

Original Article

Intradiscal injection of crocin retards lesion induced intervertebral disc degeneration

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Received January 5, 2016; Accepted March 18, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: Intervertebral disc degenerative diseases have become a serious social health problem. However, there is no clinical treatment effectively retarding the progression of intervertebral disc degeneration. Crocin, which is extracted from saffron, has been proved to exert anti-inflammatory and anti-catabolic effects on intervertebral disc in vitro and ex vivo. In the present study, effects of crocin on lesion induced rat intervertebral disc degeneration were investigated. As shown by results, intradiscal crocin injection rescued the MRI signal loss of nucleus pulposus in T2-weighted images. The excessive mRNA expression of MMP-3 and ADAMTS-5 induced by puncture lesion was significantly suppressed by crocin injection. Meanwhile, crocin treatment partly rescued the impaired mRNA expression of type II collagen and aggrecan. Obviously, crocin injection maintained the normal morphology of disc and alleviated lesion induced degeneration related histological changes reducing histological scores. Immunohistochemical assay showed that the upregulation of MMP-3 and ADAMTS-5 protein expression in nucleus pulposus was also inhibited by crocin treatment. According to biochemical assay, crocin injection suppressed the lesion induced decrease of proteoglycan content in nucleus pulposus, which is consistent with mRNA expression analysis. Our results further prove the protective effects of crocin on intervertebral disc degeneration, suggesting that crocin is promising for retarding the progression of intervertebral disc degeneration.

Keywords: Crocin, intervertebral disc degeneration model, extracellular matrix, magnetic resonance imaging

Introduction

Low back pain, as a global health problem, causes serious social and economic burden [1]. Intervertebral disc degeneration is believed to be the most important contributor to low back pain and other spine degenerative diseases [2, 3]. Intervertebral disc degeneration is highly correlated to degeneration related inflammatory responses, which are characterized by excessive degradation and impaired synthesis of extracellular matrix (ECM) in intervertebral disc, especially in nucleus pulposus (NP) [4]. The imbalance between catabolism and anabolism of ECM leads to deterioration of biological and biomechanical function of NP, resulting in intervertebral disc degeneration [5].

The main constituents of ECM in NP include type II collagen (collagen II) and proteoglycan. In NP, distribution of type II collagen presents an amorphous arrangement, helping to maintain the characteristic viscoelasticity of NP [6]. Aggrecan, the most common proteoglycan

making up half of NP dry weight, is responsible for the hydrophilic nature and high water content of NP, whose magnetic resonance imaging (MRI) signal is high in T2-weighted images [7]. In intervertebral disc degeneration progression, both type II collagen and proteoglycan are excessively degraded by degradative enzymes, mainly including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) enzyme family [6]. Previous researches have demonstrated the upregulation of MMP-3 and ADAMTS-5 in degenerated intervertebral disc, suggesting their role in ECM catabolism [8, 9].

However, current treatments for intervertebral disc degenerative diseases are limited to conservative treatment (e.g., physical therapy and oral analgesics) and surgical intervention, which can only ameliorate symptoms without addressing the etiological problem [10-12]. Despite many studies focusing on intervertebral disc regeneration, growth factor and other

Table 1. Sequences of primers used in quantitative real-time PCR

Gene	Primer sequences (5'-3')	
MMP-3	Forward	TTTGGCCGTCTCTCCATCC
	Reverse	GCATCGATCTTCTGGACGGT
ADAMTS-5	Forward	CCGAACGAGTTTACGGGGAT
	Reverse	TGTGCGTCGCCTAGAACTAC
Aggrecan	Forward	CAGATGGCACCTCCGATAC
	Reverse	GACACACCTCGGAAGCAGAA
Collagen-II	Forward	GGCCAGGATGCCCGAAAATTA
	Reverse	ACCCCTCTCTCCCTTGTCAC
β -actin	Forward	AACCTTCTTGAGCTCCTCCG
	Reverse	CCATACCCACCATCACACCT

regenerative treatment has not been applied to clinical practice [1, 13]. Moreover, few studies focus on effects of natural compounds extracted from herbal medicine on intervertebral disc, whose anti-inflammatory bioactivities may be promising in retardation of intervertebral disc degeneration.

