Author's response to reviews

Title: In Vivo Experimental intervertebral disc Degeneration Induced by Bleomycin in the Rhesus Monkey

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Author's response to reviews:

MS ID: 1753467155132848
Reviewer's report
Title: In Vivo Experimental intervertebral disc Degeneration Induced by Bleomycin in the Rhesus Monkey
Version: 2
Date: 7 July 2014
Reviewer: Thorsten Guehring
Reviewer's report:
This manuscript is of interest as it follows a slowly progressive disc degeneration model in large animals. However, compulsory revisions should include follow items:

1. How can the authors really be sure that bleomycin in fact blocks nutrition as mentioned in their manuscript? Did the authors do pre-experiments on this item? Can the authors exclude for example a toxic reaction of bleomycin?

Answer: Thanks a lot for your question. Bleomycin (BLM), one of the most common sclerosants, has been proved to be safe and effective in the treatment of vascular malformations (VMs) [1-3]. Although the precise mechanisms induced by BLM still need to be clarified, evidence is now emerging that treatment with BLM affects the adhesion molecules of the endothelium and destroys intercellular interactions [4,5], and destroys the endothelial cells, resulting in sclerostenosis of
the lumen and leading to narrowing or occlusion of the vessels, resulting in "devascular effect" with low complication rates[6,7].

We did the preliminary experiment on rabbits before this study. We injected PYM into the subchondral bone (1mm above and below the endplate) adjacent to the IVDs of rabbits. The x-ray angiography and digital subtraction angiography showed that the contrast medium concentrated in the vertebral body, although some of them dissipated with time into the general circulation(Fig.1). The histological results showed less organized fibrocartilage lamellae of annulus fibrosus and wavy arrangement of cartilage endplate(Fig.2,3). However, due to the limited medical condition, it is difficult to perform anesthesia for large animals, such as rhesus monkeys in the fluoroscopic suite of our research center. So we did not perform the x-ray angiography and digital subtraction angiography during operation in this study.

The "devascular effect" of PYM was also supported by the results of real-time PCR, which showed that the expression of vWF in the PYM group significantly decreased in comparison with the sham and vehicle control groups. Von Willebrand factor (vWF), a glycoprotein involved in blood coagulation, is mainly synthesized by capillary endothelial cells. vWF is expressed specifically in endothelial cells[8]. It is commonly used as an marker for capillary endothelial cells (EC)[9], and capillary density[10]. So, this result predicted that the capillary density of cartilaginous endplate in the PYM group decreased compared with the control groups.

Bleomycin (BLM) has been proved to be safe and effective in the treatment of vascular malformations (VMs)[1-3,6,7]. In this study, all the rhesus monkeys recovered very soon after operation and no complications induced by BLM (such as nausea, vomiting, skin necrosis, et al.[11]) were found among the animals.

Figure 1. (A),(B) The x-ray angiography and digital subtraction angiography showed that the contrast medium concentrated in the vertebral body of L5 after injecting contrast into the subchondral bone adjacent to the inferior endplate. (C),(D) The x-ray angiography and digital subtraction angiography showed that the contrast medium concentrated in the vertebral body of L6 after injecting contrast into the subchondral bone adjacent to the superior endplate.

Figure 2. The histological results of L5 inferior cartilage endplate and annulus fibrosus preoperatively, 4 and 6 weeks after operation. (A) Norma L5 cartilage endplate and annulus fibrosus preoperatively(HE×200). (B) The annulus fibrosus showed less organized fibrocartilage lamellae 4 weeks after operation. (C) The cartilage endplate showed a wavy arrangement, and the organization of fibrocartilage lamellae in the annulus fibrosus become more disorderly.

Figure 3. The MRI and histological changes of L5/6 intervertebral disc 3 months after operation. (A) Coronal FST2WI showed hypointense signal of L5/6 intervertebral disc. (B) The wavy arrangement of cartilage endplate and disorder orginazation of fibrocartilage lamellae became more severe 3 months after operation.
References for this part:

2. In the discussion the authors mentioned the important aspect of notochordal disc cells, and in line 291 they state that "notochordal cells were still observed". How did they do this by histology? This does not appear in the results section at all.

