

A Momentary Impact Injury of Vertebral Endplates, even without Structural Disruption, Initiates Disc Degeneration In Vitro.

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Abstract

Background It has been acknowledged that the intervertebral disc degeneration (IDD) is associated with an aberrant cell-mediated response to structural failures, such as vertebral burst fracture, radial fissures, and endplate fracture. However, whether a momentary impact injury of the endplates without structural disruption is sufficient to initiate disc degeneration remains elusive. This study was to further evolve an in vitro momentary impact injury model of IDD and to investigate if a momentary impact load of the endplates without structural disruption could initiate IDD. Methods. Rats spinal segments (from L1/2 to L5/6, n=54) were harvested and randomly assigned into three groups: Control (n=18), Low Impact (12 J/cm³, n=18) and High Impact (25 J/cm³, n=18). Samples in both of the impact groups were subjected axial momentary impact load using a custom-made apparatus, and cultured for 14 days. The degenerative process was investigated by using histomorphology and real-time PCR. Results: The discs in both of the impact groups showed significant degenerative changes at 14 days, both of which showed much higher histological scores and up-regulation of the catabolic (MMP-9, MMP-13) genes transcription than that of the control group (P < 0.05). The discs with endplate fracture compared to that with intact endplate also showed strongly up-regulated catabolic (MMP-9, MMP-13) genes transcription, and more significant degenerative changes based on the histological scoring (P < 0.05). Conclusion: This study demonstrated that a momentary impact load (12 J/cm³) on the spinal segments of the rats could initiate IDD at 14 days after injury and not only endplate fracture but also a momentary impact injury without structural disruption could also promote IDD.

Background

Intervertebral disc degeneration (IDD) is strongly associated with low back pain (LBP)[1,2], which accounts for about \$100 billion in annual medical expenditure of the United States[3]. Degeneration of the disc is usually attributed to aging, genetic, mechanical, nutritional factors, the environment and individual behavioral factors et al[4,5].

Cinotti et al., for instance, demonstrated degenerative changes in the annulus and nucleus, such as decreases in amounts of water, proteoglycans and cells as well as significant morphological alterations[6]. A growing body of evidence suggested that the disc degenerative process could be mainly associated with an aberrant cell-mediated response to structural failures, such as vertebral burst fracture, radial fissures, herniation, and endplate fracture[6-8]. Dudli et al. reported that fracture of vertebral endplates, but not equienergetic impact load, could promote disc degeneration in vitro[8].

Recently, researchers found that, of the people who operated high-speed craft (HSC) and were frequently subjected to high-speed impacts, the incidence of low back injuries was higher than general population[9]. However, whether a momentary impact injury of the endplate without structural disruption, which is common seen in the clinic[10], is sufficient to initiate disc degeneration is still controversial. This could be ascribed to the lack of an appropriate model.

Since a genuine in vivo impact injury model is ethically challenging, the availability of an experimental animal model that consistently reproduces the disease after a momentary impact injury would facilitate the investigations of post-traumatic degenerative process. Although several animal trauma models have been established[11-14], the different nature of injuries causes different emphasis on matrix remodeling, apoptosis, and inflammation and hence poses the question of clinical relevance, in particular as none of them mimic the clinical situation. Therefore, the goal of this study was to further evolve a momentary impact injury model in vitro and to investigate if a momentary impact injury of the endplate without structural disruption could initiate IDD. We hypothesize that not only structural disruption of the endplate but also a momentary impact injury without structural impairment can initiate post-traumatic disc degeneration.

Methods

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) if not stated otherwise.

Full-Organ IVD Culture Model and Trauma Induction. The animal study was also approved by the institutional review board and animal care committee of the Sun Yat-sen University (2013A-204).

Animal Groups and impact loading

Thirty Sprague-Dawley rats (0.4-0.5kg, male, 6-months-old) obtained from Sun Yat-sen University animal facility were euthanatized with 10% chloral hydrate at a dose of 5ml/kg via intra-peritoneal administration. Subsequently, fifty-four Spinal segments (IVD/endplate with approximately 3mm of the adjacent vertebral bodies) from L1/2 to L5/6 (3/animal) were isolated less than four hours, and flushed with 0.9% NaCl containing 50µl/ml penicillin. The segments were trimmed transversely by bone saw (IsoMet, Buehler, Lake Bluff, IL) with the cranial and caudal cutting planes parallel to each other at the height of 7.08 ± 0.21 mm, and perpendicular with respect to the cranial/caudal axis of the segment. The segments were then randomly assigned into three groups: Control (n=18), Low Impact (12 J/cm^3 , n=18) and High Impact (25 J/cm^3 , n=18). The specimens were then subjected to momentary impact load using a custom-made apparatus, which guaranteed axial load. The impact force was recorded using a piezoelectric loadcell (Kistler) (Fig. 1A). Pilot-experiment revealed 25 J/cm^3 as the threshold energy for endplate failure, at which the endplate was expected to fracture in half of the specimens. The height of each specimen was calculated as the vertical distance between the two vertebral cross sections, and recorded before and after impact loading. Based on the pilot-experiment, the specimens with a height decrease more than 10% indicated endplate fracture, whereas less than 10% decrease indicated endplate intact.