Crocus sativus L. (or saffron), which belongs to Iridaceae, is often used as a spice for food preparations and an anodyne or tranquilizer in Chinese traditional medicine. Crocin is the main constituent responsible for the multiple bioactivities of saffron among the extracts. Our previous research have demonstrated the anti-inflammatory and anti-catabolic effects of crocin on rat intervertebral discs in vitro and ex vivo [14]. The present study aim to further test the effects of crocin on rat intervertebral disc in vivo, using a lesion induced intervertebral disc degeneration model. However, to analyze effects of crocin on early stage of lesion induced intervertebral disc degeneration, the rat intervertebral disc degeneration model is modified from previous studies with earlier time point of injection and shorter degeneration induction duration [15, 16]. In the present study, crocin was injected into stabbed intervertebral disc and the effects of crocin on lesion induced intervertebral disc degeneration were analyzed by magnetic resonance imaging (MRI), mRNA expression, histological and biochemical assays.

Materials and methods

Animal model

Three-month-old Sprague-Dawley rats were provided by Experimental Animal Center of Shanghai Ninth People's Hospital. This study

was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Experiment Committee of Shanghai Jiao Tong University School of Medicine. The surgery was carried out as previously described with modification [17]. To be brief, under general anesthesia, rat tail was palpated to determine the disc levels. Tail skin and all surgical instruments were sterilized to avoid infection. After a small skin incision was made, a 20-gauge (G) needle was inserted into the middle of the appropriate disc, through the AF into the NP of Co4/Co5 and Co6/Co7 until AF on the opposite side was ruptured. Then the needle was rotated 360° and held for 30 seconds. The Co5/Co6 level remained intact with only skin incision as the control. Immediately after disc puncture, 2 μ l of crocin (5M, Sigma-Aldrich, St. Louis, MO, USA) diluted in normal saline (NS) was injected into NP of Co4/Co5 using a micro-syringe attached to a 31-G needle. Likewise, NP of Co6/Co7 was injected with 2 μ l of normal saline. For all the experiments, rat discs were divided into three groups: the intact control group; the lesion with NS alone injection group; the lesion with NS and crocin injection group.

MRI procedure

14 days after puncture, under general anesthesia, rats were laid prone and all tails were assessed by MRI. All MRI measurements were carried out on a clinical 3T whole-body MR scanner (MAGNETOM Trio, Siemens Healthcare, Erlangen, Germany) using a dedicated coil for small animal. 10 serial T2-weighted sagittal images covering the entire disc area of each tail were obtained with the following parameters: spin-echo sequence, fat saturation on, repetition time/effective echo time = 3000 ms/30 ms, field of view = 50×100 mm, Matrix = 192×384, slice thickness = 0.6 mm, slice spacing = 0.06 mm, number of slices = 10, number of averages = 1, scan duration = 9 minutes and 35 seconds. The signal intensity of NP region in T2-weighted images was calculated as gray value using Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA, USA). Five serial T2-weighted sagittal images of each disc were analyzed for mean gray value of entire disc, and all discs were assessed for each group.

