# Peer Review File

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**Reviewer A**

Comment 1:

Intervertebral disc herniation is still an enigma and several approaches try to solve problems and stimulate the healing process. Therefore, basically, paper is dealing with an interesting and investigation-worthy topic. But several aspects of the manuscript are concerning that have to be strongly considered to make it a significant scientific paper. Especially the methods of isolation and verification NP cells should be optimized to improve the understanding of what you did. Authors have to shorten the manuscript to the main and significant statements. Moreover, try to hew to the basic requirements for preparing a manuscript.

Reply 1:

Thanks for your kind comment. The manuscript has carefully re-written and the answers to your comments are listed below.

Comment 2:

Introduction

Above all, statements in this paragraph are o.k. But in summary, presented background regarding the studied issue could be given more accurate. Controversy that exists regarding the main problem could be worked out more clearly to the readership. I agree with your statements but you could do a better job in shortening the 'Introduction' to the main statements regarding the presented problem and controversies to make it more authoritative. In its current version, the introduction is too verbose and does not rule the controversy exactly. Please try to reduce this section to approximately one page in being more authoritative and limited in scope. This will make the paper more readable and, most important, it will create significant interest on the studied issue. The aim/purpose and hypothesis are stated.

Reply 2:

Thanks so much for your kindly and helpful comments. We have modified the "Introduction" part to make it more clearly and logical as advised.

Changes in the text:

Page 4-6, line 58-103

Intervertebral disc herniation (IDH) is a major cause of low back pain (LBP), which is a leading contributor to years lived with a disability worldwide (1). The nucleus pulposus (NP), which consists of NP cells (NPCs) and an abundant extracellular matrix, is located at the center of the intervertebral disc and bears most of the axial compression pressure of the spine. Disc samples and clinical images from IDH patients are commonly characterized by damage to the annulus fibrosus (AF), the outer layer of the disc, and the herniation of NP tissue inside the disc. Consistent with the low signal intensity observed on T2-weighted magnetic resonance imaging (MRI), immunohistochemical staining of IDH NP samples typically reveals a lack of collagens and proteoglycans (PGs), which are major components of the NP matrix, and decreasing numbers of NPCs (2).

Apoptosis, also known as programmed cell death (PCD), is a programmed and orderly cell death process induced to maintain cellular homeostasis. Previous studies have reported high rates of NPC apoptosis in vitro and the high level of apoptotic markers in NP samples from both IDH patients and animal models (3, 4).

Some proinflammatory factors, such as nitric oxide, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), can induce cell apoptosis via various pathways. For example, TNF-α can bind to TNF receptor 1 (TNFR1), a membrane receptor that triggers a downstream caspase activation cascade, inducing apoptosis in NPCs (5). Therefore, TNF-α may serve as a key initiating factor in disc degeneration (6).

Insulin-like growth factor-1 (IGF-1) is a natural polypeptide hormone that plays an important role in growth stimulation during childhood and is involved in tissue regeneration in adults (7). Previous studies have demonstrated that IGF-1 is critical to the maintenance of disc homeostasis, promoting both cell proliferation and the synthesis of extracellular matrix in a dose-dependent manner (8, 9). The major functional receptor for IGF-I, IGF-1R, is a receptor tyrosine kinase that can activate the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway (10, 11) and then serves as a natural stimulator of cell growth and proliferation and a potent inhibitor of apoptosis in NPCs (12).

Exosomes are a group of membranous, nano-sized particles originating from endosomes which contain bioactive lipids, proteins, and nucleic acids. One group of important exosomal cargoes, known as microRNAs (miRNAs), are small, non-coding RNAs that function as negative gene expression regulators. Many studies have reported the protective role played by exosomal miRNAs that are derived from the cells involved in disc degeneration diseases, such as IDH (13-16). Recently, we reported that the exosomal miR-27a derived from autophagy-activated healthy NPCs could repress IL-1β-induced NP matrix degradation by targeting matrix metalloprotease 13 (MMP-13) (17).

However, whether exosomes released from NPCs under pathological conditions, such as inflammation, can affect the proliferation and apoptosis of normal NPCs remains largely unknown. In this study, we investigated the effects of exosomes derived from TNF-α-stimulated NPCs on the induction of apoptosis in normal NPCs and the relationship between IGF-1 expression and exposure to these exosomes.

Comment 3:
Methods
Concerns regarding methodology substantially limit enthusiasm for the work described in this manuscript. Key details such as NP cells isolation and verification are missing, and collagen II is not the unique marker, there are many markers for NP (e.g., Krt 8, Krt 18, Krt 19 and T). Without these details, it is impossible to assess the rigor and quality of the manuscript.

Reply 3:
Thanks for your kindly comments. We have added the key details of NP cells isolation as advised. Although collagen II and aggrecan are not unique markers of NP, they represent the cartilage-like characteristics of NP tissue. These markers are widely used for NP cells identification in many articles, as well as in our previous study. Besides, the isolation process of NP tissue is well-established and easy to repeat. Our experience from previous studies make sure the primary culture of NP cells is ok in this study. [1, 2]
Change in text:
Page 6, line 111-113
The NP tissue was separated from the lumbar disc, without the outer AF layer and cartilaginous endplates though microsurgery, followed by digestion with 0.1% collagenase II for 4 h at 37°C.

Comment 4:
Results
Too many subjective statements. Please objectively describe your findings.

Reply 4:
Thanks for your kind comment. We have deleted subjective statements and modified the “Result” part as advised.

Changes in the text:
Page 14, line 287-291
After 36 h treatment, the cell viability of the 10 ng/mL TNF-α treatment group was 75.5% ± 1.9%, whereas the viability of the 30 ng/mL TNF-α treatment group was only

61.7% ± 1.7%. The cell viability of the 5 ng/mL TNF-α treatment group was not significantly different from that of the control group (Fig. 2A). We also analyzed whether TNF-α could stimulate exosome release by NPCs.

