

## Original Article

# Protection of mangiferin on hydrogen peroxide-induced injury in nucleus pulposus cells through inhibition of oxidative stress and the endothelial nitric oxide synthase pathway

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**Abstract:** Mangiferin, a xanthone glucoside, exists in various plants including *Mangifera indica* L, which exhibit a protective effect on osteogenic chondrocyte injury via anti-inflammatory and anti-oxidant activities. The objective of this study is to examine the protective effect of mangiferin on hydrogen peroxide ( $H_2O_2$ )-induced oxidative stress in nucleus pulposus (NP) cells. NP cells were exposed with 200  $\mu M$   $H_2O_2$  for 6 hours to establish the injury model. Cell cytotoxicity, apoptosis, oxidative stress, and the protein expression of the eNOS and Bax/Bcl-2 were then investigated after treatment. We found that mangiferin (10, 20, and 40  $\mu M$ ) treatment could protect damaged cells through inhibition of  $H_2O_2$ -induced cytotoxicity and apoptosis of NP cells. Furthermore, mangiferin protected against  $H_2O_2$ -induced oxidative response through down-regulating MDA, GSH, and up-regulating SOD and CAT. Additionally, mangiferin was found to have inhibitory effects on eNOS and nitric oxide production. Furthermore, expression levels of cleaved caspase-3 and Bax were up-regulated in the  $H_2O_2$  group, which were significantly reversed by mangiferin. In conclusion, mangiferin could protect NP cells against  $H_2O_2$ -induced injury probably via inhibiting oxidative stress and the eNOS signaling pathway.

**Keywords:** Mangiferin, oxidative stress, nucleus pulposus cells, apoptosis, Bax

## Introduction

Degenerative spinal disorders caused by lumbar disc degeneration (IDD), such as lumbar spinal stenosis, lumbar spondylolisthesis, and lumbar disc disease, have gained increasing attention with the changes of human longevity and lifestyle [1]. The incidence of IDD is increasing, especially in young patients, and the pathological mechanism still remains unknown [2, 3]. Nucleus pulposus (NP) cells play an important role in maintaining the stability of intervertebral disc. Excessive apoptosis of NP cells can accelerate IDD [4, 5]. Therefore, inhibition of apoptosis, oxidative damage, and inflammatory response in disc NP cells may be a critical point for mitigating lumbar disc degeneration. Mangiferin is a four-hydroxypyridine carbon glycoside, which belongs to double benzene pyridine ketones [6]. Substantial evidence has revealed that mangiferin possesses several physiological

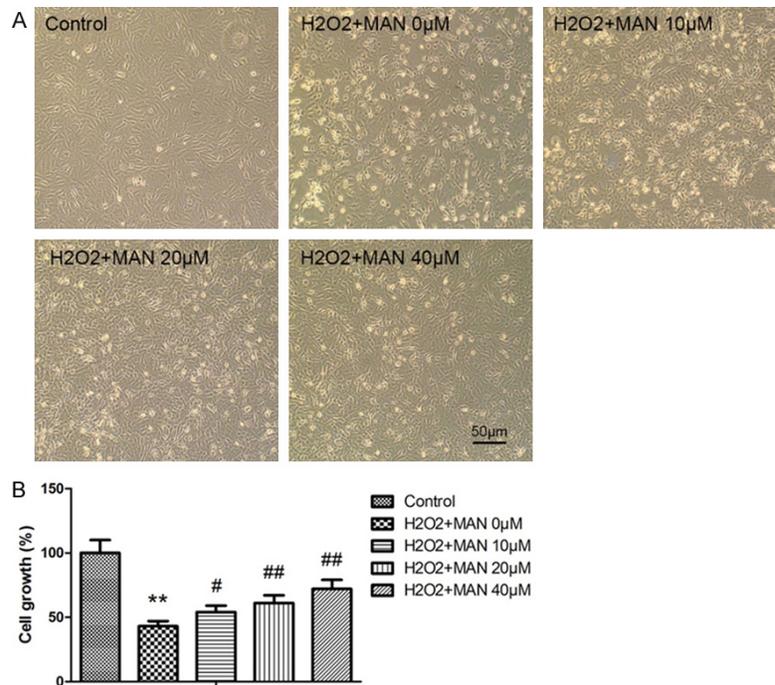
and pharmacological effects, including anti-inflammatory, anti-oxidative, cell apoptosis regulating, and anti-diabetic [7-9] functions. However, to the best of our knowledge, few studies have been performed to understand the anti-oxidant role on NP cells *in vitro*. The objective of the present study was to investigate the protective effect of mangiferin on  $H_2O_2$ -induced oxidative stress in nucleus pulposus (NP) cells, and to elucidate the underlying mechanisms.