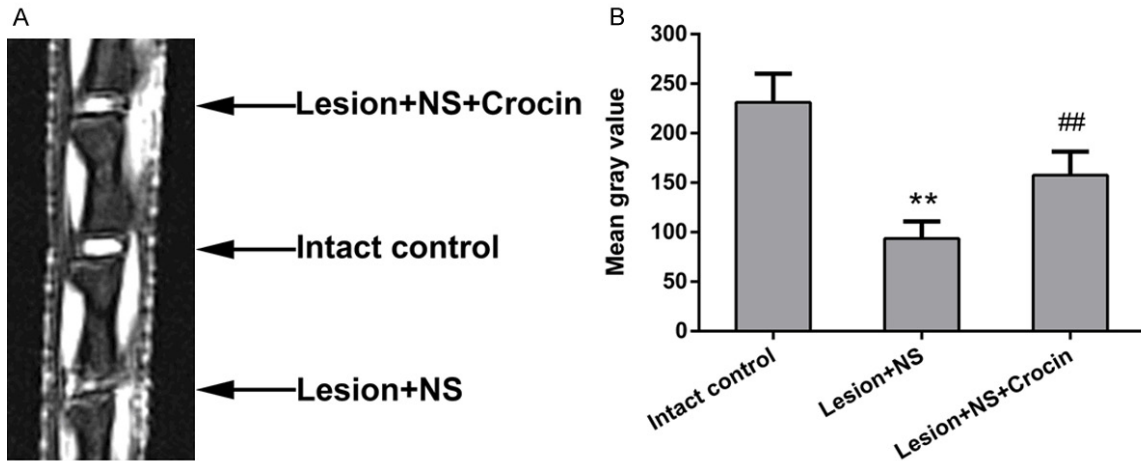


Figure 1. MRI analysis 14 days after puncture. A. Representative T2-weighted midsagittal image. B. Quantitative analysis of the gray value of NP region in T2-weighted images. Results were expressed as mean $\pm$ standard deviation. ** $P < 0.01$ compared with the intact control group; ## $P < 0.01$ compared with the lesion with NS alone injection group.

Quantitative real-time polymerase chain reaction

After MRI procedure, total RNA of NP was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Reverse transcription was carried out with 1 μ g of total RNA using a reverse transcriptase kit (Takara Bio, Inc., Otsu, Japan) for first-strand complementary DNA (cDNA) synthesis. Quantitative real-time polymerase chain reaction (qPCR) was conducted with a SYBR Premix Ex Taq kit (Takara Bio, Inc.) and an ABI 7500 Sequencing Detection System (Applied Biosystems/Life Technologies, Foster City, CA, USA) according to the manufacturer's instructions. Relative gene expression was measured using the $\Delta\Delta C_t$ method with β -actin as an internal control. The primer sequences are presented in **Table 1**.

Histological analysis

After MRI procedure, rats were euthanized and discs from each group were harvested for histological analysis. Discs were fixed in 4% paraformaldehyde, and then decalcified in EDTA for 14 days. The decalcified discs were embedded in paraffin for sectioning. Serial mid-sagittal sections of discs (5- μ m thick) were obtained to prepare slides. All mid-sagittal sections were stained with hematoxylin-eosin and Safranin O-fast green to assess the degeneration of IVD. All H&E staining and Safranin O-fast green staining slides were imaged under transmitted light illumination using a microscope (LEICA

DM4000 B; Leica Microsystems). The histological sections were graded by blinded observers using a histologic grading scale established previously [17]. The expression of MMP-3 and ADAMTS-5 was detected by immunohistochemistry (IHC) using respective first antibodies (1:100, Abcam, Cambridge, MA, USA) and a horseradish peroxidase-conjugated secondary antibody (1:50; Dako, Glostrup, Denmark), followed by color development with diaminobenzidine tetrahydrochloride (DAB, Dako). The results of IHC were quantified in mean density with the IPP version 6.0 software (Media Cybernetics, Bethesda, MD, USA).

Biochemical analysis

After MRI procedure, NP tissue was isolated from disc and then digested with papain at 37°C for 48 h. Proteoglycan content of papain digests was determined by the dimethylmethylene blue (DMMB) assay as previously described [18]. The dsDNA (double-strand DNA) content of NP tissue was determined using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA).

Statistical analysis

All results were expressed as mean $\pm$ standard deviation. The statistical analysis was performed with a one-way analysis of variance (ANOVA) for multiple comparisons using SPSS 19.0 (IBM, Inc., NY, USA). A value of $P < 0.05$ was considered to indicate a statistically significant difference.