Page 15, line 306-307
We examined whether the IGF-1 signaling pathway was involved in the TNF-Exo-induced apoptosis of NPCs.

Page 15, line 311-312
We investigated whether TNF-Exos induced NPC apoptosis and inhibited the IGF-1 signaling pathway through the delivery of exosomal miRNAs.

Page 17, line 349-352
Then, we investigated whether exosomal miR-16 could induce NPC apoptosis through the downregulation of IGF-1/IGF-1R signaling and the subsequent inhibition of the PI3K/Akt pathway.

Page 17, line 355-357
The suppression of the PI3K/Akt pathway was also demonstrated by examining the ratios of phosphorylated PI3K and Akt, which were reduced in the miR-16 mimic-transfected group compared with the PBS control group.

Page 17, line 357-359
Notably, the IGF-1 expression level and the phosphorylated PI3K ratio were slightly increased in the mimic-NC transfected group (Fig. 5C).

Page 18, line 373-376
Because it is not possible for validating our in vitro findings in live human discs, we collected 7 NP samples from extrusion and sequestration patients and compared the expression levels of miR-16, IFG-1, IFG-1R, and apoptotic markers between herniated and non-herniated NP tissue.

Page 18, line 384-385
As shown in Table S1, the samples we collected in this study were all Pfirrmann Grade IV or V, which could be considered advanced stages of disc degeneration (20).

Page 19, line 391-394
In addition, significantly increased expression levels of the apoptotic markers Bax and caspase-3 as well as reduced expression levels of the anti-apoptotic marker Bcl-2 were

observed in herniated NP samples compared with those in non-herniated NP samples (Fig. 7C).

Comment 5:
Discussion
This part is one more weak link of the paper and makes it difficult to consider it for publication.
First of all, the section should begin with "Principle results of the study are …" Please confirm/not confirm your hypothesis. You have to compare and discuss your results with previous studies in the literature, despite the fact that they are rare. Especially, you have to address strengths and limitations of your study more significantly. Limitations are significant. Therefore, please try to define whether you proved your statements or whether you could show only a tendency towards something.

Reply 5:
Thanks for your kind comments. The discussion section has been rewrote in the reversed manuscript, in which the comparisons of previous studies and limitations were added.

Change in text:
Page 19-20, line 408-411
In the present study, we found that exosomes release was increased by TNF-α pretreatment of rat NP cells. Such exosomes could induce apoptosis of normal NP cells via transmitting miR-16, which interacted with the 3'-UTR of IGF-1 and IGF-1R mRNA.

Page 25, line 522-525
We searched and validated the miRNA-mRNA regulation relationship only in rat NPCs but not in human NPCs. Therefore, the conclusion we had in the present study might be somehow different in human NPCs.

Page 25, line 537-540
However, we didn't perform in vivo animal experiment in the present study. The effect of exosomes derived from abnormal NPCs in rat intervertebral discs awaited further study by intra disc injection of such exosomes and the following imaging examinations as well as molecular biological experiments.
Comment 6:
Limitations
Add limitations

Reply 6:

Thanks for your kind comment. We discussed the limitation of this study in the discussion section from page line to page line. However, we added more discussion about limitations in the reversed manuscript which we didn't notice in the origin version.

Change in text:

Page 25, line 522-525

We searched and validated the miRNA-mRNA regulation relationship only in rat NPCs but not in human NPCs. Therefore, the conclusion we had in the present study might be somehow different in human NPCs.

Page 25, line 537-540

However, we didn't perform in vivo animal experiment in the present study. The effect of exosomes derived from abnormal NPCs in rat intervertebral discs awaited further study by intra disc injection of such exosomes and the following imaging examinations as well as molecular biological experiments.

Comment 7:

Conclusions

This part is too verbose and universal. Try to give an exact conclusion that you derived from your data.

Reply 7:

Thanks for your kind and useful comment. We simplified the conclusion section to highlight our major findings in this study.

Change in text:

Page 26, line 544-549

In conclusion, the proinflammatory factor TNF-α was able to stimulate the exosomal release of exosomes including miR-16 from NPCs in rats, whichfurther induce the apoptosis of normal NPCs through repressing the anti-apoptotic IGF-1/IGF-1R pathway (Fig. 8). Our work revealed a new mechanism through which inflammation and exosomes participate in intervertebral disc degeneration and suggested that cell-to-cell communications mediated by exosomes play important roles in the maintenance of disc homeostasis.

Comment 8:

Figures and Tables

Figure 7 C no magnification and higher magnification of image should be add. The background is not normalized.

Reply 8:

Thanks for your kind and useful comment. We added scale bar on the right corner of last picture. Besides, we noticed the unusual high background of some slides of Bax, Caspase-3 and TNF-α. Therefore, we performed the IHC staining of involved slides again. New representing pictures and statistical diagrams were added to replace the old one.

Moreover, we ignored to explain the black arrows in the legend of Figure7. The black arrows pointed to the cell clusters of NP which were believed to be the marker of degenerated discs, consisting of both apoptotic and proliferated NP cells. The figure legend has been corrected.

Change in text:

Page35, line 737-739

The black arrows pointed to the cell clusters of NP which were the markers of degenerated discs. Scale bar = 50 μm.

**Reviewer B**

Comment 1:

General: Zhang et al. examined the role of exosomes on nucleus pulposus cell apoptosis pathways, revealing a link between TNF-alpha, exosome release, and apoptosis. The manuscript is well written and provides interesting and impactful results, improving our understanding of apoptotic processes in inflammatory situations in IVDs.