## Materials and methods

### *Animals and cell culture*

Healthy male Sprague-Dawley rats (8 weeks old, obtained from the Animal Resource Center of The Second Affiliated Hospital of Inner Mongolia Medical University) weighing  $250 \pm 30$  g were euthanized by an overdose of 200 mg/kg pentobarbital. NP cells were harvested from the L2-L5 lumbar intervertebral discs as

# Mangiferin attenuates oxidative stress and the endothelial nitric oxide synthase



**Figure 1.** Effect of mangiferin on the cell growth of NP cells following  $H_2O_2$ -induced oxidative damage. A. Morphological observation of NP cells following  $H_2O_2$ -induced oxidative damage (Inverted phase contrast microscope  $\times 40$ ). B. Quantitative analysis of the cell growth of NP cells.  $**P < 0.01$ , compared with the control group;  $\#P < 0.05$ , compared with the 0  $\mu M$  mangiferin group;  $##P < 0.01$ , compared with the 0  $\mu M$  mangiferin group. Control, control group, MAN, mangiferin.

described in a previous study [Oh CD, Im HJ, Suh J, Chee A, An H, Chen D. Rho-associated kinase inhibitor immortalizes Rat nucleus pulposus and annulus fibrosus cells: establishment of intervertebral disc cell lines with novel approaches. *Spine (Phila Pa 1976)* 2016; 41: E255-61]. The NP cells were then incubated in 10% FBS containing DMEM/F-12 at a humidified atmosphere. Before mangiferin treatment, the NP cells were pre-cultured with fresh medium containing 200  $\mu M$   $H_2O_2$  for 6 hours.

### Cell cytotoxicity

NP cells were transferred at  $5 \times 10^3$ /well into 96-well plates and incubated with mangiferin (Nanjing traditional Chinese medicine Institute of Chinese Material Medica) at different concentrations (0, 10, 20, and 40  $\mu M$ ) for another 24 h in a 5%  $CO_2$  humidified atmosphere. Then, 10  $\mu L$  of CCK-8 dye (Beyotime Institute of Biotechnology) was used to evaluate the cell cytotoxicity in accordance with CCK-8 instructions.

### Detection of apoptosis by flow cytometry

NP cells were transferred into 6-well plate at  $1 \times 10^6$ /well and incubated with mangiferin at different concentrations (0, 10, 20, and 40  $\mu M$ ) for another 24 h in the above conditions. NP cells were then washed and incubated with 500  $\mu L$  binding buffer (Chemicon International, Inc. Billerica, USA). Annexin V-FITC and PI were added with dark as suggested in accordance with the kit protocol. Then, the cells were analyzed using FACS can flow cytometer (BD Biosciences, San Jose, CA).

### Oxidative stress measurement

NP cells were transferred into 6-well plate at  $1 \times 10^6$ /well and incubated with mangiferin at different concentrations (0, 10, 20, and

40  $\mu M$ ) for another 24 h in the above conditions. The levels of MDA, SOD, CAT, and GSH were valued by commercial ELISA kit (KeyGEN, Nanjing, China).

