# Atrial natriuretic peptide protects vertebral endplate chondrocytes against $H_2O_2$-induced apoptosis and oxidative stress through activation of the Nrf2/HO-1 signaling pathway

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**Abstract.** The present study aimed to investigate the effect of atrial natriuretic peptide (ANP) on cell apoptosis and oxidative stress in $H_2O_2$-induced vertebral endplate chondrocytes (EPCs), and to assess the associated mechanisms involved. Cell viability and apoptosis were evaluated using the Cell Counting Kit-8 method and TUNEL assay, respectively. In addition, the scavenging capability was detected using various enzymatic assays, and the quantity of nitric oxide (NO) and malondialdehyde (MDA), and activity of superoxide dismutase (SOD) were assessed. The expression levels of apoptosis-related proteins, activation of the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway induced by $H_2O_2$ and the effect of treatment with ANP on vertebral EPCs were detected by western blotting. The results revealed that ANP protected EPCs from $H_2O_2$-induced cell damage. $H_2O_2$-induced intracellular MDA was decreased by ANP, and the levels of SOD and NO were increased in the presence of ANP. ANP also inhibited the $H_2O_2$-induced alterations in the expression levels of cleaved-caspase-3, Bax and Bcl-2. Finally, ANP blocked $H_2O_2$-induced oxidative stress through activating the Nrf2/HO-1 signaling pathway. These findings suggested that ANP may effectively protect EPCs through inhibition of $H_2O_2$-induced oxidant injury and cell death by activating the Nrf2/HO-1 signaling pathway.

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*Abbreviations:* ANP; atrial natriuretic peptide; EPCs, endplate chondrocytes; NO, nitric oxide; MDA, malondialdehyde; SOD, superoxide dismutase; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; DMSO, dimethyl sulfoxide

*Key words:* ANP, EPCs, cell apoptosis, oxidative stress, Nrf2/HO-1 signaling pathway

## Introduction

Atrial natriuretic peptide (ANP) is a precursor protein that is produced by atrial myocytes and then secreted into the plasma. It is distributed in the heart, pituitary gland, lung, adrenal gland, and other peripheral tissues and organs, and possesses numerous bioactivities (1). For example, ANP acts as a diuretic, natriuretic and vasodilator protein, stimulates vasodilation, fluid egress, increased glomerular filtration and salt/water excretion (2), and blocks the release and/or actions of several hormones, including angiotensin II, aldosterone and vasopressin (3). There have been numerous reports demonstrating that ANP is a potential candidate for the treatment of heart failure (4), nephrosis (5) and other conditions. In addition, ANP has been reported to exert a protective effect on oxidant injury (3,6,7).

Humans are frequently exposed to oxidative stress and detrimental environmental factors, such as $H_2O_2$, endotoxin, UV light and heavy metals. Under such stressful conditions, oxidative stress is generated, which disturbs the cellular processes, and as a consequence leads to cell apoptosis, organ injury and disease (8,9). Cell apoptosis has often been evaluated by the measurement of the expression levels of several major apoptosis-associated proteins, including Bcl-2, Bax and caspase-3. The Bcl-2 and caspase families are the most common indices used in cell apoptosis research. Bcl-2 and Bax are the most important regulatory genes associated with apoptosis, and have opposite functions; these genes ultimately activate the caspase protein family and lead to chondrocyte apoptosis (10). Bcl-2 inhibits cell apoptosis and promotes cell survival, whereas Bax and caspase-3 elicit apoptosis and evoke cell death by promoting the release of cytochrome *c* (11). As a result, if the Bax/Bcl-2 ratio is modulated it can affect activation of the intrinsic apoptotic pathway. Notably, decreased Bax/Bcl-2 ratio and caspase-3 abrogated cell apoptosis (12).

Under oxidative stress, cell-protective mechanisms are activated to fight against oxidant injury and to protect cells. Single cells and organisms may adapt to harmful oxidative stress conditions via the activation of stress-related factors. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcription factor responsible for regulating the antioxidant response (13,14). The multifunctional regulator

Nrf2 is regarded as a cell-protective factor that can regulate the expression of anti-oxidant, anti-inflammatory and detoxifying proteins. Activation of the Nrf2 pathway composes a cell-protective system that improves cell viability under harmful conditions (15,16). To some extent, the main characteristics of Nrf2 are simulated by Nrf2-dependent genes and associated proteins, such as heme oxygenase-1 (HO-1), which besides removing toxic heme, produces biliverdin, iron ions and carbon monoxide (17). HO-1 and its products play a beneficial role in protecting against oxidant injury, inhibiting cell death, modulating inflammation and other cell toxicities (13,18-20).