However, several concerns were raised while reading the manuscript. I feel that a major revision addressing my comments could improve the overall quality of the manuscript. The authors should structure the manuscript better, not mixing up the introduction, methods, results, and discussion. It is not appropriate to wait until the results section to provide some methods used or to discuss results in the results section.

Overall, the following points should be addressed:

Reply 1:

Thanks for your kind comment. We have carefully modified the full text according to your sincere advice one by one.

Comment 2:

1) Abstract: please add information that the NPC cells were obtained from rat and human IVDs

Reply 2:

Thanks for your kind and useful comment. We have added the information of rat-origin NP cells and also modified the “Abstract” part.

Change in text:

Page 2, line 28-31

In this study, we examined whether exosomes released from nucleus pulposus (NP) cells (NPCs) under inflammatory conditions could induce normal NP cell apoptosis in rats and its underlining mechanism.

Comment 3:

2) introduction: please add information on the relevance of this study at the end of the introduction section. Why is this study important?

Reply 3:

Thanks for your comment and question. The mechanism of intervertebral disc degeneration remains unknown. We have studied the crosstalk between NP cells to try to explore the mechanism and found the exosomal miR-27a derived from autophagy-activated healthy NPCs could repress IL-1β-induced NP matrix degradation by targeting matrix metalloprotease 13 (MMP-13). This study aims to investigate whether exosomes released from NPCs under pathological conditions, such as inflammation, can affect the proliferation and apoptosis of normal NPCs, which can help us understand the crosstalk between the NP cells during the process of degeneration better.

Change in text:

Page5-6, line 96-102

However, whether exosomes released from NPCs under pathological conditions, such as inflammation, can affect the proliferation and apoptosis of normal NPCs remains largely unknown. In this study, we investigated the effects of exosomes derived from TNF-α-stimulated NPCs on the induction of apoptosis in normal NPCs and the relationship between IGF-1 expression and exposure to these exosomes. This study may help us understand the relationship between inflammation and apoptosis in the pathology of intervertebral disc degeneration.

Comment 4:

3)Introduction: P5L108-P6L113: These are results/discussions and should not be part of the introduction

Reply 4：

Thanks for you kindly and helpful comments. We have deleted this part as advised.

Change in text:

Page 5, line 98-100

In this study, we investigated the effects of exosomes derived from TNF-α-stimulated NPCs on the induction of apoptosis in normal NPCs and the relationship between IGF-1 expression and exposure to these exosomes.

Comment 5:

4)Methods: P6L118-121: why did you not used bovine IVD discs obtained from local ambassadors instead of euthanizing n=24 living rats? The 3R principle calls for "replacement" of the methodology to decrease the number of animals in medical research to a minimum. What is the reason for using rat IVDs for the experiments?

Reply 5:

Thanks for you kindly and helpful comments. Our findings relied largely on the in vitro experiments using rat NP cells. Rat NP cells are widely used in studies of disc degeneration field. Our group also published several articles focused on disc degeneration using rat NP cells or IVD samples. [1, 2] Besides, all NP cells used in this study were 2nd passage which required frequent primary culture of rat NP cells.

Change in text: None

Comment 6:

5) Methods: P6L125: how many cells were seeded in a T25 culture flask? "proper density" is not sufficient information to replicate the experiments.

Reply 6:

Thanks for your kind comments. We have added details instead of just "proper density".

Change in text:

Page 6, line 114-118

The NPCs were seeded at the density of $3 \times 10^4$ cells/mL in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Thermo Scientific)-high glucose supplemented with 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific) and antibiotics (1% penicillin/streptomycin).

Comment 7:

6) Methods: no information regarding detaching the cells was provided. Please add how you detach cells from the flask. How did you count the cells? The methods part should provide sufficient information so that others can replicate your experiments.

Reply 7:
Thanks for your kind comments. We have added details of detaching and counting the NP cells as advised.

Change in text:
Page 6, line 121-122
Upon reaching 70-80% cell confluency, the cells were harvested using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Invitrogen) for further experiments.

Page 7, line 123-124
Cell density was determined by brightfield cell counter following the instruments (DeNovix, USA) of manufactures.

Comment 8:
7)Methods: P6L125-126: high glucose or low glucose DMEM? Information also needed in subsequent sections (e.g., P7L144, P8L170)

Reply 8:
Thanks for your kind comments. In our study, we use high glucose DMEM. We have added the information in the whole text.

Change in text:
Page 6, line 116
Dulbecco's modified Eagle's medium (DMEM; Hyclone, Thermo Scientific)-high glucose
Page 7, line 149
DMEM-high glucose

Comment 9:
8)P7L138-142: you also used DAPI for IF. It is not mentioned in the IF methods section.

Reply 9: Thanks for your kind comments. We added the use of "DAPI" in the method section.
Change in text:
Page6, line 137-138
The samples were then washed with PBS, mounted in glycerol gel which contained DAPI for nuclei staining (Beyotime, China),

Comment 10:
9)Methods: P7L133: how many cells per well were seeded?

Reply 10: Thanks for your kind comments. We added the cell density we seeded in the method section.

Change in text:
Page7, line 129-131
Cells were seeded at the density of $3 \times 10^4$ cells/mL and cultured in 6-well plate. Upon reaching 70-80% cell confluence, NP cells were fixed with 4% paraformaldehyde for 15 min, and washed 3 times with phosphate-buffered saline (PBS).

Comment 11:
10) Methods: P11L231: please provide more information regarding the human samples (i.e., age, sex, pfirrmann grade if degeneration was present)

Reply 11: Thanks for your kind comments. We provided these information in Table S1.

Change in text:
Page12, line246-247
The sex, age and Pfirrmann grade based on MRI characteristics were recorded. (Table S1)
Comment 12:
11)Methods: P12L231: why did you not only use human samples? Why was it necessary to also use rat IVD cells? The results from human IVDs would be more relevant as they could be directly translatable to humans, whereas results found in rats maybe will not be found in humans.