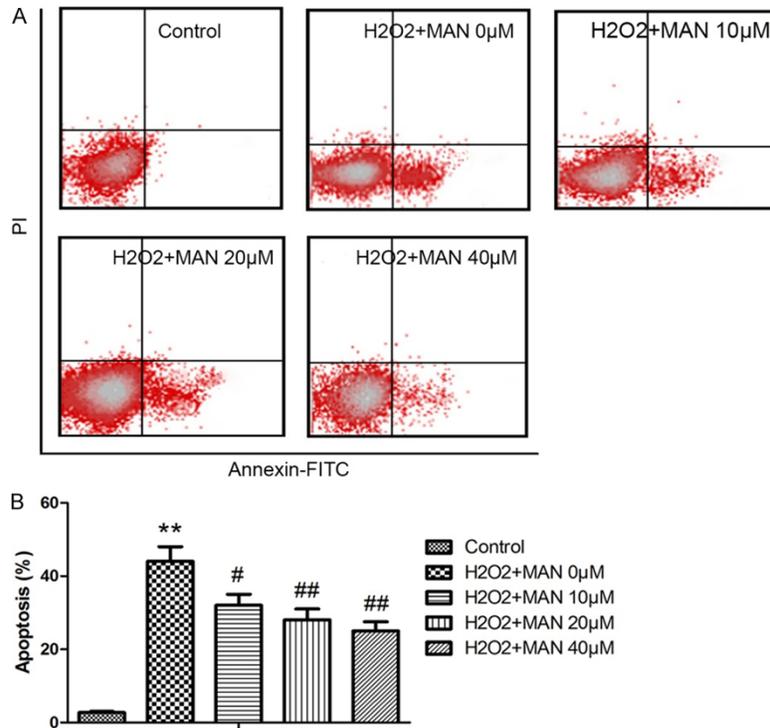
### Measurement of eNOS activity and NO production

NP cells were transferred into 6-well plate at  $1 \times 10^6$ /well and incubated with mangiferin at different concentrations (0, 10, 20, and 40  $\mu M$ ) for another 24 h in the above conditions. The eNOS activity and NO production of the NP cells were detected by Nitric Oxide Synthase Assay kit (Beyotime Institute of Biotechnology) and Griess reagent (Promega Corp., Madison, WI, USA), respectively.

### Western blot analysis

NP cells were transferred into 6-well plate at  $1 \times 10^6$ /well and incubated with mangiferin at different concentrations (0, 10, 20, and 40  $\mu M$ ) for another 24 h in the above conditions. Total protein concentration was valued by BCA assay

## Mangiferin attenuates oxidative stress and the endothelial nitric oxide synthase



**Figure 2.** Effect of mangiferin on apoptosis of NP cells following H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. A. The apoptosis of NP cells was quantified by flow cytometry after H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. B. Quantitative analysis of the apoptosis proportion of NP cells. \*\**P*<0.01, compared with the control group; #*P*<0.05, compared with the 0 µM mangiferin group; ##*P*<0.01, compared with the 0 µM mangiferin group. Control, control group, MAN, mangiferin.

**Table 1.** Anti-oxidative stress effects of mangiferin on the content of MDA and activities of CAT, SOD and GSH-px

Groups	MDA (nmol/mg protein)	CAT (U/mg protein)	SOD (U/mg protein)	GSH-px (U/mg protein)
Control	2.74±1.22	68.68±4.37	162.23±9.46	7.06±0.71
H <sub>2</sub> O <sub>2</sub> +MAN 0 µM	8.79±1.16**	19.52±2.45**	72.40±8.79**	1.14±0.86**
H <sub>2</sub> O <sub>2</sub> +MAN 10 µM	7.51±1.48#	26.89±3.67#	83.52±9.51#	3.82±0.52#
H <sub>2</sub> O <sub>2</sub> +MAN 20 µM	5.82±1.24##	36.81±2.86##	97.43±7.47##	4.13±0.37##
H <sub>2</sub> O <sub>2</sub> +MAN 40 µM	4.62±1.09##	50.19±4.05##	116.67±9.66##	4.51±0.43##

\*\**P*<0.01, compared with the control group; #*P*<0.05, compared with the 0 µM mangiferin group; ##*P*<0.01, compared with the 0 µM mangiferin group. Control, control group; MAN, mangiferin; MDA, Malondialdehyde; CAT, Catalase; SOD, Superoxide dismutase; GSH-px, Glutathione peroxidase.