Answer: Thanks for your question. Histologically, notochordal cells are distinguished from their chondrocyte-like neighbors in the nucleus pulposus by morphology. The presence of notochordal cells has been reported: large vasculolated and smaller, spindle-shaped cells[1,2]. We have added the black
arrow in the Figure 3E, which indicated the notochordal cells, and add
information in the “Results” section.

Although it has been reported that the notochordal cells (NCs) are lost in human
discs during adolescence, which is also when discs begin to show degenerative
signs [3], the fate of notochord cells during disc development and aging is still a
subject of debate. Risbud et al. found that notochordal cell were observed in
adult human nucleus pulposus tissue samples, even during disc degeneration[4],
by evaluating the gene expression of brachyury, which was one of the marker
genes for notochordal cells in the nucleus pulposus. Supporting this finding,
Hoyland et al. showed that in the degenerate human nucleus pulposus,
brachyury expression remained constant[5]. However, the differences in
expression of marker genes between notochordal cells and the other cell
lineages are still controversial[5]. Thus, although it is still possible that
development of disc disease is linked to loss of a subpopulation of notochordal
cells, either by death, or by de-differentiation to a modified phenotype[6], more
definitive studies using molecular genetic approaches are required. In this study,
due to the limited sample number, we did not analyze the gene expression of
gene markers for notochordal cells, so further studies are warranted to
investigate the cell types in the nucleus pulposus of rhesus monkeys.

References for this part:
1. Zavala G, Vázquez-Nin GH. Analysis of nuclear ribonucleoproteic structures
during notochordal cell differentiation and maturation in chick embryos. Anat Rec
2. Hunter CJ, Matyas JR, Duncan NA. The notochordal cell in the nucleus
pulposus: a review in the context of tissue engineering. Tissue Eng 2003,
models useful for studying human disc disorders/degeneration? Eur Spine J
4. Risbud MV, Schaer TP, Shapiro IM. Toward an understanding of the role of
notochordal cells in the adult intervertebral disc: from discord to accord. Dev Dyn
5. Minogue BM, Richardson SM, Zeef LA, Freemont AJ, Hoyland JA.
Transcriptional profiling of bovine intervertebral disc cells: implications for
identification of normal and degenerate human intervertebral disc cell
transformation of notochordal nucleus pulposus to chondrogenic and

3. The authors should explain why they chose the specific target genes for the
gene expression analysis. Did they consider using further genes as well as
protein expression analysis?
Answer: Thanks for your question. Intervertebral disc degeneration(IVDD) is
primarily in the process of extracellular matrix (ECM) destruction and cellular aging[1]. The two main components of extracellular matrix (ECM) in the intervertebral disc are collagen and proteoglycan (PG)[2]. Aggrecan is a major type of PG in the intervertebral disc (IVD) containing side chains of glycosaminoglycan and aggregates with hyaluronic acid[3]. The collagen fibers provide a strong framework in support of the cells and highly hydrated PG aggregates in the IVD. Type # collagen is a primary component of collagens in IVD. In degenerative articular cartilage in the nucleus pulposus, type # collagen synthesis is downregulated, while the expression of type# collagen is enhanced for repair[4,5]. So, we evaluated the gene expression of aggrecan, type # and # collagens in this study.

Aggrecanases and matrix metalloprotease (MMP) Which are upregulated by proinflammatory cytokines, such as interleukin 1#, # and tumor necrosis factors[6,7], can lead to ECM turnover in several types of tissue[8,9]. MMPs (especially MMP-3) are reported to be active in disc degeneration[10,11]. MMPs display stronger activity than aggrecanases in nucleus pulposus. Multiple studies have shown that the major proteolytic enzymes regulating articular cartilage degeneration are aggrecanase-1 and -2[12,13], also known as ADAMTS-4 (A disintegrin-like and metalloproteinase with thrombospondin type#motifs, 4) and ADAMTS-5[14]. So, we also analyzed the gene expression of MMP-3, ADAMTS-4 and ADAMTS-5 in this study.