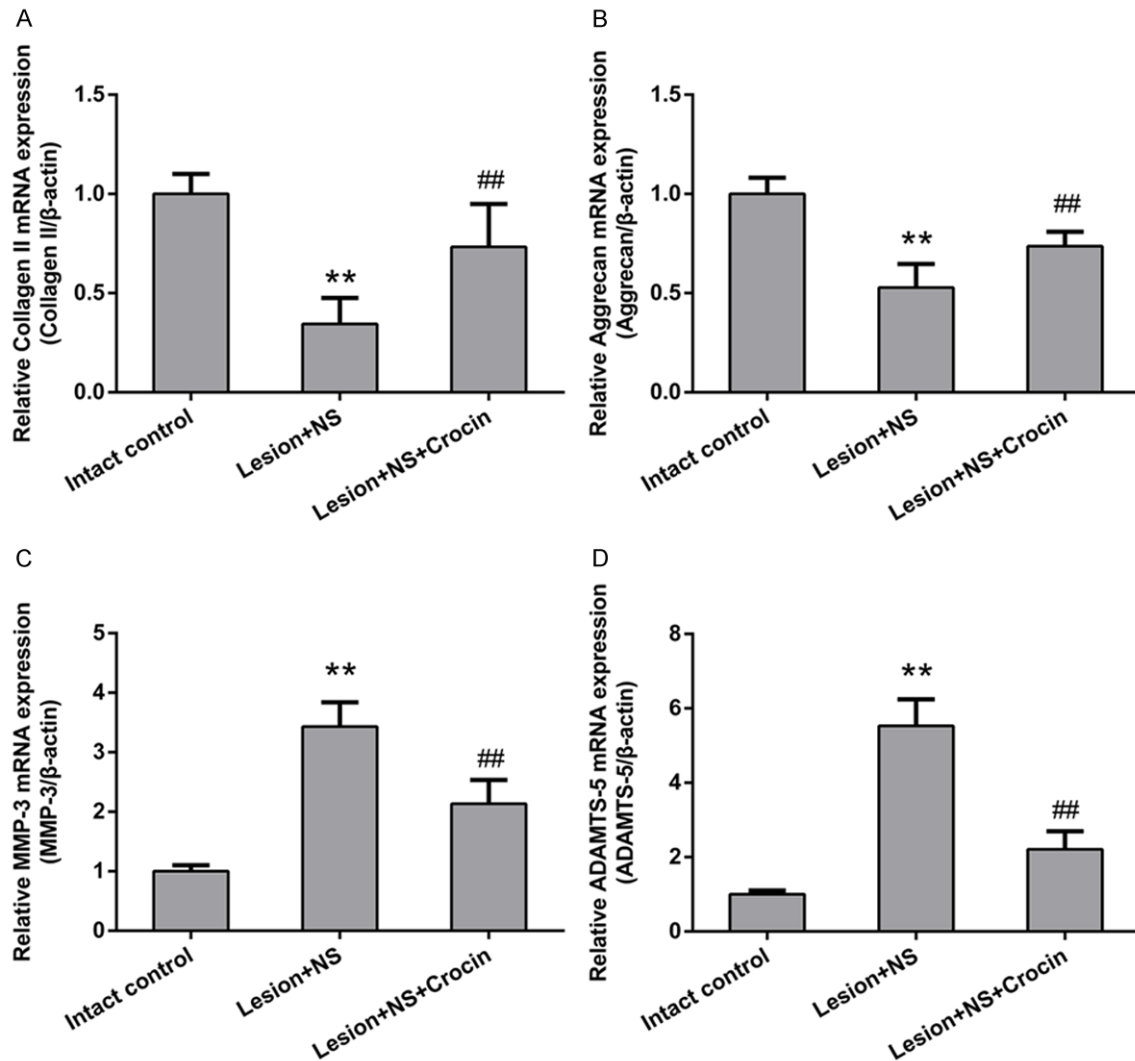


Figure 2. Quantitative real-time PCR analysis. A. mRNA expression of type II collagen. B. mRNA expression of aggrecan. C. mRNA expression of MMP-3. D. mRNA expression of ADAMTS-5. Results were expressed as mean $\pm$ standard deviation with β -actin as an internal control. ** $P < 0.01$ compared with the intact control group; ## $P < 0.01$ compared with the lesion with NS alone injection group.