After impact, samples were washed with 0.9% NaCl containing 50µl/ml penicillin for three times, and then cultured at 37°C, 5% CO₂ in DMEM (Dulbecco's Modified Eagle Medium, DMEM) with 2% fetal calf serum, 1% Pen/Strep, 50mg/ml ascorbate-2-phosphate and 0.1% Primocin. Half of the samples were collected at

day 7, and the other half were collected at day 14 for histology and mRNA analysis. The assignment of the specimens in each group was shown in Table 1.

Histology

The specimens for histology were fixed with 4% paraformaldehyde for 24 hours at 4°C, then transferred to a sealed vial containing a solution of 70% ethanol and decalcifying agent for at least 30 days. The specimens were then sequentially dehydrated, split down the mid-sagittal plane, and embedded in paraffin for histology sectioning. Serial sections were cut in the transverse plane at 8 µm with a microtome (HM360, Microm International AG, Switzerland), and then stained with hematoxylin & eosin (H&E), safranin O/fast green dyes (Fisher, Scientific, Pittsburgh, PA) by standard procedure and photographed under 40-200x magnification (Nikon Eclipse, Ti, Nikon, Tokyo, Japan). All the sections were imaged under bright field and cross-polarized light.

The changes of the intervertebral disc degeneration were investigated. The disc degeneration assessment scoring system that we developed based on our prior work [15,16] was used to assess the annulus fibrosus, the cellularity of the nucleus pulposus, and the matrix of the nucleus pulposus through sections (Table 2). The histomorphometry assessment was performed by an orthopedic researcher (L.S), who was blinded to the different treatments between groups. All histologic sections were reviewed one month after the first examination to determine the intraobserver reliability. The average score of the two measurements for each specimen was used for the statistical analysis.

Real-time PCR analysis

Followed by the RNeasy Mini Kit (Qiagen Inc., Duesseldorf, Germany), total RNA was extracted from the specimens using Trizol reagent (Ambion, Carlsbad, CA, USA). We selected seven genes as marker for degenerative changes [8,11] (Table 3). The cDNA synthesis was performed as described previously [17]. Each gene expression was quantified by real-time PCR using CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold change in comparison with the control group.

Statistical Analysis

SPSS 25.0 software (SPSS Inc., Chicago, IL) was used for univariate analysis of variance. The data between groups were compared by using t-test. The results of histological scores were analyzed using the Wilcoxon signed ranks test, with a confidence interval of 95%. To assess intraobserver reliability, we used the intraclass correlation coefficient for average and single measurement. The agreement of intraclass correlation coefficient was rated as follows: 0 to 0.4, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.8, substantial agreement, and 0.81 to 1.00, excellent agreement [17]. Statistical significance was indicated at $P \leq 0.05$.

Results

The impact time was less than 0.008Sec for both the Low Impact and the High Impact groups (Fig.1B, D). Significant higher Peak Load ($162\pm 17\text{N}$) and Loading Rate ($49,700\pm 1,752\text{N/Sec}$) were found in the High Impact group than the Low Impact group ($105\pm 4\text{N}$ and $25,430\pm 4753\text{N/Sec}$, respectively) as shown in Figs. 1C and 1E.

With the applied trauma-protocol, there was no specimen with endplate fracture in the Low Impact group. In the High Impact group, endplate fracture occurred in 9 specimens, which has been confirmed by CT scan and histological evaluation (Fig.2). After impact injury, the average height of specimens in the Low and High Impact groups decreased $3.49\pm 0.62\%$ and $7.93\pm 1.34\%$ respectively, both of which showed significant differences in comparison with the control group ($P < 0.05$, Fig.3).

Histological findings

The intervertebral discs in the control group appeared normal. The nucleus pulposus contained abundant cells and sounded by large zones of acellular matrix, and the annulus fibrosus showed normal organization of fibrocartilage lamellae (Fig.4A, F, K). At day 7, the discs in the Low Impact and High Impact groups showed no significant degenerative changes (Fig.4B,C,G,H,I,M), although both of the histological scores were higher than that of the control group (Fig.5A). There was also no significant difference in the histological score between the discs with endplate fractures and that with intact endplates in the High Impact groups ($P > 0.05$, Fig.5B).

At day 14, the discs in both of the Impact groups showed significant degenerative changes, where the nucleus pulposus comprised relatively few cells (Fig.4D,E,I,J), with less deeply stained proteoglycans (Fig.4D,E) relative to the control group. The annulus fibrosus showed less organized fibrocartilage lamellae, as compared with the control group, and the collagen fibers formed a wavy arrangement (Fig.4N,O). Both of the histological scores in the Low and High Impact groups were significantly higher than that of the control group ($P < 0.05$, Fig.5A). There was no significant difference in the histological score between the Low and High Impact groups ($P > 0.05$, Fig.5A). However, the average histological score of discs with endplate fracture was much higher than that with intact endplate in the High Impact group ($P < 0.05$, Fig.5B). The intraclass correlation coefficient was 0.923 for a single measurement, which showed strong agreement.