(KeyGEN). Then, equal amounts of protein were transferred to PVDF (Bio-Rad). anti-eNOS (SAB1305369; 1:1,000; Sigma-Aldrich), anti-Bcl-2 (sc-492; 1:1,500; Santa Cruz Biotechnology, Inc, Santa Cruz), anti-Bax (sc-493; 1:500; Santa Cruz Biotechnology, Inc) and anti-β-actin (1:5000, BestBio Inc) were used to incubate with PVDF for 6 hours. Then, secondary

antibodies were used to incubate with PVDF for 3 hours at a humidified atmosphere. ECL western blotting system (Amersham Biosciences, UK) was used to quantify the relative protein expression.

### Caspase-3 measurement

NP cells were transferred into 6-well plate at 1×10<sup>6</sup>/well and incubated with mangiferin at different concentrations (0, 10, 20, and 40 µM) for another 24 h in the above conditions. Caspase-3 activity was detected by a colorimetric assay kit (Beyotime) with the wavelength of 405 nm at 37 °C.

### Statistical analysis

Data are expressed as mean ± standard deviation and was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Statistical significance of the experimental results was calculated using ANOVA test followed by the Bonferroni t test. *P*<0.05 was considered statistically significant.

## Results

### Cytotoxicity

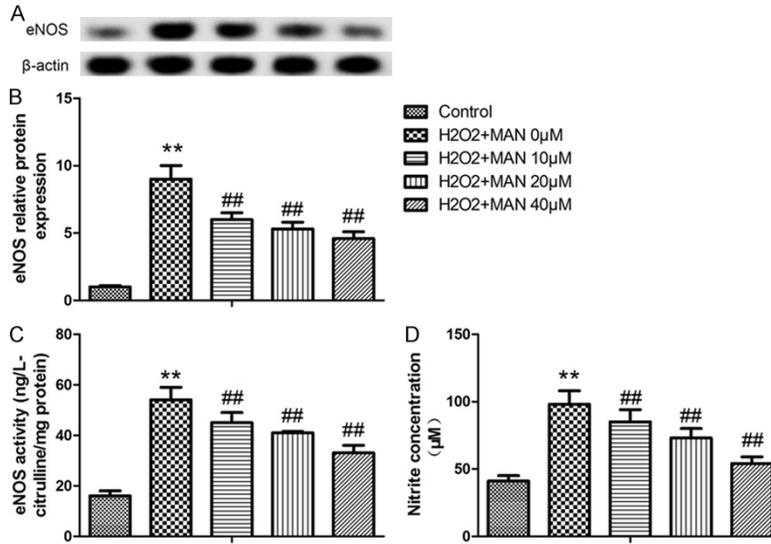
As shown in **Figure 1**. Growth of NP cells was effectively inhibited by 200 µM H<sub>2</sub>O<sub>2</sub> as compared to the control group. Treatment with mangiferin significantly

increased growth of NP cells in a dose-dependent manner (*P*<0.05), compared with the model group (**Figure 1**).

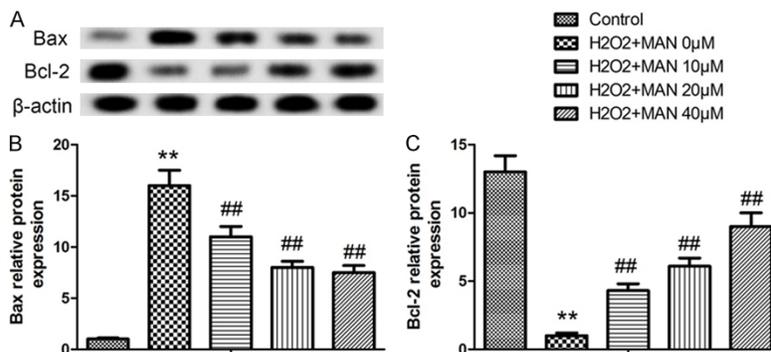
### Apoptosis rates

As shown in **Figure 2**, apoptosis in NP cells was markedly induced by 200 µM H<sub>2</sub>O<sub>2</sub>. Mangiferin treatment (10, 20 and 40 µM) significantly sup-