Results

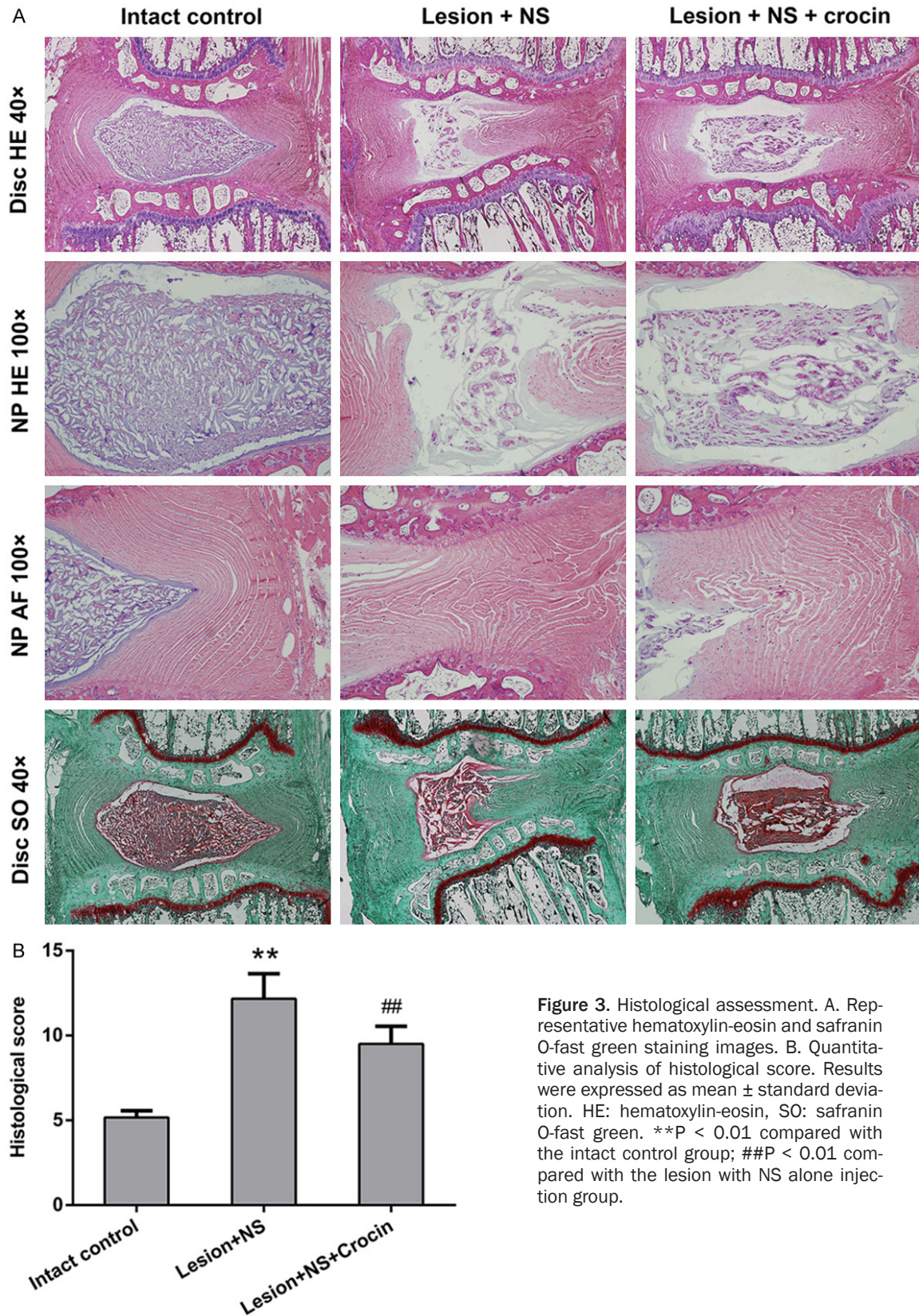
MRI analysis

14 days after puncture, as shown in the representative T2-weighted midsagittal images, signal intensity of NP region in lesion with NS alone injection group was lower than that in the intact control group, while crocin injection increase the signal intensity compared to lesion with NS alone injection group (**Figure 1A**). In T2-weighted images, the gray value of NP region in lesion with NS injection group was significantly decreased ($P < 0.01$, **Figure 1B**). However, compared with lesion with NS alone

injection group, the gray value of NP region in the lesion with NS and crocin injection group was significantly increased ($P < 0.01$, **Figure 1B**). Considering that signal intensity of NP region in T2-weighted images represents water content of NP, these results demonstrate the protective effects of crocin on lesion induced intervertebral disc degeneration.

mRNA expression of type II collagen, aggrecan, MMP-3, ADAMTS-5

Compared to lesion with NS alone injection group, group with crocin injection showed a significant increase in mRNA expression of type II



collagen compared with lesion with NS alone injection group ($P < 0.01$, **Figure 2A**), which was

significantly decreased by puncture lesion compared with the control group ($P < 0.01$,