Real-time PCR

Both of the Low and High impact injuries caused an up-regulation of the catabolic (MMP-9, MMP-13) genes transcription in comparison with the control group after injury, especially in the Low Impact group at day 14 ($P < 0.05$, Fig.6A,B). The pro-inflammatory (IL-6) gene transcription was also strongly up-regulated in the Low Impact group at day 7 ($P < 0.05$, Fig.6C); however, a subsequent decrease of gene transcription was noticed at day 14. There was no significant differences in the IL-6 gene transcription in the High Impact group at day 7 and day 14 ($P > 0.05$, Fig.6C). For the anabolic (TGF- β , Col1 α 1, Col3 α 1) genes, col1 α 1 and col3 α 1 were strongly up-regulated at day 7 in the High Impact group but reverse to down-regulation at day 14 (Fig.6D,E). Although col3 α 1 was also up-regulated at day 7 and day 14 in the

Low Impact group, there was no significant difference in comparison with the control level. Gene expression of TGF- β was not changed in both the Low and High Impact groups(Fig.6F).

In comparison with the discs with intact endplate, the catabolic (MMP-9, MMP-13) genes transcription were strongly up-regulated at day 7 in the discs with endplate fracture($P < 0.05$, Fig.7). Although the regulations of anabolic (TGF- β , Col1 α 1, Col3 α 1) genes transcription was also much higher but did not reach significance. Due to the limited number of samples at day 14, we could not find any significant difference in each gene expression between the disc with an intact endplate and that with endplate fracture (data were not shown).

Discussion

We aimed to develop an in vitro impact injury model that could mimic the clinical situation to some extent, and to investigate if a momentary impact injury without structural disruption could initiate intervertebral disc degeneration. Therefore, histomorphometric and biological analysis after in vitro trauma were performed on spinal segments with different level of impact loading. The results showed that, not only structural disruption of the endplate but also a momentary impact injury without structural impairment could initiate post-traumatic disc degeneration.

Although intervertebral disc degeneration(IDD) related to impact injury in the clinic is not uncommon, whether and how it influences the onset of the IDD changes is still not well documented. In order to investigate the post-traumatic degenerative process, in vivo and in vitro models have been developed. Animal trauma models are usually divided into three main classes: stab incision models, overload models, and endplate perforation models[13]; however, none of them mimic the clinical situation. In this study, we designed a dropped-weight apparatus for the sterile induction of impact injury and an accompanying injury of the adjacent intervertebral disc, using different level of energy. The histomorphological findings showed that the proteoglycans in nucleus pulposus decreased significantly and the fibrocartilage lamellae showed less organized in the low- and high-impact injury groups, compared with the control groups. These changes were consistent with the previous studies showing loss of proteoglycans and less organized collagens during the initial phase of disc degeneration [19-21].

The degenerative changes in the IVDs, such as the composition of extracellular matrix (ECM), loss of disc cells, proteoglycans and water content, have been suggested to be the consequence of an up-regulation of catabolic matrix metalloproteinases (MMPs) and pro-inflammatory (IL-1 β , TNF- α , IL-6) gene transcriptions[22-26]. Consistent with the previous reports, we found that mRNA expressions of catabolic (MMP-9, MMP-13) genes were up-regulated in the impact injury groups, especially at day 14 in the low-impact group. The pro-inflammatory (IL-6) gene was also up-regulated at day 7 in both the low and high-impact groups. The correlation between inflammatory gene regulation and catabolic gene transcription may indicate interactions between their transcription regulations. It has been reported that the activity of MMPs is not limited to matrix cleavage; they also modulate the inflammatory response[27], which may explain why degenerative discs were also sensitive to pro-inflammatory stimuli[28]. It has been reported

that TGF- β was a major up-stream regulator of collagens and proteoglycans[29]. However, TGF- β gene transcription was not affected in this study. The remodeling response of TGF- β may be compensated by the inhibiting effect of the pro-inflammatory cytokines[30]. This needs to be verified by further study.

It is well known that, the nutritional pathways that into the nucleus pulposus of human intervertebral discs are mainly by diffusion through the central portion of the end-plate from these marrow space cartilage contacts[11]. Thus, a functional intact endplate is crucial to transport nutrients and waste products by diffusion[31,32]. Dudli et al.[8] characterized the process of disc degeneration using an in vitro full-organ model, and elucidated that burst endplates, but not equienergetic loading promoted disc degeneration. However, Chan et al.[33] reported that, impact load which was generally sub-traumatic, could also cause cumulative damage and injury to the lumbar spine. In the current study, we also found that discs with intact endplate in the impact groups also showed degenerative signs based on the histomorphological findings and the catabolic (MMP-9, MMP-13) genes transcription, although it was not so significant as that with endplate fracture. This indicates that not only endplate fracture but also a single impact injury can initiate degenerative changes of intervertebral discs.