Von Willebrand factor (vWF), a glycoprotein involved in blood coagulation, is mainly synthesized by capillary endothelial cells. vWF is expressed specifically in endothelial cells[15]. It is commonly used as an marker for capillary endothelial cells (EC)[16], and capillary density[17]. In order to evaluate the influence of Bleomycin (BLM) on the capillary density in the cartilage endplate, we analyzed the quantitation of gene expression of von willebrand factor (vWF) in the cartilage endplate using the same method as mentioned in our manuscript. We have added this content in the “Real-time PCR” part in the M#M.

It has been reported that the cytokines TNF-#, IL-1#, IL-6 and IL-8 increased significantly in aged and/or degenerated IVDs[18-20]. So, we analyzed the quantitations of gene expression of inflammatory cytokines (TNF-#, IL-1# and IL-6) in the intervertebral discs using the CDNA we kept before, and supplemented the results and figures, and added some discussion in the “Discussion” section (The sixth paragraph of “Discussion”, blue color). Thanks for your review.

References for this part:


Level of interest: An article whose findings are important to those with clinic.

Reviewer's report
Title: In Vivo Experimental Intervertebral Disc Degeneration Induced by Bleomycin in the Rhesus Monkey
Version: 2Date:10 July 2014
Reviewer: Dafu Chen
Reviewer's report:
Minor Essential Revisions
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Needs some language corrections before being published
Statistical review :Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests: I declare that I have no competing interests

This is an interesting manuscript. The authors reported a new method to establish an early ischemic, progressive intervertebral disc degeneration model by injection of bleomycin into subchondral bone adjacent to the intervertebral discs of rhesus monkeys. The radiological and histological findings of this work showed that subchondral bone injection of bleomycin could induce mild, slowly progressive disc degeneration due to the block of the nutrient pathway that into the intervertebral disc. This work addresses a significant issue of establishing a
slowly progressive disc degeneration model, which is important for the study related to the pathophysiology and therapies of intervertebral disc degeneration. Although this manuscript was well written, there are still some deficiencies and grammar errors need for minor essential revisions or discretionary revisions.

items:
1. In the abstract, the authors should show the full name of the abbreviation of PBS.
Answer: Thanks for your suggestions. We have added the full title of PBS in the abstract.

2. In the abstract, the results showed the inverse correlation between histological score and T1# values of NP and AF, however, the authors did not depicted them in the method section. The authors should supplement the information in the method section according to the consistence between the methods and results section.
Answer: Thanks for your suggestions. We have added the information in the method section: The correlation between histological score, GAGs and T1# values were also analyzed.

3. Page 9, line 146: What pulse sequence is used for T1# MRI? Please provide details. The authors should also show more details of T1# values, so the readers could understand it more easily.
Answer: Thanks for your question. We have added more details of T1# in the “MRI imaging analysis”. The T1#-weighted images were acquired using spin-lock pulses followed by a spin-echo acquisition. The time of spin-lock (TSL) in this study was 2/15/30/45 ms. Using the Siswin software, we mapped these data and exported the data to mapping images.

Answer: Thanks for your question. We used the same method of anesthesia as we used in the first surgery. We have added the details in the manuscript.

5. Figure 2. It is difficult to differentiate the lines between groups. Please use different colors to make it clearer. And according to Fig 2B, 2C, which graph represented the nucleus pulposus? and annulus fibrosus? It is not clear. The authors should revise the title of the longitudinal coordinates of the graphs in order to facilitate the readers.
Answer: Thanks for your suggestions. We have drawn figure 2 again according to the suggestions.

