programs of qRT-PCR: 95°C for 30 s, then 40 cycles of 95°C for 5 s, and finally 60°C for 34 s. All PCRs were performed in triplicate. The relative number of transcripts was calculated using the $2^{-\Delta\Delta Ct}$ formula.

Cell Counting Kit 8 (CCK-8) assay

CCK-8 was used to assess NPC proliferation. The NPCs were seeded in 96-well plates at a density of 2×10^3 per well. After 36 h, 10 μL of CCK-8 reagent was added to each well and incubated for 4 h at 37°C away from light. A histogram of cell proliferation activity was created based on its OD value at 450 nm.

Protein analysis

NPCs and exosomes were lysed, and the protein concentration was measured by BCA. The lysates were centrifuged, then subjected to SDS-PAGE and transferred to PVDF membranes, and the membranes were blocked with 5% skim milk for 1 h at room temperature. After washing with 1xTBST buffer, the primary antibody was added and incubated at 4°C overnight. Then, the membrane was washed 3 times in 1×TBST buffer and the second antibody was added and incubated at room temperature for 2 h. The protein expression level was analyzed by digital gel imaging (Chemi Imager 4000, Alpha Innotech USA). Human serum exosomes (Umibio Co., Shanghai, China) served as the positive control protein (PC).

Statistical analysis

All experiments were independently repeated 3 times. The final data were presented as mean±SD. All statistical analyses were performed using Origin 9.1 or SPSS 20.0. GLM-repeated measures and LSD test were used to determine the statistical significance of results. A p value <0.05 was considered statistically significant.

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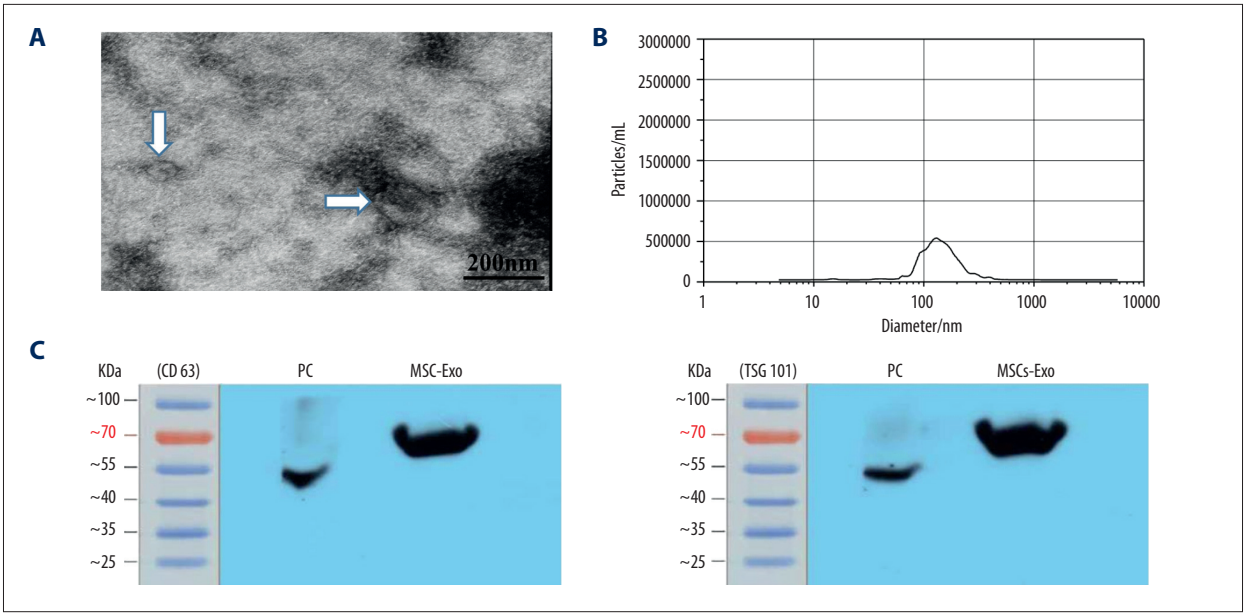

Figure 1. Characterization of BMSC-derived Exo. (**A**) Cup-shaped vesicles with a size of 124 nm and 78 nm were observed by TEM. (**B**) The Exo particles produced an absorption peak in the region of about 125 nm, slightly larger than the expected size of Exo. (**C**) Exo markers TSG101 and CD63 were identified by Western blot analysis.