Reply 12:
Thanks for your kind comments. The reason why we used rat IVD cells was that healthy human samples sometimes were difficult to acquire so we used rat IVD cells instead. To ensure the stable source of NPCs to perform this study, the SD rats we used were all raised under the same conditions, were of similar ages, and were of the same gender. In fact, results found in rats maybe will not be found in humans, so we had already written limitations at the “Discussion” part.

Change in text:
None

Comment 13:
12)Methods: P12L249-250: So, you had 24 biological replicates (rats)+ 7 biological replicates (humans), and each had three technical replicates, right?

Reply 13: Thanks for your kind comments. 24 rats were used for primary culture of NP cells in this study. 7 human NP tissues were used in this study. Each experiment had three biological replicates.

Change in text: None

Comment 14:
13) Methods: statistics section should also include the normality test and the nonparametric tests if the normality assumption was not fulfilled.

Reply 14:
Thanks for your kind and helpful comments. We have added the statistics methods as advised.

Change in text:
Page 13, line 263-264
Normality test and the nonparametric tests were also used if the normality assumption was not fulfilled.

Comment 15:
14)Methods: no information about TNF-alpha pretreatment was provided. How long were the cells incubated with TNF alpha, the concentration of TNF-alpha, etc.? Instead, parts of this methodology are presented in the results section (P13L272-285).

Reply 15: Thanks for your kind and helpful comments. We provided information about TNF-alpha pretreatment in the method section of exosomes isolation part.

Change in text:
Page8, line 150-151
The medium was collected after 36 h of 10ng/mL TNF-α pretreatment,

Comment 16:
15)Methods: no information on cell viability test was provided in the methods section. Instead, one line mentions the use of a cell-counting kit in the legend of Figure 2(P36L757)

Reply 16:
Thanks for your kind comments. We have added the cell viability test in the methods section.

Change in text:
Page 7-8, line 140-146
**Cell viability test**
Briefly, NPCs (density: $3 \times 10^3$ cells per well) were seeded in a 96-well plate. The cell viability was examined by using a Cell Counting Kit-8 (CCK-8; Beyotime, China) following the manufacturer's protocols. The NPCs were treated with different concentration of TNF-α for 36h.Optical density (OD) was measured at a wavelength of 450nm using a Gen5 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Comment 17:
16)Results: Figure 2: what is your theory when looking at the results of the nanoparticle tracking analysis (NTA): why is a higher concentration of TNF-alpha (30ng/mL) associated with a lower exosome release in NPCs compared to the 10ng/mL group? This was not discussed in the discussion section.

Reply 17: Thanks for your kind comments. We analyzed the cell viability following different dosages of TNF-alpha pretreatment before the NTA analysis of exosome amount. The CCK8 assay indicated that 30ng/ml pretreatment of TNF-alpha for 36h would significantly decreased the cell viability of NP cells. That might be the reason why a higher concentration of TNF-alpha (30ng/mL) associated with a lower exosome release in NPCs compared to the 10ng/mL group.

Change in text: None

Comment 18:
17)Results: Figure 2: which cells were used here (human or rat cells)? Please provide the number of biological and technical replicates (sample sizes) in legends when statistical analyses are presented. Here it says n=3, but this is not further explained. Are these technical or biological replicates? Information on both technical and biological replicate should be provided in figure legends. Additionally, it should be clear whether it is human or rat cells. Each figure should stand on its own.

Reply 18: Thanks for your kind comments. We added the spice (rat) before the cells we used in all figure legends. To note, we said "All biological experiments were

independently repeated three times." in the method of statistics. In all figure legends, n=3 represented three biological replicates.

Change in text:

Page31, line670-671

Figure 2. The effects of tumor TNF-α on cell viability and exosome release from rat NPCs.

Page32, line679-680

Figure 3. Exosomes derived from TNF-α-treated rat NPCs induced NPC apoptosis and repressed IGF-1 expression.

Page32, line689-690

Figure 4. miR-16 is abundant in exosomes from TNF-α-pretreated rat NPC, regulating both IGF-1 and IGF-1R in normal rat NPCs.

Page34, line701-702

Figure 5. Exosomes from miR-16 mimic-transfected rat NPC repressed IGF-1 and IGF-1R and the downstream PI3K/Akt pathway in normal rat NPCs.

Page34, line715-717

Figure 6. miR-16 inhibitor transfection of TNF-α-pretreated rat NPCs decreased the levels of exosomal miR-16 and blocked the pro-apoptotic effects via the inhibition of the PI3K/Akt pathway.

Comment 19:

18)Results: Figure 3: quality of Figure3 is low, and it is not sufficiently readable. Please improve quality. Further, provide information, whether this is human or rat cells, and provide information on biological and technical replicates (this should also be done in all other figures). Figure 3A: I don't know fully understand the statistics here. In the main text (P14L289-290), you write that "incubation with TNF-Exos induced NPC apoptosis…". However, from the Bar chart, it seems that the apoptosis rate (%) is significantly decreased for the TNF-exos group, indicating that the rate of apoptosis (I guess this is the number of apoptotic cells divided by all cells) decreased?

Reply 19: Thank you for pointing out our mistake. We corrected this mistake in the reversed manuscript and re-upload the Figure3A with better quality. Besides, all NP cells used in this study were obtained by primary culture of rat NP. This was described in the method section.

Change in text: Figure 3A re-uploaded

Comment 20:
19) Results: P15L320-322: was this comparison significant? It is not clear from Figure 4B as there 2 two different graphs for cells and exos, and this comparison compares cells with exos.
If you also compared each exos group with each cell group, it would have been wise to conduct a two-way ANOVA with a posthoc test to account for multiple comparisons. If you have done this, this should be mentioned in the statistics section.