Taken together, an in vitro intervertebral disc degeneration model with impact injury has been developed and applied to demonstrate that not only structural disruption of the endplate but also a single impact injury without structural impairment can initiate post-traumatic disc degenerative changes, which are confirmed by histological changes such as less deeply stained proteoglycans and less organized fibrocartilage lamellae. This is also accompanied by the induction of collagenases (MMP-9, MMP-13) and up-regulation of pro-inflammatory (IL-6) gene transcription, which are recognized to be involved in disc degeneration.

However, there are several limitations to the study. Firstly, there are some biomechanical and anatomic differences between the spine of the rats and that of the human. In addition, it is difficult to compare the momentary impact energy exactly to that of human falling. Thus, this animal model just mimics the clinical situation of falling to some extent; however, the objective of this study was to evolve an in vitro impact injury model of IVD and to investigate the roles of a momentary impact injury of the endplates without structural disruption on the process of intervertebral disc degeneration (IDD), and the results may provide some clues for us to investigate the initiation of disc degeneration after momentary axial impact injury of endplates, even without structural failure.

To be honest, drawing conclusions from animal models as well as from in vitro study is always problematic. The increased gene transcription that demonstrated with quantitative PCR, may not essentially induce a functional gene product, as mechanisms like post-transcriptional modifications or gene silencing may interfere. With matrix metalloproteinases, just a small aspect of the characteristics of disc degeneration was studied. Finally, due to the limited number of samples in this study, we did not focus on the difference in the gene expression between the discs with intact endplate and that with endplate fracture. So, further studies are warranted to verify the results of the current study.

Conclusions

This current study demonstrates that a momentary impact loading (12 J/cm³) on the spinal segments of rats could initiate intervertebral disc degeneration (IDD) at 14 days after injury and not only endplate impairment but also a momentary impact loading without structural disruption could also promote IDD.

Abbreviations

IVD: Intervertebral disc; IDD: Intervertebral disc degeneration; LBP: low back pain; MMP: matrix metalloproteinase; TGF- β : Transforming growth factor- β ; Col1 α 1: Collagen-1 α 1; Col3 α 1: Collagen-3 α 1; DMEM: Dulbecco's Modified Eagle Medium; H&E: hematoxylin & eosin; GAPDH: glyceraldehyde 3-phosphate dehydrogenase;

Declarations

Ethics approval and consent to participate

The animal study was also approved by the institutional review board and animal care committee of the Sun Yat-sen university (2013A-204).

Consent for publication

All the authors consent to publish this manuscript

Availability of data and materials

Applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZS drafted the manuscript substantially. XW carried out the acquisition and interpretation of data. XP and GC carried out the radiological measurements, the acquisition and interpretation of data. XL and JY carried out the histological assessments. ZL, SL and XW carried out the sample collection and the experiment of Real-time PCR. WY carried out the statistical analysis. FW and WY carried out the design of this study, animal model establishment and gave final approval of the version to be published. All authors read and approved the final manuscript.