Results

Isolation and characterization of Exo derived from BMSCs

The morphology of BMSC-derived Exo appeared as cup-shaped vesicles with a size of about 100 nm, as observed by transmission electron microscopy (Figure 1A). Particle size and distribution of the isolated Exo were analyzed by the Nanoparticle Tracking Analyzer and produced an absorption peak in the region of about 125 nm, slightly larger than the expected size of Exo (Figure 1B). The expression of the known exosomal markers TSG101 and CD63 was confirmed by Western blot analysis (Figure 1C). The above results identified these particles collected from MSC culture medium as BMSC-Exo.

Effect of the different pH values on extracellular matrix components and proliferation activity of NPCs

As observed by inverted phase-contrast microscopy, NPCs in Group A had triangular or short fusiform shape, with high proliferation capacity (Figure 2). Nuclear chromatin fragmentation, shrinkage, and the formation of cell vacuoles were observed in Group B and C (Figure 2) but not in Group A (Figure 2). Quantifications of cellularity in each group were provided: Group A (1.35×10^5), Group B (6.20×10^4), Group C (4.81×10^4); Group D (2.63×10^6), Group E (9.63×10^5), and Group F (1.24×10^6).

The results of Western blot analysis showed that the expression of cleaved caspase-3 and MMP-13 in Group B and C was significantly higher compared with group A (cleaved caspase-3: $p<0.05$ versus Group A, $p<0.05$ versus Group B; MMP-13: $p<0.05$ versus Group A, $p<0.01$ versus Group B). However, the expression of collagen II was significantly lower in Group C compared with B ($p<0.05$ versus Group B), and the expression of aggrecan was also significantly lower in Group B and C compared with Group A ($p<0.05$ versus Group A; $p<0.01$ versus Group B) (Figure 3). These findings suggest that collagen II and aggrecan expression are suppressed in an acid environment, whereas the expression of matrix-degrading enzyme MMP-13 is upregulated, accompanied by increased NPC apoptosis, as shown by increased cleaved caspase-3.

The proliferative activity of NPCs in Group B significantly decreased compared with Group A ($p<0.01$ versus Group A). Compared with Group B, the proliferative activity of cells further decreased in Group C ($p<0.01$ versus Group B) (Figure 4). Thus, the pH range of 5.9–6.1 was chosen as the appropriate acidic environment in the subsequent experiments.

Effect of the different amounts of Exo on the proliferative activity of NPCs

Next, we examined the effect of the different amounts of Exo on the proliferative activity of NPCs in pH 5.9–6.1 medium. As shown in Figure 5, the proliferative activity of NPCs was increased along with increasing amounts of Exo. The proliferation of NPCs was significantly increased at an amount of BMSC-Exo equivalent to 10 μg of BMSC-Exo protein ($p<0.01$ versus 0 μg BMSC-Exo protein), peaked at 20 μg of BMSC-Exo protein, and subsequently slightly reduced when an amount of