Oxidative stress has been reported to be associated with intervertebral disc degeneration (IDD) (21). The endplate plays a critical role in biomechanical integrity and disc nutrition (22), and endplate chondrocytes (EPCs) are vitally important to intervertebral discs under physiological and pathological conditions (23). Notably, although ANP has been reported to exert beneficial effects, including protective effects against oxidant injury, to the best of our knowledge, the protective effect of ANP on EPCs and the associated mechanisms involved have not been reported and are poorly understood. Therefore, on the basis of these reports, it was hypothesized that ANP may serve an important protective role in $H_2O_2$-induced EPC injury and apoptosis through activation of the Nrf2/HO-1 signaling pathway.

## Materials and methods

*Drug and materials*. ANP (purity, >99%) was obtained from National Institutes for Food and Drug Control. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich; Merck KGaA. Brusatol (a specific Nrf2 inhibitor) was purchased from MedChemExpress. GAPDH (cat. no. sc-365062), Bax (cat. no. sc-7480), Bcl-2 (cat. no. sc-7382), Lamin-B1 (cat. no. sc-374015), Nrf2 (cat. no. sc-365949) and HO-1 (cat. no. sc-136960) antibodies were purchased from Santa Cruz Biotechnology, Inc. Cleaved (C)-caspase-3 (cat. no. ab2302) was obtained from Abcam. DAPI (cat. no. C1002) was obtained from Beyotime Institute of Biotechnology.

*Cell culture*. EPCs were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences [EPCs were differentiated from ATDC5 cell line (24) in The Cell Bank of Type Culture Collection of Chinese Academy of Sciences]. It has been shown that the ATDC5 cell line is a useful *in vitro* model for examining the multistep differentiation of chondrocytes. Undifferentiated ATDC5 cells proliferate rapidly until they reach confluence, at which point they undergo growth arrest. When treated with insulin, transferrin and sodium selenite, confluent ATDC5 cells re-enter a proliferative phase and form cartilaginous matrix nodules (mature chondrocytes) (24). The EPCs were seeded into 10-cm culture plates at a density of $1x10^5$ cells/plate. The complete medium was changed every other day, and the first two and three passages of EPCs were used in subsequent experiments. Cells were grown as monolayers in DMEM/F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich; Merck KGaA) and antibiotics (1% penicillin/streptomycin). Cells were incubated at 37˚C in a humidified atmosphere containing 5% $CO_2$.

*Cell treatment*. EPCs were treated with different concentrations of $H_2O_2$ (50, 100, 200, 300 and 400 $\mu$M), and the control (0 $\mu$M) group was treated with DMSO, for 48 h at 37˚C. Subsequently, ANP (300 nM) was administrated to $H_2O_2$-treated cells for 48 h at 37˚C. Blank control cells were treated with DMSO (model group) and Saline solution was applied to $H_2O_2$-treated cells serving as a saline group. In addition, brusatol (0.3 $\mu$g/ml) was used to treat $H_2O_2$-treated cells for 48 h at 37˚C. Finally, brusatol (0.3 $\mu$g/ml) was applied in the ANP (300 nM)-treated cells for 48 h at 37˚C, which served as the ANP + Brusatol group.

*Cell viability assay*. As previously reported (25), the viability of EPCs was investigated using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc.) in accordance with the manufacturer's protocol. Firstly, the EPCs ($1x10^4$ cells/well) were seeded in 96-well plates and incubated at 37˚C for 24 h. Cells were then treated as aforementioned. At the predetermined time, the cells were washed three times with PBS, and 100 $\mu$l DMEM/F12 containing 10% CCK-8 was added and incubated for another 2 h. Finally, the absorbance was measured at 470 nm using a microplate reader and the relative survival rate was calculated as follows: Cell viability=absorbance of test cells/absorbance of blank control cells. Each sample was performed in triplicate and the mean was calculated.

*TUNEL assay*. The TUNEL method was used to measure cell apoptosis. Previous studies have used the TUNEL method to determine cell viability as described previously (26). VECTASHIELD mounting medium containing DAPI (1.0 $\mu$g/ml; Vector Laboratories, Inc.) was used for visualization of nuclei. EPCs cultured with $H_2O_2$ with or without ANP were harvested after 12 h and transferred to six-well plates. Cells ($1x10^4$) were then fixed in 4% paraformaldehyde (freshly prepared) for 15 min and incubated with 0.1% Triton X-100 for 10 min at 4˚C. At each step, cells were washed three times with aseptic PBS. Finally, cells were incubated and stained with the TUNEL detection kit (cat no. G7130; Promega Corporation) for 1 h at 37˚C in the dark. Apoptotic cells were detected using a fluorescence microscope (Olympus Corporation) and counted in seven random fields.