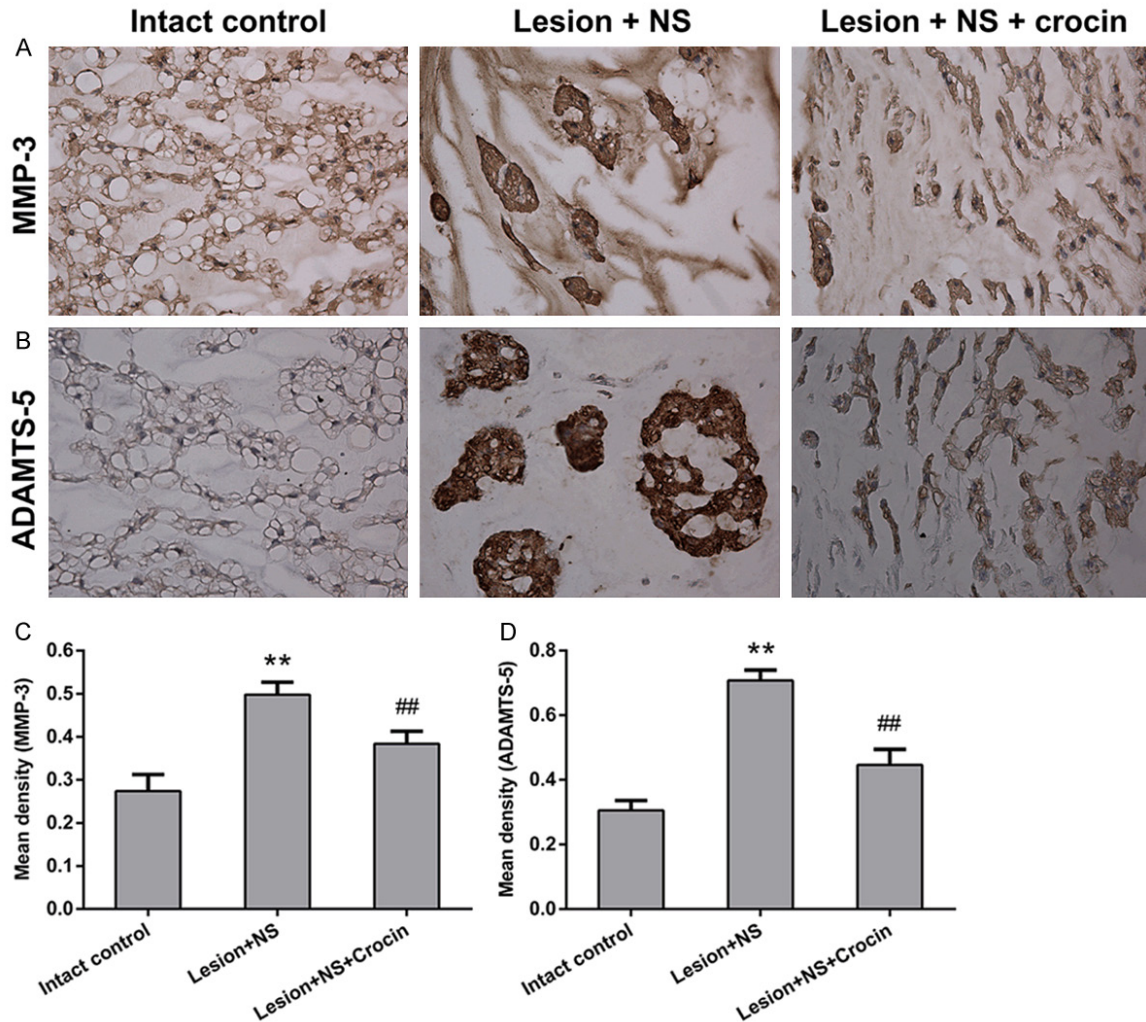


Figure 4. Immunohistochemical qualitative and quantitative assay (magnification 400×). A. MMP-3 expression in NP. B. ADAMTS-5 expression in NP. C. Quantitative analysis of MMP-3 expression. D. Quantitative analysis of ADAMTS-5 expression. Results were expressed as mean $\pm$ standard deviation. ** $P < 0.01$ compared with the intact control group; ## $P < 0.01$ compared with the lesion with NS alone injection group.