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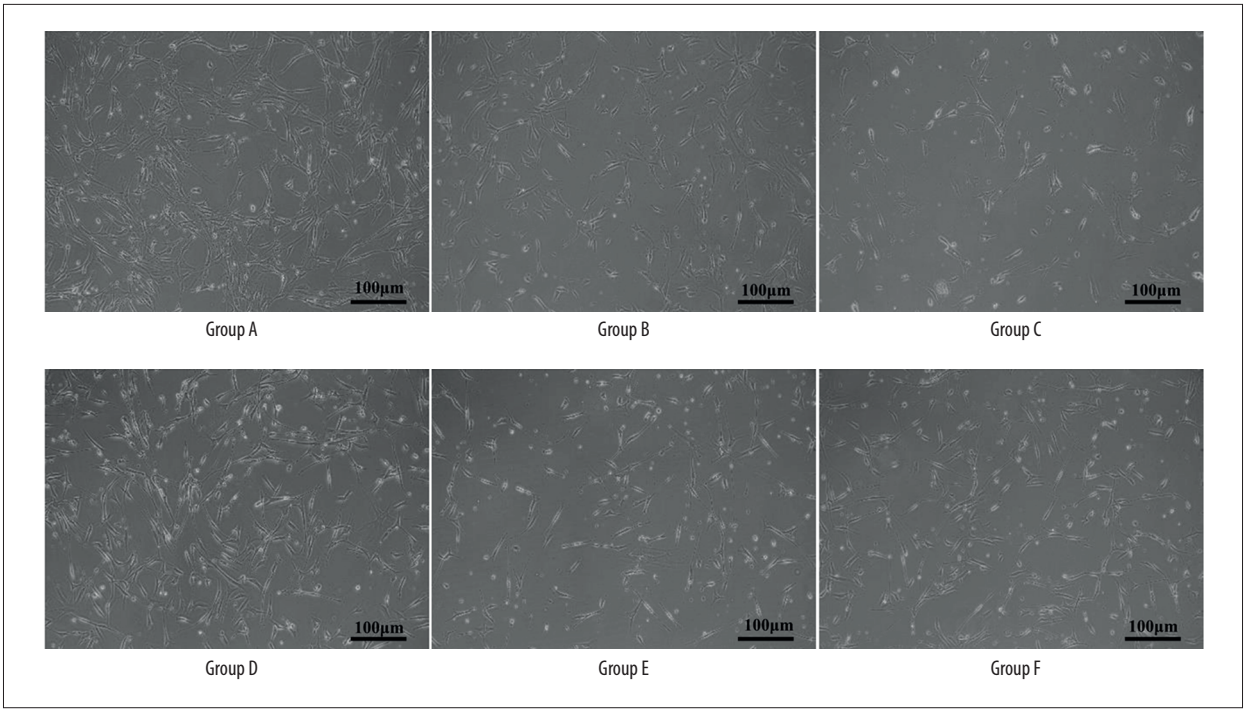

Figure 2. The cellular morphology of NPSS. NPCs in each group shaped as triangle or short fusiform, with high cell viability in Group A and D. The number of NPCs slightly decreased in Group B, and obviously decreased in Group C. The number and viability of NPCs were enhanced in the NPCs treated with BMSC-Exo (Group F).

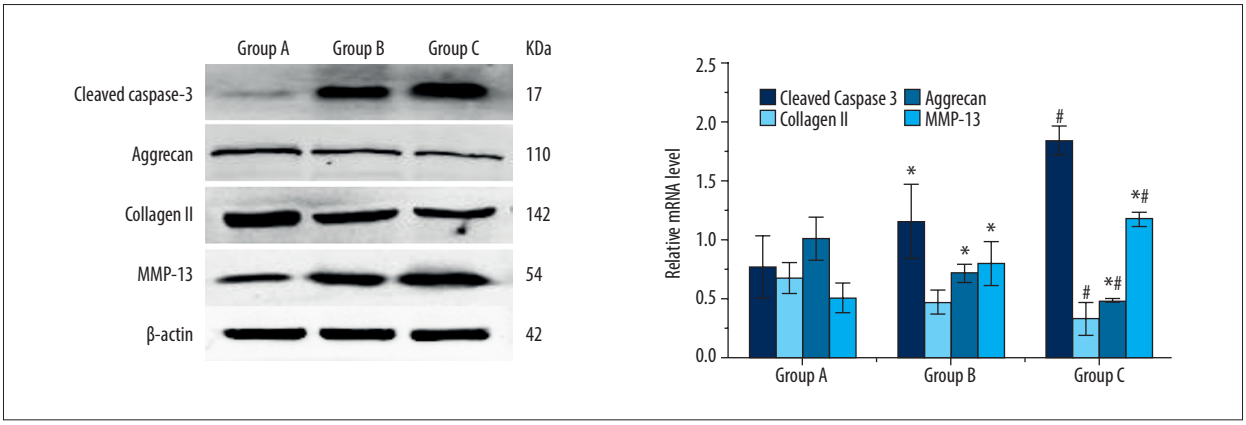

Figure 3. Effect of the different pH on expression of cleaved caspase-3, aggrecan, collagen II and MMP-13. The expression of cleaved caspase-3 and MMP-13 was significantly increased in Group B and C. The expression of collagen II and aggrecan was significantly decreased in Group B and C. Bars, mean values±SD of three independent experiments. * p<0.05 *vs.* Group A; # p<0.05 *vs.* Group B.