*Western blot analysis*. The expression levels of signaling pathway proteins were detected by western blotting, which has been previously described (19,26,27). EPCs were incubated with ANP (300 nM) and/or $H_2O_2$ or the negative control brusatol (0.3 $\mu$g/ml) for 48 h at 37˚C. The blank control group was incubated with DMSO. After incubation and aspiration of the culture solution, cells were washed twice with PBS. Total proteins were extracted using RIPA buffer [FUJIFILM Wako Pure Chemical Corporation; containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 and 0.1% SDS] on ice for 30 min with occasional swirling to disperse the lysate evenly. The specific nuclear proteins were extracted using a NE-PER™ nuclear extraction kit (cat. no. 78835; Thermo Fisher Scientific, Inc.) following the

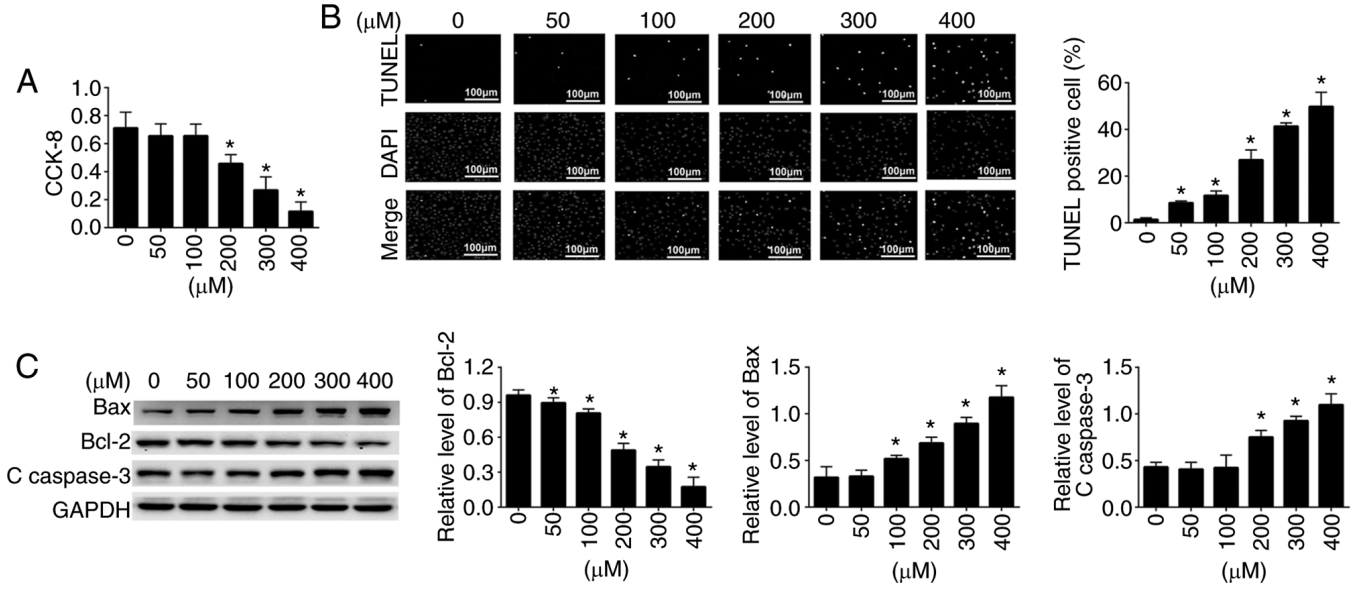


Figure 1. $H_2O_2$ induces the apoptosis of EPCs. (A) Cytotoxicity of various concentrations of $H_2O_2$ on EPCs was determined after 24 h using CCK-8 assay. The viability of EPCs was decreased in a dose-dependent manner. (B) TUNEL assay was performed in EPCs to detect cell apoptosis. It was observed that cell apoptosis was markedly increased in the $H_2O_2$-treated group in a dose-dependent manner. (C) Protein expression levels of Bax, Bcl-2 and C-capase-3 in EPCs in control and $H_2O_2$-treated cells. Protein expression levels of C-caspase-3 and Bax (pro-apoptotic) were increased, whereas those of Bcl-2 were decreased. Data are presented as the mean ± SD. *P<0.05 vs. the blank control group. EPCs, endplate chondrocytes; CCK-8, Cell Counting Kit-8; C-, cleaved.

manufacturer's instructions. Cellular protein lysates were collected and protein concentration was quantified using a BCA kit (cat. no. A045-3-2; Nanjing Jiancheng Bioengineering Institute). Proteins (40 $\mu$g/lane) were separated by SDS-PAGE on 10% gels and transferred onto nitrocellulose membranes. The blots were blocked with 5% non-fat milk in Tris-buffered saline for 2 h at 25˚C and then probed with primary antibodies against C-caspase-3 (1:500), Bax (1:1,000), Bcl-2 (1:1,000), GAPDH (1:500), Nrf2 (1:200), HO-1 (1:1,000) and Lamin B1 (1:1,000) at 4˚C overnight. Subsequently, membranes were incubated with the respective secondary antibodies (cat. no. ab6802; 1:3,000; Abcam) according to the appropriate protocols. The antibody signals were then detected with Chemistar High-sig ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). Each sample was analyzed three times. Protein expression levels were semi-quantified by densitometric analysis using Quantity One software (version 4.6.6; Bio-Rad Laboratories, Inc.)