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Figures

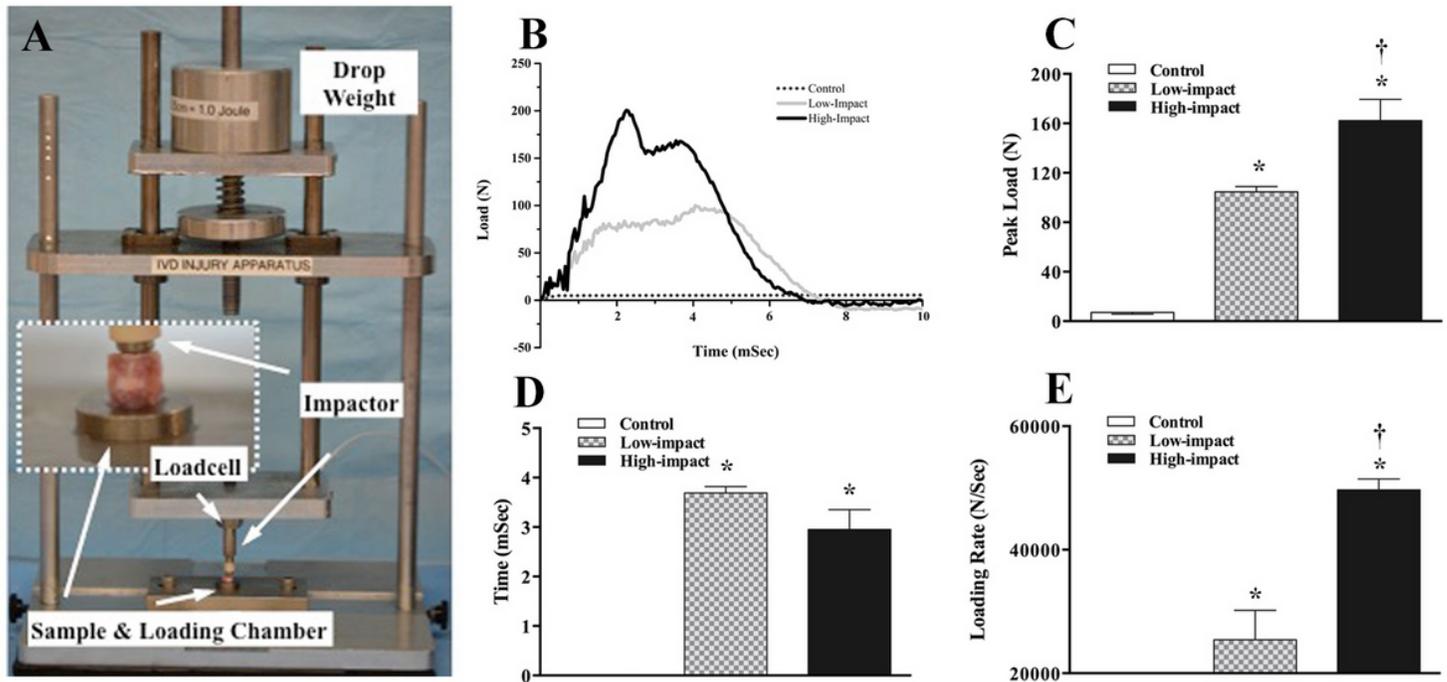


Figure 1

An ex vivo impact injury model for intervertebral disc (IVD) degeneration. A IVD Impact Apparatus; B Impact load profile versus time, C Peak load, D Time to reach Peak Load, and E Loading Rate in the Control, Low Impact and High Impact groups(* $P \leq 0.05$ vs. Control group; † $P \leq 0.05$ vs. Low Impact group).



Figure 2

The micro-CT imaging and histology image of a spinal segment with endplate fracture. A,B The sagittal and transverse view of the micro-CT showed the fracture of vertebral endplate. C,D The H&E and B safranin O staining demonstrated the fracture of vertebral endplate (EP), combined with an osseous disc herniation(DH).