25 or 30 μg of BMSC-Exo protein was added compared with that of 20 μg protein. Thus, we used 20 μg of BMSC-Exo protein in the subsequent experiments.

Protective effect of Exo on NPCs in acidic environment

Consistent with the above-mentioned observations, the expression of cleaved caspase-3 and MMP-13 significantly increased, whereas collagen II and aggrecan expression significantly decreased in NPCs cultured at pH 5.9–6.1 (Group E) compared with those cultured at pH 7.1–7.3 medium (Group D) (Figure 6, p<0.05 versus Group D). The mRNA expression of caspase-3, aggrecan, collagen II, and MMP-13 showed a similar trend to that of the protein expression changes at pH 5.9-6.1 environment (caspase-3, collagen II, MMP-13: p<0.05 versus Group D; aggrecan: p<0.01 versus Group D), as evidenced by qRT-PCR (Figure 7). More importantly, when BMSC-derived Exo equivalent to 20 μg of BMSC-Exo protein was added into NPCs

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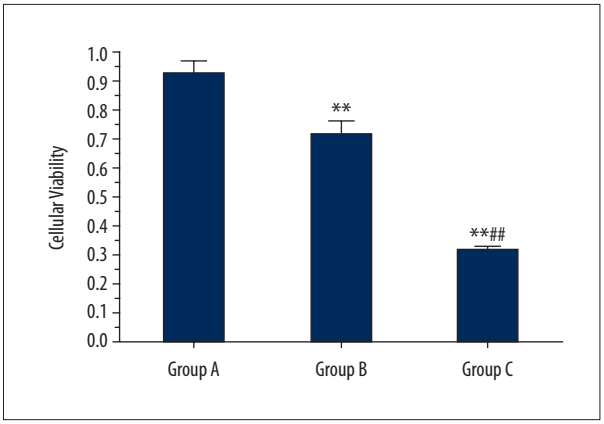

Figure 4. Effect of the different pH on proliferation activity of NPCs. Proliferation activity of NPCs was determined by Cell counting Kit 8. NPC proliferation was significantly decreased in Group B and C compared with Group A. Bars, mean values±SD of three independent experiments. ** p<0.01 *vs.* Group A; ## p<0.01 *vs.* Group B.

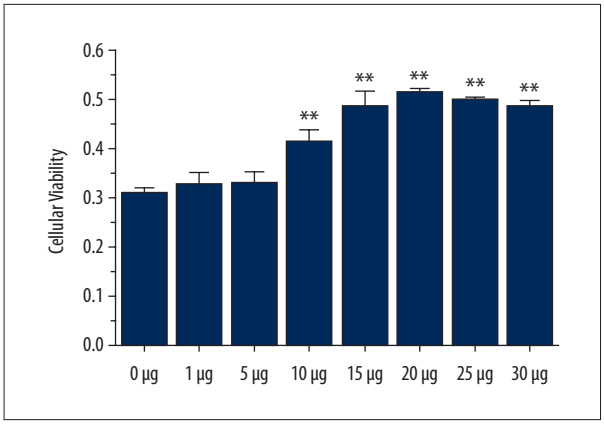

Figure 5. Effect of the different concentrations of Exo on proliferation activity of NPCs. Proliferation activity of NPCs was determined by Cell Counting Kit 8. BMSC-derived Exo promoted NPC proliferation in a concentration-dependent manner, and NPC proliferation activity reached the maximum at 20 μg BMSC-Exo. Bars, mean values±SD of three independent experiments. ** p<0.01 *vs.* 0 μg.