Reply 20: Thanks for your kind comments. We analyzed these 4 miRNAs expression in NP cells treated by PBS (control) or TNF-alpha as well as exosomes collected from NP cells treated by PBS or TNF-alpha respectively. Therefore, the comparison was performed either between two groups of cells or two groups of exosomes but not between cells and exosomes.

Change in text: None.

Comment 21:
20)Results: Figure 5: please explain control, mimic-NC exo, and mimic exo in the figure legend.

Reply 21: Thanks for your kind comments. We explained these abbreviations in the figure legend.

Change in text:
Page34, line 711-713
Control: NPCs were not treated with anything, mimic-NC exo: exosomes from NPCs transfected with miR-16 mimics-NC, mimic exo: exosomes from NPCs transfected with miR-16 mimics.

Comment 22:
21)Results: P17L361-363: these are discussions and should not be present in the results section. Please transfer to the results sections to discuss the results found in P17L358-360.

Reply 22:
Thanks for your kind and useful comments. We have deleted this part and transferred these discussions in the results sections to the discussion part as advised.

Change in text:

Page 17, line 349-352

Then, we investigated whether exosomal miR-16 could induce NPC apoptosis through the downregulation of IGF-1/IGF-1R signaling and the subsequent inhibition of the PI3K/Akt pathway.

Comment 23:

22) Results: P17L367: how long pretreated? This is methodology and should be available in the methods section.

Reply 23: Thanks for your kind and useful comments. We added this information in the text.

Change in text:

Page17, line 362-364

To further validate the role played by exosomal miR-16 in the induction of NPC apoptosis, miR-16 inhibitors were transfected into NPCs following pretreatment of 10 ng/mL TNF-α for 36h.

Comment 24:

23) Results: Figure 6: please explain TNF NPC exo, NC NPC eco, and inhibitor NPC exo in figure legends. Each figure should stand on its own.

Reply 24: Thanks for your kind comments. We explained these abbreviations in the figure legend.

Change in text:

Page35, line 724-727

TNF-NPC-Exo: exosomes from TNF-α-pretreated NPCs, inhibitor-NC NPC-Exo: exosomes from TNF-α-pretreated NPCs transfected with miR-16 inhibitor-NC, inhibitor NPC-Exo: exosomes from TNF-α-pretreated NPCs transfected with miR-16 inhibitor.

Comment 25:

24) Results: P18L377-391: this is introduction and no results. Please transfer to the introduction section. P18L392-395: this is methods and no results. It is appropriate to start with “As shown in Figure 7A…” (P18L395), which are the actual results.

Reply 25:

Thanks for your kind and useful comments. We have modified the text as advised.

Change in text:

Page 17, line 355-357

The suppression of the PI3K/Akt pathway was also demonstrated by examining the ratios of phosphorylated PI3K and Akt, which were reduced in the miR-16 mimic-transfected group compared with the PBS control group.

Comment 26:

25) Results: P19L403-416: these are discussions, and no results

Reply 26:

Thanks for your kind and useful comments. We have deleted the text as advised.

Change in text:

Page 18, line 373-376

Because it is not possible for validating our in vitro findings in live human discs is not possible, we collected 7 NP samples from extrusion and sequestration patients and compared the expression levels of miR-16, IFG-1, IFG-1R, and apoptotic markers between herniated and non-herniated NP tissue. As shown in Figure 7A, the herniated NP tissue from an extrusion patient presented a relatively clear boundary from non-herniated tissues in both the MRI and surgical specimens.

Comment 27:

26)Results: P19L416-418: this should be mentioned in the methods section. (see comment 10)

Reply 27: Thanks for your kind comments. We provided these information in Table S1.

Change in text:

Page12, line246-247

The sex, age and Pfirrmann grade based on MRI characteristics were recorded. (Table S1)

Comment 28:

27) Results: P20L427-430: this is a discussion and no results.

Reply 28:

Thanks for your kind and useful comments. We have deleted the text as advised.

Change in text:

Page19, line391-394

In addition, significantly increased expression levels of the apoptotic markers Bax and caspase-3 as well as reduced expression levels of the anti-apoptotic marker Bcl-2 were observed in herniated NP samples compared with those in non-herniated NP samples (Fig. 7C).

Comment 29:

28) Discussion: P27L581-582: please add information that this was in rat NPCs

Reply 29:

Thanks for your kind comments. We have added the information that the in-vitro findings are in rats.

Change in text:

Page 27, line 581-582

These in vivo findings indirectly supported our in vitro results in rats.

Comment 30:

29) Discussion: Figure 8: please explain abbreviations (i.e., MVB) and expand the figure legend explaining what your working hypothesis is. Each figure should stand on its own.

Reply 30: Thanks for your kind comments. We briefly explained our working hypothesis in the figure legend.

Change in text:

Page35-36, line743-746

Inflammation increases expression level of miR-16 in rat NPCs and also stimulates the exosomes release. The exosomal miR-16 transmitted to normal NPCs sequently induce the cell apoptosis via inhibition of IGF-1&IGF-1R/PI3K/AKT pathway. MVB: multivesicular bodies

Comment 31:

30)Discussion: P27L585-587: this statement should be carefully revised as the results do not fully support it. You did not have studied intervertebral disc degeneration in the present study.

Reply 31: Thanks for your kind comments. As introduced in the abstract, NPCs apoptosis is an important factor of intervertebral disc degeneration. However, we noticed that it might be better to modified our statement regarding cell apoptosis rather

than intervertebral disc degeneration here.

Change in text:
Page26,line547-549
Our work revealed a new mechanism through which inflammation and exosomes participate in NPCs apoptosis and suggested that cell-to-cell communications mediated by exosomes play important roles in the maintenance of disc homeostasis.