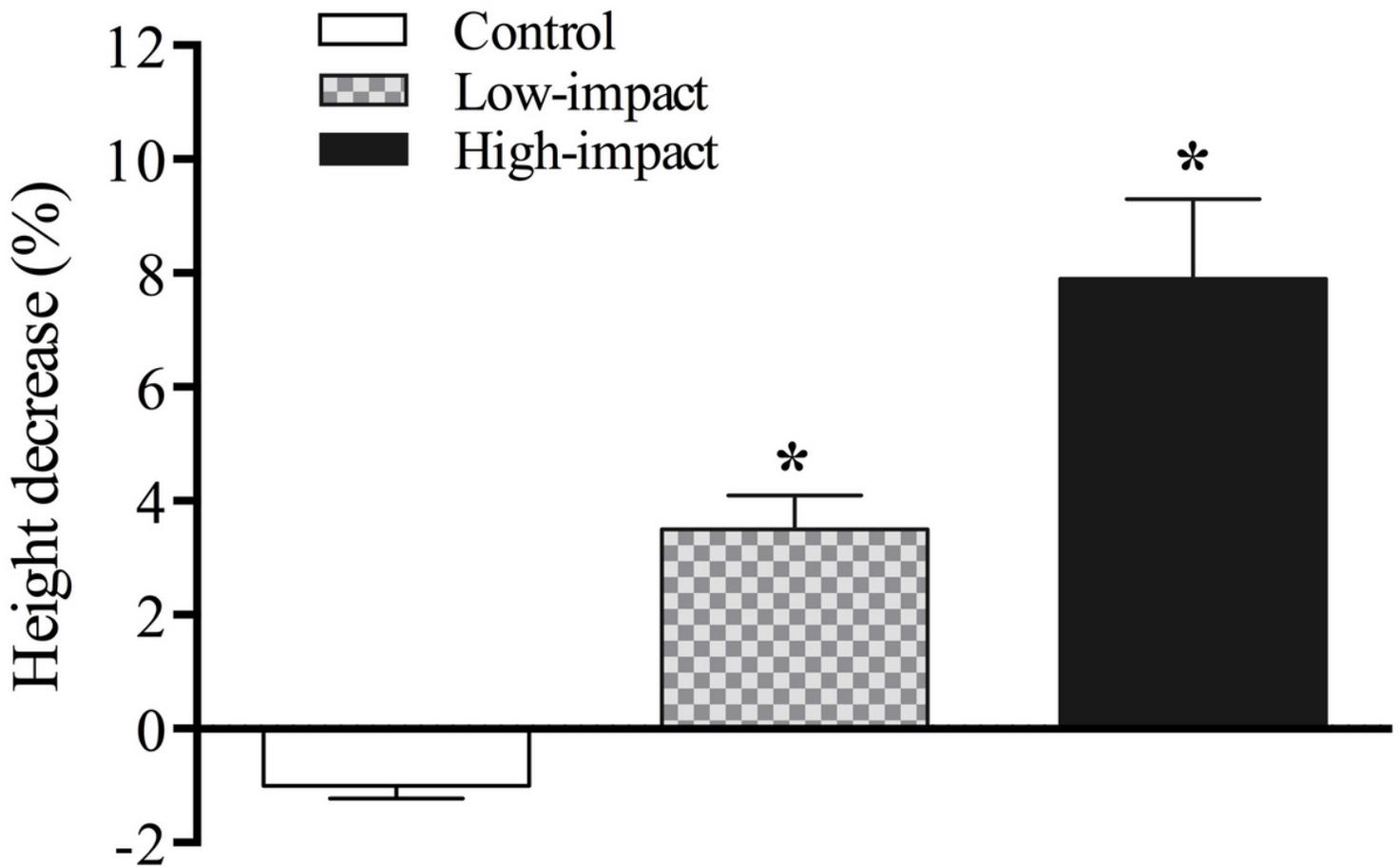


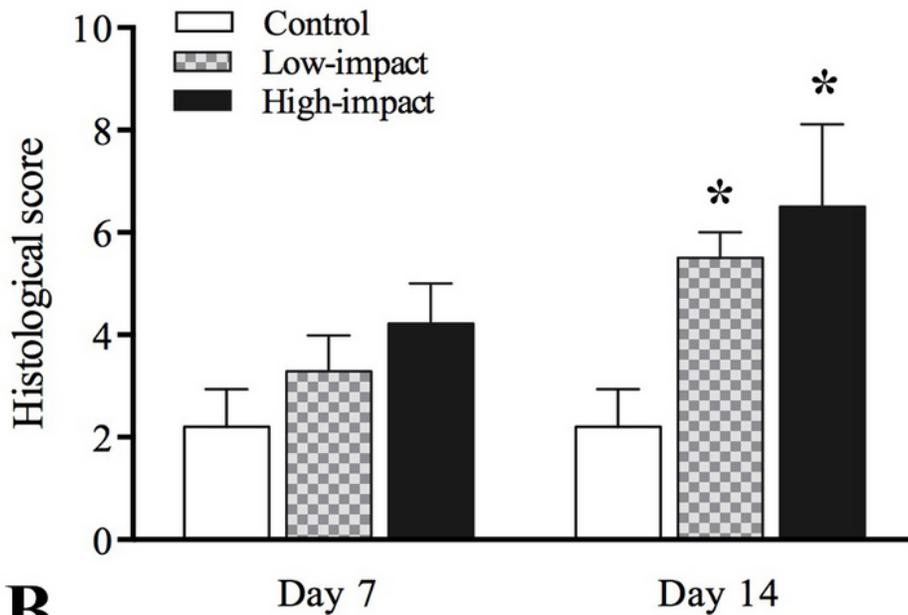
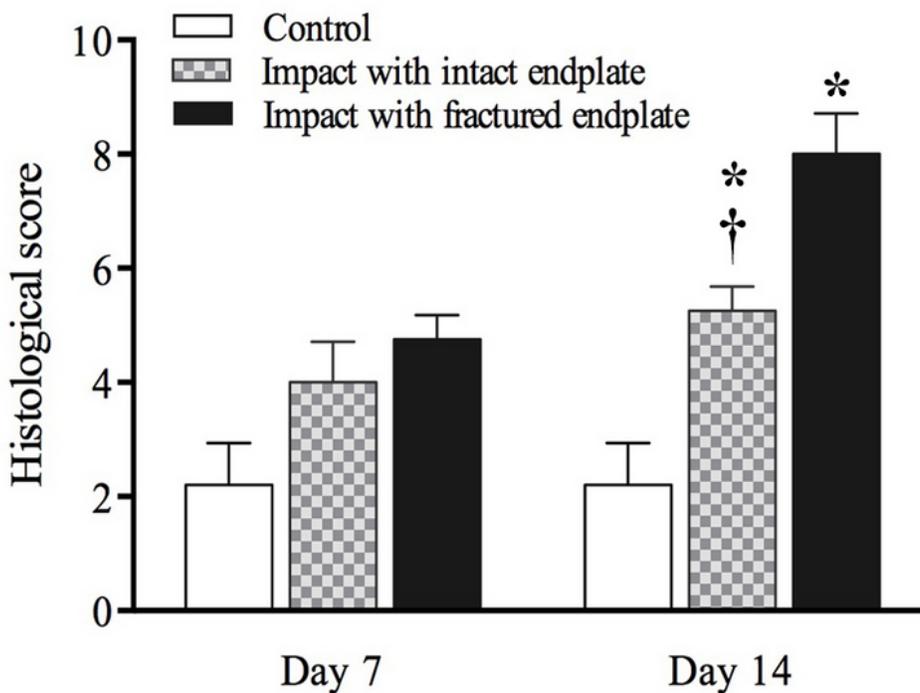
Figure 3

This graph shows the mean percentage of decrease of sample height between groups. There was a significant decrease in the height changes of High-impact group in comparison with the Control and the Low-impact groups (* $P < 0.05$ vs Control and Low-impact groups).