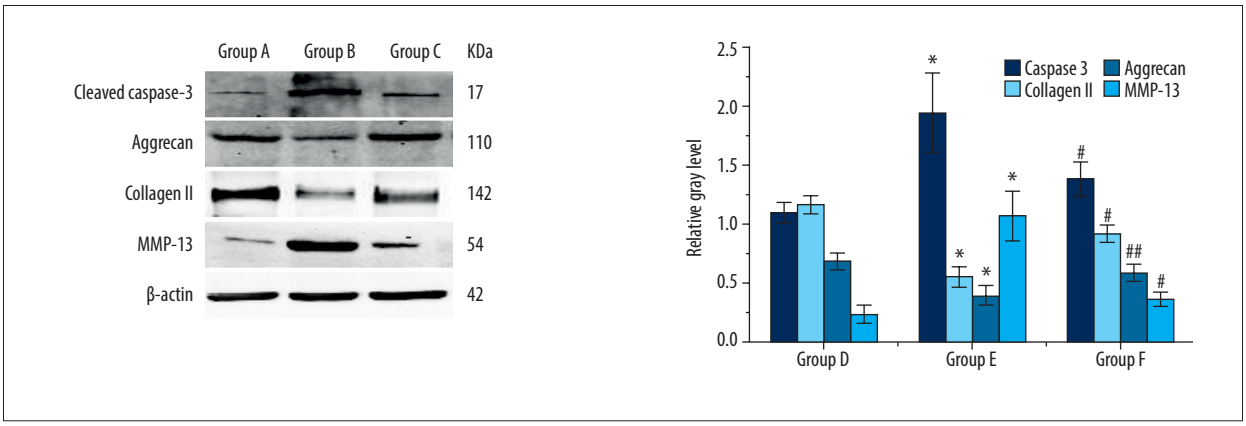

Figure 6. Effect of BMSC-derived Exo on expression of cleaved caspase-3, aggrecan, collagen II and MMP-13. The expression of cleaved caspase-3, aggrecan, collagen II and MMP-13 was detected by Western blotting. Compared with Group D, the expression of cleaved caspase-3 and MMP-13 significantly increased, and the expression of collagen II and aggrecan significantly decreased in Group E. BMSC-derived Exo (Group F) significantly decreased the expression of cleaved caspase-3 and MMP-13 and increased the expression of collagen II and aggrecan in acidic pH. Bars, mean values±S.D. of three independent experiments. * p<0.05 *vs.* Group D; # p<0.05, ## p<0.01 *vs.*s Group E.

cultured in pH 5.9–6.1 medium (Group F), the upregulation of cleaved caspase-3 and MMP-13 and downregulation of collagen II and aggrecan induced by acidic pH were significantly reversed, as shown by Western blot analysis (Figure 6). Similar result was obtained by qRT-PCR, showing that BMSC-derived Exo attenuated the effect of acidic pH on the expression of caspase-3, aggrecan, collagen II, and MMP-13 (Figure 7). These results suggest that BMSC-derived Exo facilitates the expression of extracellular matrix components and reduces matrix degradation by inhibiting the expression of matrix-degrading enzyme MMP-13.

Discussion

In recent years, the incidence of LBP has been increasing with aging of the population, and has become an economic and social problem [2,24]. Previous studies showed that IVDD is an important cause of LBP, and the pathogenesis of human IVDD is related to abnormal mechanical loading and increased inflammation by cytokines. A previous study demonstrated that a high concentration of inflammatory cytokines can accelerate disc degeneration, and NPC apoptosis and extracellular matrix degradation may be responsible [25]. The pH of human IVDs ranges from 7.1 in healthy IVDs to lower than 6 in degenerated IVDs [26]. Although

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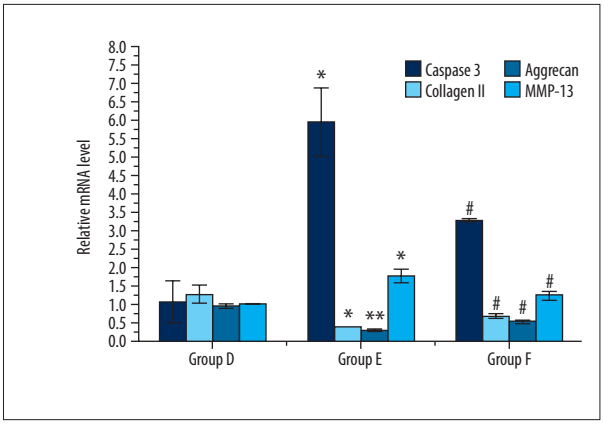

Figure 7. Effect of BMSC-derived Exo on mRNA expression levels of caspase-3, aggrecan, collagen II and MMP-13. The mRNA expression of caspase-3, aggrecan, collagen II and MMP-13 was analyzed by qRT-pCR. In acidic pH (Group E), caspase-3 and MMP-13 mRNA expression significantly increased, mRNA levels of collagen II and aggrecan decreased. BMSC-derived Exo (Group F) reduced caspase-3 and MMP-13 mRNA level and increased collagen II and aggrecan mRNA expression in acidic pH. Bars, mean values±SD of three independent experiments. * $p<0.05$, ** $p<0.01$ *vs.* Group D; # $p<0.05$, *vs.* Group E.