*Measurement of oxidative stress*. EPCs ($3x10^5$ cells/well) were seeded onto 6-well plates and incubated with ANP in the presence/absence of $H_2O_2$ or brusatol. Cells were then harvested and lysed by ultrasonic disruption (300 W, 15 min) on ice, and then centrifuged at 12,880 x g for 15 min at 4˚C. The supernatant was collected to detect the levels of superoxide dismutase (SOD) using a SOD assay kit (cat. no. A001-1-2), nitric oxide (NO) using a NO assay kit (cat. no. A013-2-1) and malondialdehyde (MDA) using an MDA assay kit (cat. no. A003-1-2) obtained from Nanjing Jiancheng Bioengineering Institute according to the manufacturer's recommendation and as previously described (28).

*Statistical analysis*. Each experiment was performed independently at least three times. Statistical analyses were performed using SPSS 18.0 software (SPSS, Inc.). Statistical differences among multiple groups were assessed by one-way analysis of variance, followed by Tukey's post hoc tests if there were statistical differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Effect of $H_2O_2$ on the viability and apoptosis of EPCs*. Before investigating the effect of ANP on EPC apoptosis and oxidative stress, the cytotoxic effects of $H_2O_2$ on EPCs were evaluated using the CCK-8 assay. The results are shown in Fig. 1A. Cell viability was significantly affected and impaired by exposure to different concentrations of $H_2O_2$ in a dose-dependent manner. When EPCs were incubated with increasing concentrations of $H_2O_2$, cell viability was gradually decreased (Fig. 1A); 200 $\mu$M $H_2O_2$ was selected for subsequent experiments. Subsequently, the TUNEL assay was conducted to investigate the cell death of EPCs. The results are presented in Fig. 1B. It was observed that the incidence of cell apoptosis was markedly increased in the $H_2O_2$-treated group in a dose-dependent manner compared with that in the control group. The TUNEL assay results were in agreement with the findings of the CCK-8 assay. To further investigate the apoptosis of EPCs, the expression levels of apoptosis-associated proteins, Bcl-2, Bax and C-caspase-3, were detected by western blot analysis. The expression levels of these three apoptosis-related proteins in control and $H_2O_2$ (50-400 $\mu$M)-treated EPCs are shown in Fig. 1C. Compared with those in the untreated control cells, increasing concentrations of $H_2O_2$ significantly increased the protein expression levels of Bax and C-caspase-3 (pro-apoptotic), and decreased the protein expression levels of Bcl-2 (anti-apoptotic). These results indicated that $H_2O_2$ may induce EPC death through activation of the apoptosis-related signaling pathway.

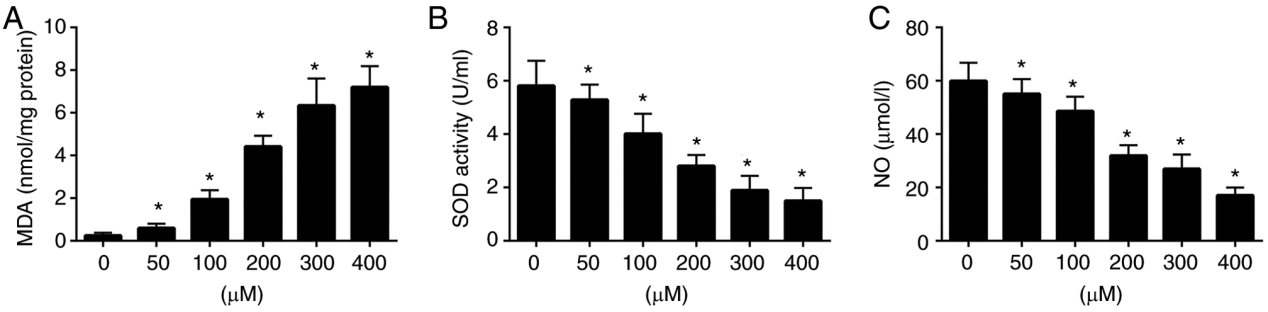


Figure 2. $H_2O_2$ induces oxidative stress in EPCs. EPCs were incubated with $H_2O_2$ (50-400 $\mu$M) for 24 h. Cells were lysed and then the levels of (A) MDA, (B) SOD and (C) NO were measured using commercial assay kits. MDA activity was increased, and SOD and NO content was decreased in $H_2O_2$-treated cells. Data are presented as the mean ± SD. *P<0.05 vs. blank control group. EPCs, endplate chondrocytes; MDA, malondialdehyde; SOD, superoxide dismutase; NO, nitric oxide.