# Mangiferin attenuates oxidative stress and the endothelial nitric oxide synthase



**Figure 3.** Effect of mangiferin on the level and activity of eNOS as well as NO concentration of NP cells following H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. (n=5, Mean ± Standard Deviation). A. Representative images of immunoblots with antibodies against eNOS of NP cells following H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. eNOS: 133 kDa; β-actin: 36 kDa. B. Quantitative analysis of the protein level of eNOS of NP cells following H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. The data were normalized to the loading control β-actin. C. Measurement of eNOS activity. D. NO production was detected spectrophotometrically by measuring its metabolite, nitrite. \*\*P<0.01, compared with the control group; ##P<0.01, compared with the 0 µM mangiferin group. Control, control group, MAN, mangiferin, eNOS, endothelial nitric oxide synthase; NO, nitric oxide.



**Figure 4.** Effect of mangiferin on the expression levels of Bax and Bcl-2 of NP cells following H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. A. Representative images of immunoblots with antibodies against Bax and Bcl-2 of NP cells following H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. Bax: 23 kDa; Bcl-2: 26 kDa; β-actin: 36 kDa. B, C. Quantitative analysis of the protein level of Bax/Bcl-2 of NP cells following H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. The data were normalized to the loading control β-actin. \*\*P<0.01, compared with the control group; ##P<0.01, compared with the 0 µM mangiferin group. Control, control group, MAN, mangiferin, Bax, Bcl-2-associated X protein; Bcl-2, B cell lymphoma-2.

pressed the apoptosis rate in a dose-dependent manner (P<0.05), compared with the model group (Figure 2).

decreased Bax expression in a dose-dependent manner (Figure 4B and 4C). Furthermore, the activity of caspase-3 was found to be

## Oxidative stress expression

As shown in Table 1, 200 µM H<sub>2</sub>O<sub>2</sub> markedly increased the content of MDA, and suppressed the levels of CAT, SOD, and GSH-px in NP cells (P<0.01). However, the content of MDA was significantly reduced by mangiferin, and the activities of CAT, SOD, and GSH-px were effectively increased by mangiferin in a dose-dependent manner (P<0.01), compared with the model group.

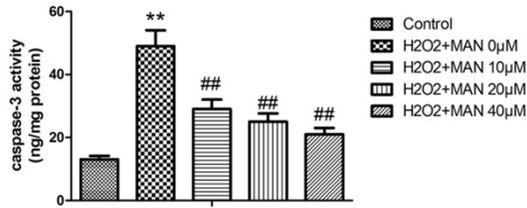
## eNOS expression as well as NO concentration

Figure 3A revealed that the protein level of eNOS was markedly induced by 200 µM H<sub>2</sub>O<sub>2</sub> (P<0.01). However, eNOS expression was effectively suppressed after mangiferin treatment (10, 20 and 40 µM) in a dose-dependent manner (P<0.01) (Figure 3B). Similar results were observed in eNOS activity (Figure 3C). Furthermore, NO production was found to be significantly increased by 200 µM H<sub>2</sub>O<sub>2</sub> (P<0.01). Mangiferin markedly reduced the nitrite level (P<0.01) in a dose-dependent manner (Figure 3D).

## Bax/Bcl-2 expression as well as caspase-3 activity

As shown in Figure 4A, there was significant decreases in Bcl-2 expression and increases in Bax expression by 200 µM H<sub>2</sub>O<sub>2</sub> induced damage (P<0.01). Mangiferin effectively increased the expression of Bcl-2 and

## Mangiferin attenuates oxidative stress and the endothelial nitric oxide synthase



**Figure 5.** Effect of mangiferin on caspase-3 activity of NP cells following  $H_2O_2$ -induced oxidative damage. \*\* $P < 0.01$ , compared with the control group; ## $P < 0.01$ , compared with the 0  $\mu M$  mangiferin group. Control, control group, MAN, mangiferin.

increased after  $H_2O_2$ -induced damage ( $P < 0.01$ ), while, mangiferin significantly suppressed caspase-3 followed  $H_2O_2$ -induced damage, as shown in **Figure 5**.