it is un clear whether the acidic disc environment is a result of the degeneration or is itself a cause of the degeneration, the harmful effect of a pathological acid environment on discs has been proven. In this study, we found that the proliferative activity of NPCs was weakened with the decrease in pH value, and matrix degradation was strengthened in an acid environment by upregulating MMP-13 expression. We used medium with different pH levels to culture NPCs, and found that medium with pH 5.9–6.7 had a dramatic impact on NPC viability and the gene expression of NPCs. This was consistent with the previous *in vivo* study.

A previous study has shown that MSC transplantation may be an effective strategy for the treatment of IVDD [9]. *In vitro* studies showed that proliferative activity of NPCs and production of proteoglycans were enhanced by co-culture of MSCs and human NPCs [27]. MSC transplantation inhibited the expression of inflammatory cytokines, thus downregulating matrix-degrading enzyme expression. This management could also prevent the apoptosis of NPCs [28]. *In vivo* studies found that production of extracellular matrix components, including aggrecan and proteoglycans, was increased after transplanting MSCs into degenerated IVD [59]. In a human clinical pilot study, 10 patients were treated by transplantation of MSCs by injecting the cells into the center of the intervertebral disc. After 12 months, the symptom of low back pain was improved, and the image examination showed that moisture in the disc was increased [30]. However, there is also controversy about the role of MSCs, as some studies found that MSCs injected into discs of IVDD animal models failed to survive. More severe disc degeneration is associated with worse survival of MSCs [31]. Based on this, some scholars pointed out that the optimal environment for survival of MSCs is above pH 6.8, but the environment in degenerated disc is often more acidic, and pH scores are often lower than pH 6.8 or even 6.2. Thus, the strategy of transplantation of MSCs is strongly affected by the pathological acidic environment [13]. Development of material with acidic resistance and suitable for NPC proliferation is needed.

Exo derived from MSCs can not only survive within the harsh hypoxic environment of the IVD, but also can obviously improve the proliferation of NPCs and increase synthesis of extracellular matrix [17]. With its recent development, Exo has been used not only for IVDD but also for other diseases. In traumatic osteonecrosis of the femoral head (ONFH), downregulation of BMSC-derived exosomal microRNA-224-3p promotes endothelial cell proliferation and angiogenesis of traumatic ONFH [9]. In addition, Exo also showed a distinct potential in modulating the immune reactions, and it can induce the islet regeneration via pancreatic and duodenal homeobox 1 [21]. However, in all these studies, the acidic environment was not involved. So, whether it still works in the pathological acidic environment is not clear until now.

Our study is the first to verify the beneficial effect of BMSC-Exo on IVDD in abnormal acidic conditions. Initially, the proliferative activity of NPCs increased along with the increasing concentration of BMSC-Exo. Then, Exo was able to prevent and mitigate NPC apoptosis through repressing caspase-3 expression and attenuating caspase-3 cleavage induced by acidic pH. Moreover, Exo effectively enhanced the extracellular matrix expression and inhibited extracellular matrix decomposition.

The potential mechanism underlying acidic pH-induced NPC apoptosis may be due to activation of acid-sensitive ion channels (ASIC) or p38 MAPK pathway [3,32–34]. Exo, as an important vesicle for intercellular communication, contains a number of proteins, mRNAs, and microRNAs that can obviously improve the proliferation of NPCs and increase synthesis of extracellular matrix [17]. It is likely that cross-reactions exist between microRNAs, ASIC, and the MAPK pathway. The molecular mechanism by which microRNAs regulate ASIC or MAPK expression or activity needs further investigation.

Conclusions

In the pathological acid environment, MSC-derived Exo promotes the expression of chondrocyte extracellular matrix, collagen II, and aggrecan, and reduces matrix degradation by downregulating matrix-degrading enzymes, protecting NPCs from acidic pH-induced apoptosis. This study reveals a potential therapeutic strategy for treatment of IVD degeneration.

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