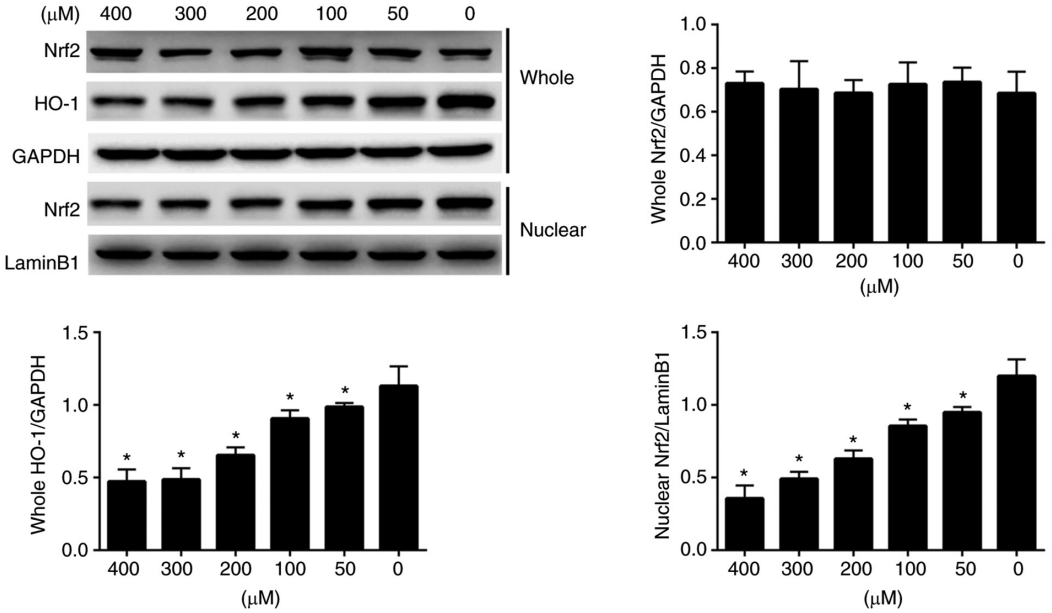


Figure 3. Expression of the Nrf2/HO-1 signaling pathway in $H_2O_2$-induced endplate chondrocytes. The expression of Nrf2 in the nucleus and the expression of HO-1 in whole cells was markedly decreased with increasing $H_2O_2$ dose. *P<0.05 vs. blank control group. Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1.

*$H_2O_2$ induces oxidative stress in EPCs*. To assess the levels of oxidative stress in $H_2O_2$-induced EPCs, the levels of related enzymes (MDA) or markers (SOD and NO) were detected using commercial assay kits. The results revealed that exposure to $H_2O_2$ markedly increased MDA activity, and decreased SOD and NO levels compared with those in the untreated group (Fig. 2). These results revealed that $H_2O_2$ induced oxidative stress in EPCs and affected the levels of associated indices.

*$H_2O_2$ inhibits the expression of proteins associated with the Nrf2/HO-1 signaling pathway in EPCs*. The expression levels of Nrf2 and HO-1 were detected in $H_2O_2$-induced EPCs by western blotting (Fig. 3). With regard to the protein expression levels of Nrf2 in whole cells, there was a slight difference among the different groups; however, this was not statistically significant. By contrast, the protein expression levels of HO-1 in whole cells were markedly lower in the $H_2O_2$-treated groups compared with those in the control group; a moderate inhibition of ~60% was achieved in response to 200 $\mu$M $H_2O_2$ compared with 0 $\mu$M $H_2O_2$. The protein expression levels of Nrf2 in the nucleus were also detected; the results indicated that the protein expression levels of Nrf2 in the nucleus were markedly decreased with increasing $H_2O_2$ dose. These findings suggested that a large proportion of Nrf2 protein had moved from the nucleus and the expression levels of the related protein HO-1 were also decreased after treatment with $H_2O_2$. These results indicated that $H_2O_2$ treatment may block the nuclear translocation of Nrf2 signaling pathway components and may inhibit HO-1 expression in whole EPCs.

*ANP activates the NrF2/HO-1 signaling pathway*. Western blotting was conducted to measure the protein expression levels of Nrf2 and HO-1 in EPCs treated with $H_2O_2$, ANP and brusatol, alone or together (Fig. 4). When compared with those in the model and saline groups, the whole Nrf2, nuclear Nrf2 and whole HO-1 protein expression levels were increased in the ANP treatment group. Conversely, the ANP-induced increase in whole Nrf2, nuclear Nrf2 and whole HO-1 protein expression