Figure 2A). The mRNA expression of aggrecan was also decreased by puncture lesion and rescued by crocin injection significantly ($P < 0.01$, **Figure 2B**). Puncture lesion caused a significant upregulation of mRNA expression of both MMP-3 and ADAMTS-5 compared with the control group ($P < 0.01$, **Figure 2C, 2D**). In group with crocin injection, mRNA expression of both MMP-3 and ADAMTS-5 were significantly lower than that in lesion with NS alone injection group ($P < 0.01$, **Figure 2C, 2D**).

Histological changes

As shown by hematoxylin-eosin and safranin O-fast green staining images (**Figure 3A**), NP of

the intact control group presented round gelatinous appearance comprising at least half of the disc area in midsagittal sections with stellar shaped nucleus pulposus cells evenly distributed. AF of the intact control group was intact with well-organized fibrous lamellae and a clear defined border existed between NP and AF. Obviously, compared with the intact control group, NP of lesion with NS alone injection group lost regular gelatinous appearance accompanied by partly replacement of fibrous tissue with weak safranin O staining (**Figure 3A**). Meanwhile, puncture lesion decreased the size of the NP, disrupted the organization of AF lamellae and interrupted the border between the AF and NP (**Figure 3A**). However, crocin

Crocin retards intervertebral disc degeneration

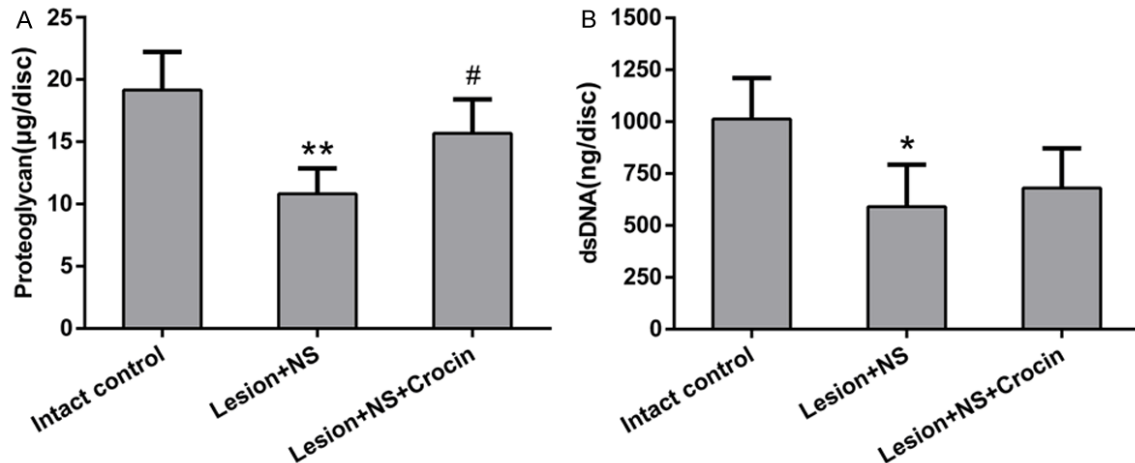


Figure 5. Biochemical analysis of proteoglycan and dsDNA content in NP. A. Proteoglycan content assay. B. dsDNA content assay. Results were expressed as mean $\pm$ standard deviation. * $P < 0.05$ compared with the intact control group; ** $P < 0.01$ compared with the intact control group; # $P < 0.05$ compared with the lesion with NS alone injection group.

injection rescued the lesion induced histological changes compared with only NS alone injection group (**Figure 3A**). Analysis of histological score shows the histological score of lesion with NS alone injection group was significantly increased compared with the intact control group ($P < 0.01$, **Figure 3B**). Consistent with MRI analysis, the histological score of lesion with crocin injection group was significantly lower than lesion with NS alone injection group ($P < 0.01$, **Figure 3B**). Through immunohistochemical qualitative and quantitative assay, it is found that crocin injection significantly decreased the protein expression of MMP-3 and ADAMTS-5 in NP compared with the lesion with NS alone injection group ($P < 0.01$, **Figure 4**), which was increased by puncture lesion compared with the intact control group ($P < 0.01$, **Figure 4**).