6. Page 15, line 232: There was an error of grammar. “at any time points” should be changed to “at any time point”.
Answer: Thanks a lot for your review. We have revised the error of grammar in the manuscript, which was shown with blue color.

7. Page 15, line 236: The authors should give the P values of the decrease of
T1# values of NP during the second 3 months.

Answer: Thanks for your suggestions. We have added the P values in the manuscript, which was shown with blue color.

Discretionary revisions

1. Table 2. The authors may need to give more details about the abbreviation of the gene names.

Answer: Thanks for your suggestions. We have showed the full title of the gene names in the “Material and Methods” section, so we do not think it is imperative to show more details about the abbreviation of the gene names in table 2. Thanks for your review.

Reviewer’s report
Title: In Vivo Experimental intervertebral disc Degeneration Induced by Bleomycin in the Rhesus Monkey

Version:2 Date:14 July 2014
Reviewer: Juquan Song

Reviewer's report:
The current study is to establish a mild intervertebral disc degeneration animal model in rhesus monkey with bleomycin injection. Authors observed that a time course of radiology and MRI image changes of IVD in rhesus monkey from 1 to 15 months after bleomycin locally injection. Compared to histological morphology and genomic changes at 15 months, they confirmed that bleomycin caused IVD degeneration in rhesus monkeys.

The animal model is very interested and provides a new tool for IVDD research. There is a concern of bleomycin application. Several procedures in method also need to be clarified with typos correction in the paper. The manuscript needs to be revised carefully and polished by a native English writer. The concerns from reviewer are listed as below and divide them into:

- Major Compulsory Revisions

1. Authors applied Bleomycin for the current animal model. First, how did authors determine the dose of bleomycin in the study? Bleomycin is associated with DNA strand breaks, with pulmonary fibrosis and lung function impairment. Are authors aware of the side effect of bleomycin in the current model? Did authors examine pathophysiologic changes of lung tissue during the current? Bleomycin diminished caveolin-1 expression in lung tissue, and caveolin-1 elevated in NP cells from human degenerate discs. Does bleomycin application potentially affect the mechanic pathway study for future therapeutic strategy development? Authors should address those concerns in the revised version.

Answer: Thanks for your questions. We determined the dose of bleomycin according to our preliminary experiment and the safe dose that is usually used in the clinical treatment of vascular malformation. Recently, the use of bleomycin, a
cytotoxic antitumor agent, has been favored for treatment of venous malformations (VMs) because it has been shown to have good clinical efficacy combined with a low side-effect profile [1-3]. It has been reported that, the safe total administered does was limited to 1.0 mg/ml in children younger than 16 years old, and in those older than 16 years old, a 1 to 10 mg of bleomycin was used per session [4]. Based on the previous studies, we injected 1 mg/ml bleomycin into the subchondral bone adjacent the intervertebral disc (IVDs) of rabbits. The results showed that it induced slowly progressive disc degeneration after 12 weeks (Fig 1, 2). So, we also used the same dosage regimen with 1 mg/ml in this study.

Dose-related pulmonary fibrosis is reported in some oncology patients receiving high cumulative doses of intravenous bleomycin [5]. A 13 percent risk of pulmonary fibrosis and 3 percent mortality rate was reported in patients receiving over 450 mg of bleomycin systemically. Below this level, the incidence of pulmonary fibrosis was 3 to 5 percent [6]. To our known, there has been no report about pulmonary fibrosis induced by bleomycin with the safe dosage of 1 mg/ml until now. In this study, although we did not examine pathophysiologic changes of lung tissue, we performed the x-ray check and blood test (such as blood gas analysis) for the rhesus monkeys during experiment, and did not find any positive signs of lung injury.