**Reviewer C**
Comment 1:
1. Title: It may need be modified a bit. For instance, "Exosomes released from TNF-α-stimulated nucleus pulposus cells inducing cell apoptosis by… in rat in vitro". In my opinion, it is necessary to add a determiner 'in rat'. As the authors did all the mechanism study in rat and the potential targeting position of miR-16-5p in IGF-1 and IGF-1R existing in rat but may not in human.

Reply 1:
Thanks for your kindly and helpful comment. We have modified the title according to your sincere advice to make the study subject clearer for readers.

Change in text:
Page 1, line 1-2
TNF-α-stimulated nucleus pulposus cells induce cell apoptosis through the release of exosomal miR-16 targeting IGF-1 and IGF-1R in rats

Comment 2:
2. Abstract:
2a. 'background': it needs to introduce a short background and aims of this research.
2b. 'methods' you need to introduce the purpose of each method you used and connect them with a logical order. For instance, 'To evaluate the effects of … in … methods were used to … Further …'.
2c. 'results' show the main result remove the method, as you have already introduced in method.

Reply 2: Thanks for your kindly and helpful comment. We reorganized this section to make the expression more logical and brief.

Change in text:
Page2, line28-31

In this study, we examined whether exosomes released from nucleus pulposus (NP) cells (NPCs) under inflammatory conditions could induce normal NP cell apoptosis in rats and its underlining mechanism.

Page2, line35-38
Given the exosomal miRNAs might be the key factors of exosomes, bioinformatics approaches and quantitative real-time polymerase chain reaction (qRT-PCR) were used to identify IGF-1-regulating micro RNAs (miRNAs), including miR-16.

Page2-3, line44-50
Results: Exosomes from TNF-α-treated NPCs induced apoptosis in normal NPCs and repressed IGF-1 expression. Exosomal miR-16 regulated IGF-1 and induced NPC apoptosis. The dual-luciferase reporter assay revealed that miR-16 binds the 3' untranslated regions (3'-UTRs) of IGF-1 and IGF-1R. Exosomal miR-16 repressed IGF-1 and the IGF-1R/phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway which therefore induced NPC apoptosis. Rescue experiments using miR-16 inhibitors further validated these findings.

Comment 3:
3. Keywords: Remove the key words ,'homeostasis' ,it is too general, and may add 'microRNA…'.

Reply 3:
Thanks for your kind comment. We have modified the key words as advised.

Change in text:
Page 3, line 55-56
Key words: exosomes, nucleus pulposus (NP) cells, apoptosis, microRNA, inflammation

Comment 4:
5. Page 3 lines 56-66.
As this study focused on proinflammation factor, TNF-a, induced NPC apoptosis and underlying mechanism. I would like suggest authors add more words to introduce the role of TNF-a in IVD degeneration (NP degeneration) instead of disc herniation. As this study included results from disc herniation patients, it can be discussed a bit in discussion section.

Reply 4: Thanks for your kind comment. We introduced the role of TNF-a in IVD degeneration and had some discussion in the introduction and discussion sections.

Change in text:

Page4, line72-77

Some proinflammatory factors, such as nitric oxide, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), can induce cell apoptosis via various pathways. For example, TNF-α can bind to TNF receptor 1 (TNFR1), a membrane receptor that triggers a downstream caspase activation cascade, inducing apoptosis in NPCs (5). Therefore, TNF-α may serve as a key initiating factor in disc degeneration (6).

Page19, line397-403

The expression levels of proinflammatory cytokines, including TNF-α, IL-1β, and lipopolysaccharide (LPS), are markedly increased in both human and animal models of degenerated disc disease compared with those in normal controls (21, 22). Proinflammatory cytokines, particularly TNF-α, can induce apoptosis in NPCs within discs, increasing the expression of proteases responsible for matrix degradation, which can accelerate the structural and biological deterioration of the intervertebral disc (23).

Change in text: None.

Comment 5:

6. Page 3 lines 69-70, reference 3-4. They cannot support your state, please cite the correct studies from peer-field. And check all the references you cited carefully below. All this paragraph, removes all the redundancy basic information of apoptosis and more focus on the relation between inflammation (TNFa but not only) and NPC apoptosis.

Materials and Methods

Reply 5: Thanks for your kind comment. We rechecked all the reference and replaced the wrong reference you mentioned above.

Change in text:

Page28, line587-588

[3] Roberts S. Disc morphology in health and disease[J]. Biochem Soc Trans. 2002, 30(Pt 6): 864-869.

[4] Bertram H, Nerlich A, Omlor G, et al. Expression of TRAIL and the death receptors DR4 and DR5 correlates with progression of degeneration in human intervertebral disks[J]. Mod Pathol. 2009, 22(7): 895-905.

Comment 6:

7. Page 9, line 189. Anti-IGF-1 antibody is a goat hosted one. How can author use an anti-rabbit secondary antibody for WB?

Reply 6:

Thank you for pointing out our mistake. We purchased primary antibody of IGF-1 from Absin company in this study after we found the one from Abcam was not good to use for some reasons. The product information has been reversed.

Change in text:

Page 9, line 193

IGF-1 (#abs119614, 1:1,000; Absin)

Comment 7:

8. Page 10, Daul-luciferase reporter assay. Add the detail (sequence or region) of cloned sequence of 3'-UTR.

Reply 7:

Thanks for your kind comment. We have supplied the sequences as advised.