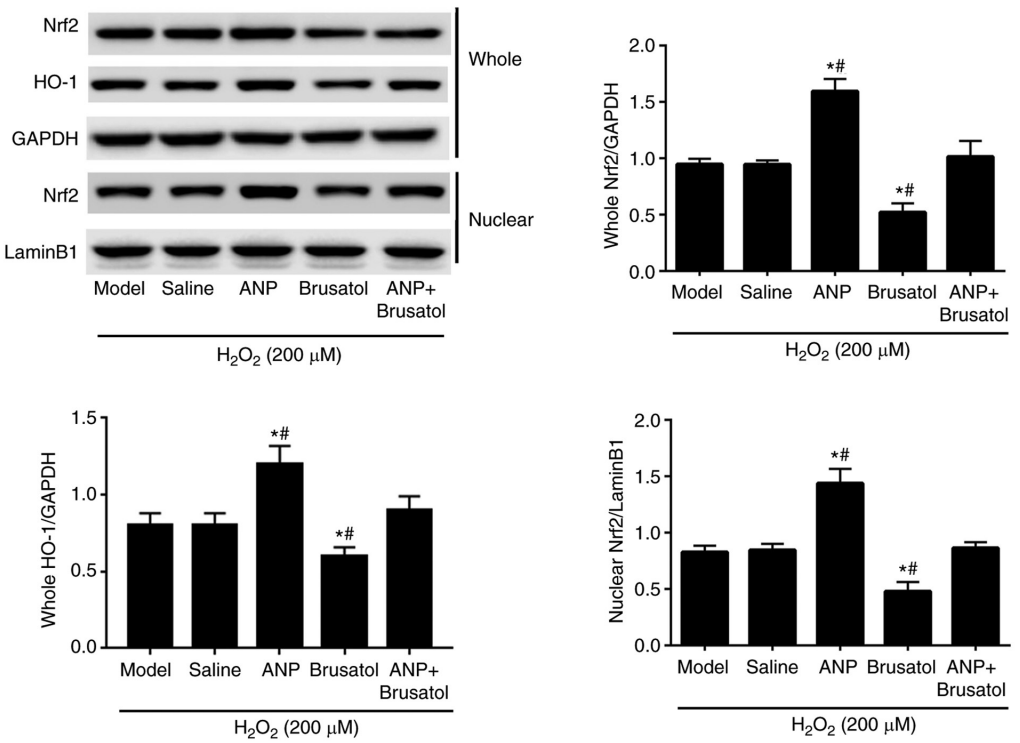


Figure 4. Protein expression levels of Nrf2/HO-1 in $H_2O_2$-treated endplate chondrocytes after treatment with, saline, ANP, brusatol and ANP + brusatol. The changes in Nrf2/HO-1 proteins caused by $H_2O_2$ treatment were blocked by ANP. Data are presented as the mean ± SD. *P<0.05, vs. saline group; #P<0.05 vs. ANP + brusatol group. Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; ANP, atrial natriuretic peptide.

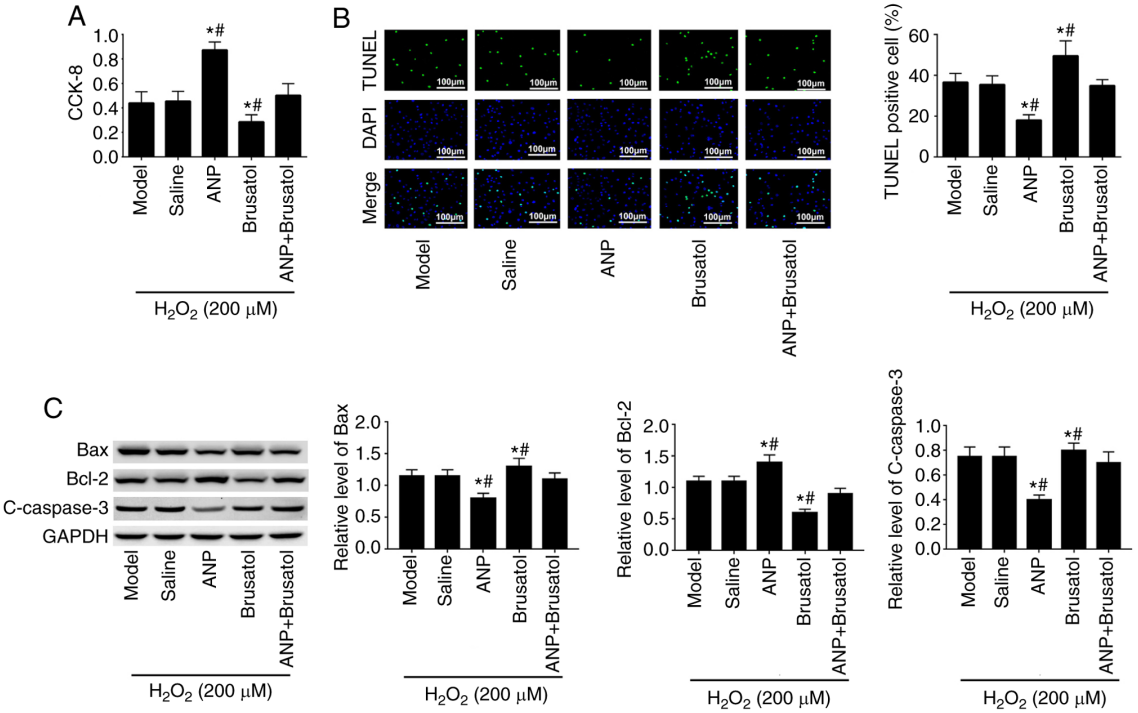


Figure 5. ANP inhibits the $H_2O_2$-induced apoptosis of EPCs. (A) CCK-8 assay was conducted to detect cell viability. (B) TUNEL assay was performed in EPCs to detect cell apoptosis. (C) Western blot analysis was used to measure the expression levels of Bax, Bcl-2 and C-capase-3 in EPCs. The results showed that $H_2O_2$-induced cell death and changes in apoptosis-related proteins were reversed by ANP pretreatment. Data are presented as the mean ± SD. *P<0.05, vs. saline group; #P<0.05 vs. the ANP + brusatol group. EPCs, endplate chondrocytes; CCK-8, Cell Counting Kit-8; C-, cleaved; ANP, atrial natriuretic peptide.

levels was offset by brusatol. Collectively, ANP treatment increased Nrf2 and HO-1 expression in $H_2O_2$-induced EPCs, and may exert protective effects against oxidant stress.