### Discussion

Symptomatic disc degeneration is becoming a scourge due to the unprecedented increase in human life expectancy. IDD diseases represent one of the main causes of low back pain in industrialized countries [10, 11]. So far, the main treatment for IDD is traction therapy, intervertebral disc displacement, spinal fusion, etc. [12]. Individuals suffering from IDD may experience permanent incapacitation from low back pain [13, 14]. In the current study, the growth of NP cells exposed in  $H_2O_2$  was significantly inhibited and cell apoptosis increased. However, after co-treatment with mangiferin, we found that fewer apoptotic cells were detected in a dose-dependent manner, which revealed that mangiferin effectively inhibited the apoptosis of  $H_2O_2$ -induced NP cells.

The present research indicates that oxidative stress exists in the process of cellular metabolism, including cell proliferation, signal transduction, apoptosis, and necrosis [15, 16]. Excessive oxidative stress induced by intracellular protein, lipid peroxidation, and nucleic acid damage as well as enzyme denaturation, which finally leads to cell apoptosis and necrosis [17, 18]. Potential mechanisms include massive  $Ca^{2+}$  entering into the mitochondria due to an inverse ion concentration gradient, accumulating inside and causing damage to mitochondria, with ion homeostasis out of unbalance, which ultimately leads to the synthesis dysfunction of ATP followed  $H_2O_2$ -induced oxidative damage [19]. In the current

study, mangiferin effectively decreased the content of MDA and increased the activities of SOD, CAT, and GSH-px following  $H_2O_2$ -induced oxidative damage. Similar research has been reported in a previous study [20]. Additionally, Viswanadh et al. proposed that mangiferin effectively enhances the activities of GSH-px, glutathione-S-transferase (GST), SOD, and CAT [21].

Abnormal production of NO is the main cause of many cardiovascular diseases. eNOS, as a rate limiting enzyme, which participates in the synthesis of NO, plays a key role in the regulation of the vascular function. Therefore, it is necessary to ameliorate the regulation of NO concentration and eNOS activity followed  $H_2O_2$ -induced oxidative damage in order to improve the NP cell function [22, 23]. The present study demonstrated that the protein expression level and the activity of eNOS together with NO concentration were all elevated by  $H_2O_2$ . However, mangiferin effectively suppressed eNOS levels and decreased the NO concentration followed  $H_2O_2$ -induced oxidative damage.

In order to explore the mechanisms of mangiferin on  $H_2O_2$ -mediated cell apoptosis, we quantified cleaved caspase-3 activity and Bax/Bcl-2 protein expression. We found that mangiferin could markedly enhance Bcl-2 expression and decrease Bax as well as the cleaved caspase-3 activity in a dose-dependent manner. Previous studies have revealed that the caspase-3 is a pro-apoptotic protein, which plays a key role in regulating mammalian cells apoptosis [24-26]. Furthermore, caspase-3 could be activated by the promotion of Bax. Bax is the major protein involved in regulating apoptosis in the Bcl-2 family, and it can promote release of cytochrome c, activating caspase, and leading to apoptosis [27, 28]. Bcl-2 can block the apoptosis effect of Bax and promote cell survival [29, 30]. A previous study has shown that mangiferin prevented MPTP-induced behavioral deficits, oxidative stress, apoptosis, dopaminergic neuronal degeneration and dopamine depletion [31]. Furthermore, mangiferin attenuates contusive spinal cord injury in rats through the regulation of oxidative stress, inflammation and the Bcl-2 and Bax pathway [32].

In conclusion, our findings have demonstrated that mangiferin exerts protective effects in NP cells by attenuating oxidative stress and cell

apoptosis. Moreover, we have provided evidence that eNOS/NO is a potential target for mangiferin in intervertebral discs. These findings suggest that mangiferin can be of potential therapeutic value in the prevention of IDD.

**Disclosure of conflict of interest**

None.

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