Change in text:

Page 11,line 212-216

IGF-1R: forward 5'-TCCTTGGATCCTGAATCTGT-3', reverse 5'-ACGTTGCCTTAGCTTCAGCC-3'

IGF-1: forward 5'- GAGGAGCCTCCCGAGGAACA-3', reverse 5'-CCTAATTTTGTCCTTTTGGG-3'

Comment 8:

9. Add the method of cell viability assay. As figure 2a, you showed a result from the cell viability assay.

Reply 8:

Thanks for your kind comments. We have added the cell viability test in the methods section.

Change in text:

Page 7-8, line 140-146

**Cell viability test**

Briefly, NPCs (density: $3 \times 10^3$ cells per well) were seeded in a 96-well plate. The cell viability was examined by using a Cell Counting Kit-8 (CCK-8; Beyotime, China) following the manufacturer's protocols. The NPCs were treated with different concentration of TNF-α for 36h.Optical density (OD) was measured at a wavelength of 450nm using a Gen5 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Comment 9:
10. Page 11. Immunohistochemistry, The same problem as WB.

Reply 9:
Thank you for pointing out our mistake. We purchased primary antibody of IGF-1 from Absin company in this study after we found the one from Abcam was not good to use for some reasons. (probably because of improper storage or freezing & melting cycles) The product information has been reversed.
Change in text:
Page 10, line 193
IGF-1 (#abs119614, 1:1,000; Absin)

Comment 10:
11. Figure 2A. Can you explain why the viability of NPCs no increase with time even in control group?

Reply 10:
Thanks for your kind comment. We noticed we mistakenly used the data from no TNF-α group at each time point as control to calculate the cell viability. In fact, it actually represented "Relative quantification of cell viability" at each time point. Beside, though we perform the CCK8 assay on a time course manner, we mostly cared about the cell viability after 36h of TNF-α treatment. Because we later chose to assess the exosomes release on 36h after TNF-α treatment of NPCs. Therefore, we only presented the CCK8 data of 36h time point in a bar plot form in the reversed manuscript. Figure2 was re-uploaded.

Change in text:
Page14, line287-291
After 36 h treatment, the cell viability of the 10 ng/mL TNF-α treatment group was 75.5% ± 1.9%, whereas the viability of the 30 ng/mL TNF-α treatment group was only 61.7% ± 1.7%. The cell viability of the 5 ng/mL TNF-α treatment group was not significantly different from that of the control group (Fig. 2A). We also analyzed whether TNF-α could stimulate exosome release by NPCs.

Comment 11:
12. As showed in figure 3A column(right), showed NPCs treated with exosome from TNF-NPC with lower rate of apoptosis it is opposite to your results.

Reply 11:

Thank you for pointing out our mistake. We corrected this mistake in the reversed manuscript.

Change in text: Figure 3A re-uploaded

Comment 12:

13. Section' miR-16 is abundant in exosomes from TNF-α-pretreated NPCs and regulates both IGF-1 and IGF-1R in normal NPCs.' Describe your result concisely (not only this part, but all the results), shortly show the necessary background, focus on the main finding. I will suggest show Figure 4A, 4C as supplementary data. It is widely reported that miR16 target IGF-1R, please cite the main articles.

Reply 12:

Thanks for your kind and useful comments. We have modified this part as advised.

Change in text:

Page 15, line 311-312

We investigated whether TNF-Exos induced NPC apoptosis and inhibited the IGF-1 signaling pathway through the delivery of exosomal miRNAs.

Page22, line 470-473

Interestingly, previous studies had reported the regulatory relationship between miR-16 and IGF-1R. It was reported that miR-16 had inhibitory effect on the proliferation and metastasis of hepatocellular carcinoma and osteosarcoma cell lines.(27-28)

Comment 13:

14. Section 'Exosomes from miR-16 mimic-transfected NPCs repressed IGF-1 and IGF-1R expression and the downstream PI3K/Akt pathway in normal NPCs'. Confused writing logic and with lot of redundancy words. Please give a simple summary of aims in the beginning, and introduce your results concisely. The section below is a good example.

Page 17 Lines 361-363, move to discussion.

Reply 13:

Thanks for your kindly comments. We have modified this part as advised.

Change in text:

Page 16-17, line 340-359

Next, we validated the role played by miR-16 in the exosome-induced apoptosis of

NPCs. NPCs were transfected with miR-16 mimics or normal control (NC) miRNA (miR-NC). To investigate the contribution of miR-16 to the pro-apoptotic effect of NPC-produced exosomes, we developed miR-16-overexpressing exosomes by transfecting NPCs with miR-16 mimics, followed by exosome isolation. The successful overexpression of miR-16 in both NPCs and exosomes was confirmed by qRT-PCR (Fig. 5A).

To detect the successful uptake of exosomal miR-16, NPCs were transfected with Cy3-labeled miR-16 mimics, followed by exosome isolation. NPCs incubated with these exosomes exhibited a granular fluorescent pattern within the cytoplasm (Fig. 5B).

Then, we investigated whether exosomal miR-16 could induce NPC apoptosis through the downregulation of IGF-1/IGF-1R signaling and the subsequent inhibition of the PI3K/Akt pathway. The incubation of NPCs with exosomes derived from miR-16 mimic-transfected-NPCs resulted in the significant downregulation of both IGF-1 and IGF-1R protein expression. The suppression of the PI3K/Akt pathway was also demonstrated by examining the ratios of phosphorylated PI3K and Akt, which were reduced in the miR-16 mimic-transfected group compared with the PBS control group. Notably, the IGF-1 expression level and the phosphorylated PI3K ratio were slightly increased in the mimic-NC transfected group (Fig. 5C).

Comment 14:

15. Section ‘Herniated human NP tissues expressed a high level of miR-16, low levels of IGF- 1 and IGF-1R, and was characterized by high apoptotic activity’. When I read this part, I thought I jumped to discussion or introduction. Please show the main finding, all the rests go to discussion.

Reply 14:

Thanks for your kindly and helpful comments. We have modified this part as advised.