Biochemical analysis

Consistent with mRNA expression analysis, the DMMB assay showed that puncture lesion caused significant loss of proteoglycan in NP compared with the intact control group ($P < 0.01$, **Figure 5A**). As expected, crocin injection effectively rescued the loss of proteoglycan in NP compared with the lesion with NS alone injection group ($P < 0.05$, **Figure 5A**), which was parallel to mRNA expression analysis. However, crocin injection exerted no significant effects on lesion induced loss of dsDNA content in NP (**Figure 5B**).

Discussion

Many studies have proved that inflammation plays an important role in intervertebral disc degeneration [19]. Accompanied by inflammatory responses, upregulation of degradative enzymes causes excessive catabolism of ECM, impairing the architecture and function of intervertebral disc [20]. Most of current researches on intervertebral disc degeneration aim to achieve degenerative disc repair and regeneration, including growth factor injection, gene delivery, anabolic drug injection and tissue engineering [1, 13, 15, 21-24]. However, different from previous researches, the present study focuses on retardation of intervertebral disc degeneration especially in early stage. Consistent with our previous in vitro and ex vivo study [14], the present results demonstrate that crocin injection effectively retards puncture lesion induced upregulation of degradative enzymes and ECM loss, suggesting that crocin may be an alternative choice in early treatment of intervertebral disc degeneration.

For clinical assessment of intervertebral disc degeneration, MRI is the most important method due to the non-invasive characteristic. With accumulation of water-absorbing proteoglycan, NP possesses a high water content which could be reflected by T2-weighted magnetic resonance images. There has been evidence that degenerative changes of the intervertebral disc

cause the signal loss of NP on T2-weighted magnetic resonance images [25, 26]. It is proved that proteoglycan concentration in NP directly correlates with the signal intensity in T2-weighted magnetic resonance images [27]. As NP is most severely affected during lesion induced disc degeneration, MRI analysis was chosen for our study [17]. In our study, crocin was proved to rescue the signal loss of NP on T2-weighted images induced by puncture lesion, suggesting that crocin inhibits lesion induced proteoglycan loss in NP.

Intervertebral disc degeneration usually correlates with impairment of ECM components expression, such as type II collagen and proteoglycan [28-30]. Our previous results show that crocin inhibits LPS induced downregulation of mRNA expression of type II collagen and aggrecan in vitro [14]. The present in vivo study further proves the protective effects of crocin on mRNA expression of type II collagen and aggrecan, suggesting that crocin protects ECM synthesis during intervertebral disc degeneration. Meanwhile, key degradative enzymes correlated with intervertebral disc degeneration were analyzed in this study [31, 32]. Consistent with our in vitro results, crocin was found to inhibit lesion induced upregulation of MMP-3 and ADAMTS-5, and this would help in retardation of ECM catabolism especially in early degeneration.

In our study, puncture lesion caused obvious degeneration related histological changes, which is similar with other intervertebral disc degeneration models [16, 17] and clinical samples [33]. Crocin injection effectively alleviated the lesion induced degeneration related histological changes. Simultaneously, protein levels of MMP-3 and ADAMTS-5 were decreased by crocin injection compared with lesion with NS alone injection, which would maintain NP morphology and ECM content. DMMB analysis demonstrates the similar effects of crocin on proteoglycan content in vivo, which can also be reflected by MRI procedure and safranin O-fast green staining. However, crocin did not significantly raise the dsDNA content in NP compared with lesion with NS alone injection group, while the similar phenomenon has also been observed in previous studies aiming at intervertebral disc regeneration with anabolic peptides

[13, 34]. Considering that the effects of crocin on disc mainly depends on its anti-inflammatory bioactivity, crocin treatment may just retard degenerative progression without definite regenerative potent. Therefore, crocin intervention should be applied at the onset or early stage of intervertebral disc degeneration.

Together with our previous in vitro and ex vivo results, the present study further prove the protective effects of crocin on intervertebral disc degeneration. With abundant sources of saffron and low extraction cost, crocin may be a promising candidate for intervertebral disc degeneration treatment, although there is still need for further researches on its biocompatibility and bioactivity in large animals.

Disclosure of conflict of interest

None.

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