Figure 4

Representative hematoxylin & eosin (H&E) and safranin O/fast green stained sagittal sections of spinal segments under (A-J) brightfield and (K-O) polarized light ($\times 200$) in different groups. A, F, K The intervertebral disc (IVD) in the control group showed that the nucleus pulposus contained abundant cells and surrounded by large zones of acellular matrix, and the annulus fibrosus showed normal organization of fibrocartilage lamellae. B, V, G, H, L, M The discs in the Low and High Impact groups at day 7 after injury showed no significant degenerative changes, although the nucleus pulposus contained less cells and the annulus fibrosus showed less organization of fibrocartilage lamellae. D, E, I, J, N, O The discs in the Low and High Impact groups at day 14 after injury showed significant degenerative changes, where the nucleus pulposus comprised relatively few cells (D, E, I, J), with less deeply stained proteoglycans, and the collagen fibers formed a wavy arrangement in the annulus fibrosus (N, O).

A**B****Figure 5**

The histological scores of the IVDs between different groups at day 7 and day 14 after injury. A: This graph showed that both of the histological scores in the Low and High Impact groups were significantly higher than that of the control group (* $P < 0.05$ vs. Control group). B: This graph showed that the average histological score of discs with endplate fracture was much higher than that with intact endplate in the High Impact group at 14 days after injury (* $P < 0.05$ vs. Control group; † $P < 0.05$ vs. High Impact group).