*ANP inhibits the $H_2O_2$-induced apoptosis of EPCs.* The cell viability and apoptosis of EPCs were measured to determine whether ANP may confer protection against $H_2O_2$-induced

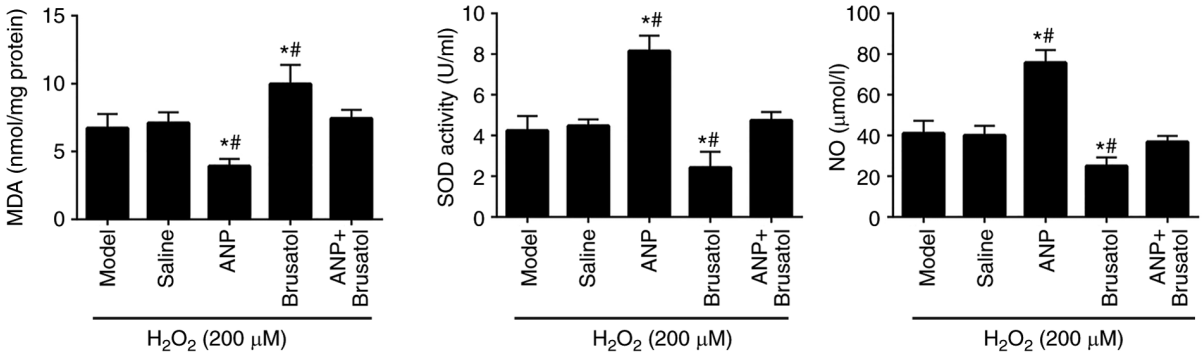


Figure 6. ANP inhibits $H_2O_2$-induced oxidative stress in EPCs. EPCs were pretreated with ANP for 2 h before the treatment with 250 $\mu$M $H_2O_2$ for 24 h. Cells were lysed and then the levels of MDA, SOD and NO were detected using commercial assay kits. The increased MDA activity, and decreased SOD and NO content in $H_2O_2$-treated cells was reversed by ANP treatment. Data are presented as the mean ± SD. $^*$P<0.05, vs. saline group; $^\#$P<0.05 vs. the ANP + brusatol group. EPCs, endplate chondrocytes; MDA, malondialdehyde; SOD, superoxide dismutase; NO, nitric oxide; ANP, atrial natriuretic peptide.

cell damage. As presented in Fig. 5A, cell viability was inhibited in brusatol-treated cells compared with that in the saline and ANP + brusatol groups, whereas this phenomenon was reversed by ANP treatment. The cell viability in the ANP-pretreated group was higher than that in cells without ANP. The results of the TUNEL assay (Fig. 5B) also revealed that ANP pretreatment decreased $H_2O_2$-induced cell apoptosis when compared with that in the saline group. Subsequently, the results of western blotting revealed that in brusatol-treated cells, the protein expression levels of Bax and C-caspase-3 were increased, whereas those of Bcl-2 were decreased; these effects were reversed by ANP treatment. Notably, when ANP + brusatol were present, the expression levels of these proteins almost returned to the same levels as the model group. Taken together, these results suggested that ANP protected EPCs against $H_2O_2$-induced apoptosis.

*ANP regulates the levels of MDA, SOD and NO in EPCs treated with $H_2O_2$.* The levels of MDA, SOD and NO were examined in ANP-treated EPCs. The results revealed that pretreatment with ANP increased the levels of SOD, which is able to eliminate $H_2O_2$, and NO, and reduced MDA content when compared with those in the model group (Fig. 6). By contrast, the Nrf2 specific inhibitor, brusatol reversed the reduced MDA, and increased SOD and NO levels compared with the ANP group. These findings suggested that ANP blocked the adverse effects of $H_2O_2$ on EPCs and may have potential antioxidant effects. These results coincided with the increased expression and nuclear translocation of Nrf2 and Nrf2/HO-1, whereas no obvious differences in enzymes were found between the saline group and ANP + brusatol group.

## Discussion

ANP has been proven to exert beneficial effects on intervertebral discs under physiological and pathological conditions (3), and has also been shown to exert a protective effect on oxidant injury (3,6,7). However, its effect and associated mechanism on EPCs is poorly understood and, to the best of our knowledge, has never been reported. Based on the previous reports regarding the beneficial and protective effects of ANP during oxidant injury, it was hypothesized that ANP may help to protect EPCs against $H_2O_2$-induced oxidant injury; therefore, a series of experiments were performed to confirm and explore its underlying mechanism.