Change in text:

Page 18-19, line 373-394

Because it is not possible for validating our in vitro findings in live human discs, we collected 7 NP samples from extrusion and sequestration patients and compared the expression levels of miR-16, IFG-1, IFG-1R, and apoptotic markers between herniated and non-herniated NP tissue. As shown in Figure 7A, the herniated NP tissue from an extrusion patient presented a relatively clear boundary from non-herniated tissues in both the MRI and surgical specimens. The RNA extraction and qRT-PCR detection of the four previously examined miRNAs, miR-452-5p, miR-190a-5p, miR-301a-3p, and miR-16-5p, were performed separately in both herniated and non-herniated tissues. Among these miRNAs, only the expression of miR-16-5p was significantly increased

in the herniated NP tissue compared with the non-herniated tissue (Fig. 7B).
As shown in Table S1, the samples we collected in this study were all Pfirrmann Grade IV or V, which could be considered advanced stages of disc degeneration (20). We performed immunohistochemistry to assess the expression levels of IGF-1, IGF-1R, and TNF-α, as well as protein markers of apoptotic activity in both herniated and non-herniated NP tissues. Both IGF-1 and IGF-1R were expressed at lower levels in herniated NP tissues compared with non-herniated NP tissues. In contrast, TNF-α had elevated expression in herniated NP tissues compared with non-herniated NP tissues. In addition, significantly increased expression levels of the apoptotic markers Bax and caspase-3 as well as reduced expression levels of the anti-apoptotic marker Bcl-2 were observed in herniated NP samples compared with those in non-herniated NP samples (Fig. 7C).

Comment 15:

16. Page 20, lines 422-423, 'In contrast, TNF-α had reduced expression in herniated NP tissues compared with non-herniated NP tissues.' This is opposite description compare to Figure.

Reply 15:

Thank you for pointing out our mistake. We corrected this mistake in the reversed manuscript.

Change in text:

Page 19, lines 389-390

In contrast, TNF-α had elevated expression in herniated NP tissues compared with non-herniated NP tissues.

Comment 16:

17. Page 20, lines 427-430. According to the results from Figure 7. TNFa and mir16 expressed higher and IGF1/IGR-1R expressed lower in herniated tissue than non-herniated part. And herniated tissue exhibited higher apoptosis level of NPC. Together with results in vitro which can only indicate TNFa induced NPC apoptosis involved in mir-16- IGF1/IGR-1R pathway. Please introduce clearer.

And another question is, as I know, the human IGF1 without targeting sequence for mir-16 in 3' UTR. Could the author explain a bit about this with your finding in Figure 7?

Reply 16:

Thanks for your kind and helpful comment. We realized this is a limitation and question remained for further study in the future for we didn't validate our conclusion using human NPCs. Beside, factors affecting IGF1 expression in vivo could be very complicated. Here, we only objectively presented our findings on immunohistochemistry slides.

We added discussion about concerns above in the reversed manuscript.

Change in text:

Page25, line 522-525

We searched and validated the miRNA-mRNA regulation relationship only in rat NPCs but not in human NPCs. Therefore, the conclusion we had in the present study might be somehow different in human NPCs.

Page25, line 537-540

However, we didn't perform in vivo animal experiment in the present study. The effect of exosomes derived from abnormal NPCs in rat intervertebral discs awaited further study by intra disc injection of such exosomes and the following imaging examinations as well as molecular biological experiments.

Comment 17:

18. Can author show the isotype IgG control of Figure 7C, as Bax and Cas-3 staining with higher background in extracellular matrix, and add explanation of black arrow in figure legend?

Reply 17:

Thanks for your kindly comment. We noticed the unusual higher expression of HRP-labeled secondary antibody signal in some parts of slides we used in Figure7C. Therefore, we performed the IHC staining of involved slides again. New representing pictures and statistical diagrams were added to replace the old one.

Moreover, we ignored to explain the black arrows in the legend of Figure7. The black arrows pointed to the cell clusters of NP which were believed to be the marker of degenerated discs, consisting of both apoptotic and proliferated NP cells. The figure legend has been corrected.

Change in text:

Page35, line 737-739

The black arrows pointed to the cell clusters of NP which were the markers of degenerated discs. Scale bar = 50 μm.

Comment 18:

19. The discussion is largely adding reference without comparing and contrasting other studies. Could you compare your finding with other research in this area instead of showing the reference and reiterating results?

Reply 18: Thanks for your kind and useful comments. We have modified this part as

advised.

Change in text:
Page22, line 470-473
Interestingly, previous studies had reported the regulatory relationship between miR-16 and IGF-1R. It was reported that miR-16 had inhibitory effect on the proliferation and metastasis of hepatocellular carcinoma and osteosarcoma cell lines.(27-28)

Comment 19:
20. Page 27 lines 584-585 as I remind before add the determiner, “in rat”.

Reply 19:
Thanks for your kind and useful comment. We have carefully modified the “Conclusion” part as advised.

Change in text:
Page 26, line 544-546
In conclusion, the proinflammatory factor TNF-α was able to stimulate the exosomal release of exosomes including miR-16 from NPCs in rats, which further induce the apoptosis of normal NPCs through repressing the anti-apoptotic IGF-1/IGF-1R pathway (Fig. 8).

**Reference**

[1] Hu S Q, Zhang Q C, Meng Q B, et al. Autophagy regulates exosome secretion in rat nucleus pulposus cells via the RhoC/ROCK2 pathway[J]. Exp Cell Res. 2020, 395(2): 112239.
[2] Zhang Q C, Hu S Q, Hu A N, et al. Autophagy-activated nucleus pulposus cells deliver exosomal miR-27a to prevent extracellular matrix degradation by targeting MMP-13[J]. J Orthop Res. 2020.