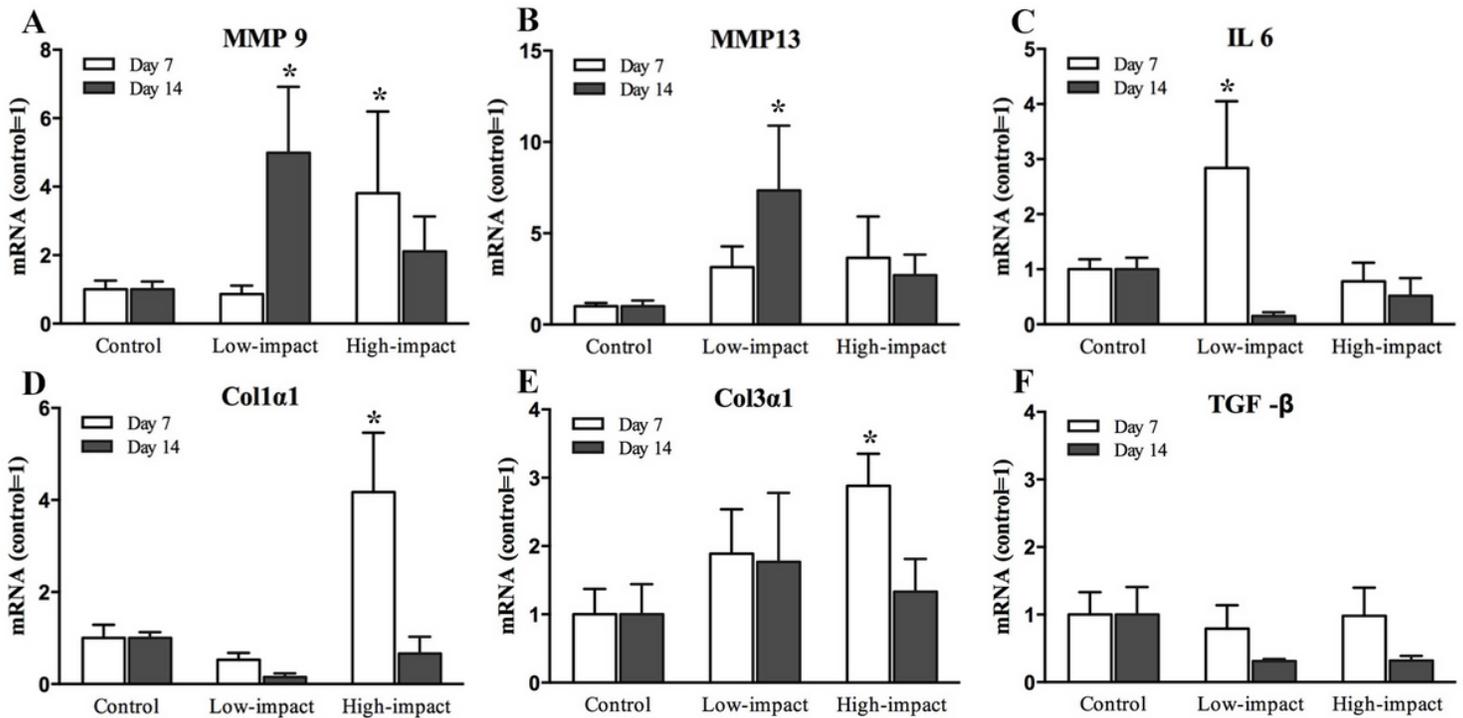


Figure 6

The genes transcription of (A) MMP-9, (B) MMP-13, (C) IL-6, (D) Col1a1, (E) Col3a1, and (F) TGF-β in the intervertebral discs of different groups. A, B Both of the Low and High impact injury caused an up-regulation of the catabolic (MMP-9, MMP-13) genes transcription in comparison with the control group after injury, especially in the Low Impact group at day 14 ($P < 0.05$). C The pro-inflammatory (IL-6) gene transcription was also strongly up-regulated in the Low Impact group at day 7 ($P < 0.05$); however, a subsequent decrease of gene transcription was noticed at day 14. D,E Col1a1 and col3a1 were strongly up-regulated at day 7 in the High Impact group but reverse to down-regulation at day 14. Although col3a1 was also up-regulated at day 7 and day 14 in the Low Impact group, there was no significant difference in comparison with the control level. F Gene expression of TGF-β was Significantly downregulated at day 14 in the High Impact groups. * $P < 0.05$ vs. Control group.

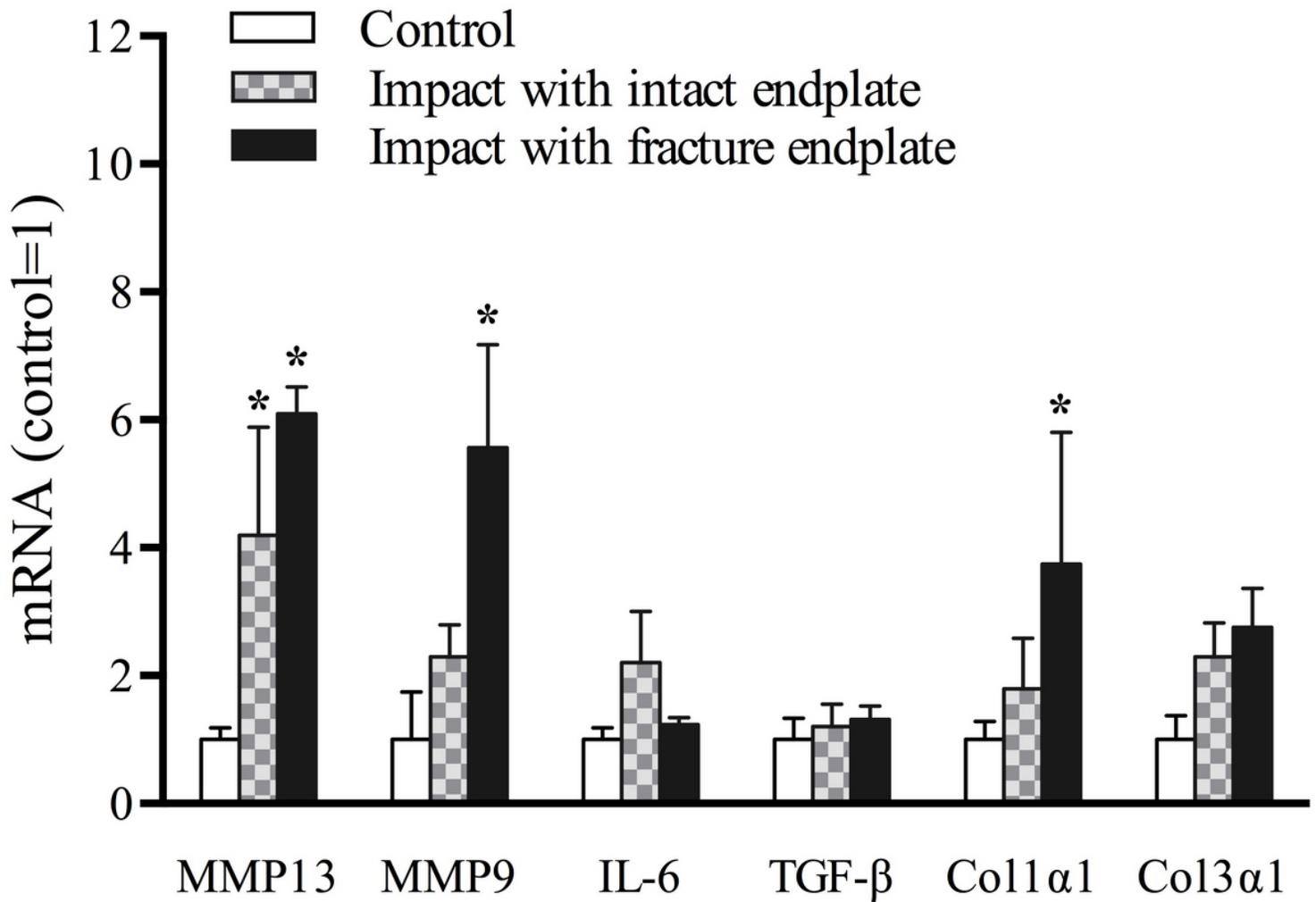


Figure 7

The comparison of genes transcription of (A) MMP-9, (B) MMP-13, (C) IL-6, (D) Col1α1, (E) Col3α1, and (F) TGF-β between the intervertebral discs with endplate fracture and that with intact endplate. * $P < 0.05$ vs. Control group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [NC3RsARRIVEGuidelinesChecklist2014BMC.pdf](#)