Actually, the essential role and expression of Caveolin-1 gene in the process of intervertebral disc degeneration is still controversial. Heathfield et al. found that NP cells from degenerate discs exhibited elevated levels of caveolin-1 [7], which suggest that Caveolin-1 may play a prominent role in the pathogenesis of IVD degeneration. However, recently Smolders et al. have found that early IVD degeneration involved down-regulation of Caveolin-1 expression, which appears to be essential to the physiology and preservation of nucleus pulposus cells. Therefore, Caveolin-1 may be regarded as an exciting target for developing strategies for IVD regeneration [8].

To answer the question from the reviewer, we repeated the real-time PCR and supplemented the results of Caveolin-1 gene expression in the IVDs of rhesus monkeys. The results showed that although Caveolin-1 gene expression was downregulated in the degenerative IVDs induced by bleomycin, there was no significant differences between bleomycin group and control groups. Some people may be concerned that Bleomycin may directly affect the cavelolin -1 gene expression. Although it has been reported that bleomycin could induce DNA damage by liberation of free radicals [9], there have been no reports about the message RNA damage induced by bleomycin. Bleomycin has been successfully used in the induction of lung fibrosis animal model, and the models are popularly used for the related research of RNAs expressions [10-12], which indicates that bleomycin does not affect the mechanic pathway study for future therapeutic strategy development.

We have added the results and related discussion in the manuscript.
Figure 1. The histological results of L5 inferior cartilage endplate and annulus fibrosus of rabbit preoperatively, 4 and 6 weeks after subchondral bone injection of bleomycin. (A) Norma L5 cartilage endplate and annulus fibrosus preoperatively (HE×200). (B) The annulus fibrosus showed less organized fibrocartilage lamellae 4 weeks after operation. (C) The cartilage endplate showed a wavy arrangement, and the organization of fibrocartilage lamellae in the annulus fibrosus become more disorderly after 6 weeks.

Figure 2. The MRI and histological changes of L5/6 intervertebral disc 12 weeks after operation. (A) Coronal FST2WI showed hypointense signal of L5/6 intervertebral disc. (B) The wavy arrangement of cartilage endplate and disorder orginazation of fibrocartilage lamellae became more severe 12 weeks after operation.

References for this part
9. Lown JW, Sim SK. The mechanism of the bleomycin-induced cleavage of


2. A recent publication from Wan ZY (Int J Clin Exp Pathol, 2014) demonstrates that TNF, IL1beta, TGF and SMAD increased in human degenerative samples. Authors might provide some data of inflammation response and TGF/smald pathway correlated with the current model.

Answer: Thanks for your suggestions. We analyzed the quantitations of gene expression of inflammatory cytokines (TNF-#, IL-1#, IL-6) and of TGF-#1 in the intervertebral discs using the CDNA we kept before, and supplemented the results and added some discussion in the “Discussion” section, which were shown with blue color words.

- Minor Essential Revisions

1. The reviewer is confused about the content of abstract which needs to be revised carefully

a. Move the objective of study (at Line 38) into the section of Background.

Answer: Thanks for your review. We have move “the objective of study,” (at line 38 )” into the section of Background.

b. There were confusions about animals with reagent rejection, please clarify and correct them. In the method at line 121, injection locations are L3-4 and L5-6; at line 124, injection volume is 2.0ml.

Answer: We have corrected the typos about injection locations in the abstract of this manuscript. At the original line 121, “L3-6” means from L3 to L6. In order to make it more clear. We changed it to ‘the vertebral bodies from L3 to L6’.

According to the injection volume, the subchondral bone adjacent to each endplate was injected with 2ml. So, Because each segment has two endplates (superior and inferior), the total volume of injection for each segment was 4.0ml, which has been shown in the abstract.

c. At line 55, correct negative symbol “r=-0.740”

Answer: Thanks a lot for your review. We apologize to you for the error. We have corrected the symbol.

d. The conclusion makes confusion between statements of a slow progressive IVDD animal model and early stage of MRI effectiveness. Please restate the conclusion.
Answer: Thanks for your review. Our study demonstrated that this method could induce a slowly progressive IVDD animal model. Because this model decreased slowly, and we just investigated the degeneration model for 15 months. So we think it was in the early stage. In order to make this expression become smoother, we deleted the words “early stage”. Thanks.

2. In the method section, there are several procedures need to be clarified.

a. 6 male and 4 female rhesus monkeys were enrolled in the study. Were there activity behaves and body weight different between two genders? If so, please provide the information.

Answer: Thanks for your review. In this study, the three groups consisted of different lumbar segments of the same animals. So the activity behaves and body weight of animal between groups were consistent.

b. Please clarify and revise the sentence of “and written informed consent was obtained from all subjects” in line 107.

Answer: Thanks a lot for your review. We made an error in this sentence. It was not written informed consent. Before enrollment, we got the written files from the animal center, which showed that each monkey was eligible and healthy. We have deleted this sentence, which made the readers feel confused. Thanks.

c. Please confirm and correct the agent delivery location and volume dose.

Answer: Thanks for your review. We have answered this question in 1b.

d. Please provide the detail of animal position when taking roentgenograms procedure.

Answer: Thanks a lot for your suggestion. The lateral views of roentgenograms for the lumbar spine were acquired for all the monkeys in this study. We have added the information in the “IVD Height Measurement” section of the “Methods” in the manuscript.

e. Please provide information of light microscopy for taking histologic images

Answer: Thanks. Actually, we have provided the information of light microscopy in the “Histology evaluation and histological scores” section of the “Methods”.

f. Is there another way such as histologic morphology to assess the number of capillaries in the cartilage endplate at 15 months directly instead of measuring vWF gene expression?

Answer: Thanks for your review. Another way to assess the number of capillaries in the cartilage endplate is to perform micro-CT of the endplate following injection of a fluorescent vascular tracer. However, due to the limited medical condition, it is difficult to perform micro-CT in our research center. So we selected to measure vWF gene expression to indirectly assess the vascular number in this study.

Von Willebrand factor (vWF), a glycoprotein involved in blood coagulation, is mainly synthesized by capillary endothelial cells. vWF is expressed specifically in endothelial cells[1]. It is commonly used as an marker for capillary endothelial cells (EC)[2], and capillary density[3]. In order to evaluate the influence of bleomycin on the capillary density in the cartilage endplate, we analyzed the
quantitation of gene expression of von willebrand factor (vWF) in the cartilage endplate using the same method as mentioned in our manuscript.

References for this part:


3. In Result section,

a. Please clarify the sentence in line 112 “7 IVDs in rhesus monkeys”

Answer: Thanks for your review. That means there are seven intervertebral discs in the rhesus monkey. In order to make it more clear. We have added the full title of IVDs in the manuscript.

b. Data presented is somewhat overlapped in figure 6 and table 3. Please reorganize data presentation form.

Answer: Thanks for your review. Figure 6 is a scatter graph, which can show the correlation between different measurements intuitively, and also can show the trend of the correlation. Table 3 not only showed the $r^2$, but also showed the statistical values. So, we think both of them are complements to each other.

c. In figure 5, please mark and specify the IVD location for each treatment

Answer: Thanks for your review. We have marked the location of lumbar vertebral body in figure 5, and specified the IVD location for each treatment in the figure legends.

Figure 5 The representative T1# maps of lumbar spine in an rhesus monkey model. Bleomycin and PBS solution was injected at L3-4 and L5-6 respectively. Preop: Preoperatively.

4. Authors claimed the current model is closely to mimic clinical patients.

According to age converting to human, it stands for young adults, is it the purpose for this model? Please justify the reason of animal aging selecting in the current study?

Answer: Thanks for your question. In order to minimize the influence of age on intervertebral disc degeneration, we selected the rhesus monkeys with limited range of age (range, 5-7 years). If we had chosen the elder monkey (over 7-9 years old), their lumbar intervertebral disc might have degenerated naturally at some degree. In order to keep all the monkey models in same line, we chosen the younger monkey in this study.

Thanks for your review.