In the present study, the oxidative stress model was induced by $H_2O_2$, and EPCs were pre-incubated in the presence or absence of ANP. The inhibitory effects of ANP on cell death were evaluated by detecting cell viability and the expression levels of apoptosis-related proteins (C-caspase-3, Bax and Bcl-2) in EPCs. The results indicated that $H_2O_2$-treated cells exhibited a higher rate of apoptosis, whereas ANP pre-incubation could significantly inhibit cell death. Furthermore, $H_2O_2$-induced C-caspase-3 and Bax activation, as well as Bcl-2 suppression, was also significantly reversed when EPCs were treated with ANP, whereas the effect of ANP was attenuated in the presence of brusatol. In consistent with this finding, previous studies have found similar results (29-31). Furthermore, it was reported that cell apoptosis was suppressed by increased Bcl-2, reduced Bax and caspase-3 activity (12). Taken together, along with the findings of other studies, the present results suggested that ANP could inhibit $H_2O_2$-induced cell apoptosis and regulate the levels of apoptosis-related proteins.

Previous studies have revealed that oxidative stress can induce cell apoptosis (32,33) and since the present results revealed that ANP could reduce $H_2O_2$-induced cell apoptosis, it was hypothesized that ANP may have a role in the regulation of several enzymes/proteins that can protect cells against oxidant injury. To explore the underlying relationship between the prevention of oxidant injury and inhibition of cell death, the levels of three essential oxidative stress-related enzymes (SOD-1, MDA and NO) were assessed (34,35). The results indicated that $H_2O_2$ induced changes in the levels of SOD, MDA and NO, and these changes were reversed by ANP.

It has been reported that under normal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, whereas under oxidative stress, Keap1 is modified by -SH groups and Nrf2 is phosphorylated. This facilitates Nrf2 dissociation from Keap1 and its translocation into the nucleus (36). The antioxidant response element can be activated by Nrf2 and the transcription of Nrf2-regulated genes (such as HO-1 and glutathione S-transferase) can be increased after binding with Maf protein. Among these genes, HO-1 enzyme activity may remove the

prooxidant molecule heme, and lead to the generation of iron ions, biliverdin and carbon monoxide (37). Biliverdin is reduced to bilirubin by the action of biliverdin reductase, and bilirubin is a potent antioxidant (38); consequently, HO-1 is generally considered as a cellular defense against oxidative stress. Notably, the expression of Nrf2-facilitated HO-1 shows an antioxidant effect (18). These findings indicated that the Nrf2/HO-1 signaling pathway may serve a significant role in oxidative stress. Based on these reports, it was hypothesized that ANP may prevent oxidative stress through regulation of the Nrf2/HO-1 signaling pathway. Western blotting was conducted to investigate the expression levels of proteins in the Nrf2/HO-1 pathway, and the specific inhibitor of the Nrf2 pathway, brusatol (39), was used in the present study as a negative control. The results revealed that ANP activated Nrf2 expression and nuclear translocation, and reversed $H_2O_2$-induced inhibition of the Nrf2/HO-1 pathway, whereas brusatol treatment blocked the inhibitory effects of ANP on cell apoptosis and oxidative stress. According to these results, it was suggested that the decreased expression of Nrf2/HO-1 in the $H_2O_2$-treated group was related to cell death and oxidative stress, and that ANP prevented oxidative stress through activating the Nrf2/HO-1 signaling pathway. Specifically, ANP promoted Nrf2 nuclear translocation and upregulated HO-1, which suggested that it may directly activate the Nrf2/HO-1 signaling pathway. As a consequence of activating the Nrf2/HO-1 signaling pathway, ANP could suppress the expression of apoptosis-related proteins, such as Bax and C-caspase 3, promote expression of the anti-apoptotic protein Bcl-2, and inhibit apoptosis and oxidant injury in EPCs.

The present study, in combination with previous reports (40,41), suggested that oxidative stress may be a crucial contributor towards cell apoptosis, and oxidative stress was related to the elevated levels of oxidative stress markers, which were affected by an intracellular oxidative and antioxidant defense mechanism in $H_2O_2$-treated EPCs. In the present study, the $H_2O_2$-induced cell apoptosis was accompanied by oxidative stress and inhibition of the Nrf2/HO-1 signaling pathway in EPCs, whereas these changes were reversed by ANP treatment. Therefore, it was suggested that the associated potential mechanisms of protection were possibly linked to activation of the Nrf2/HO-1 signaling pathway in EPCs under oxidative stress conditions, and that the Nrf2/HO-1 signaling pathway may serve a conservative role in cell viability. As a result, the protective effects of ANP on EPCs were revealed to be due to direct activation of the Nrf2/HO-1 signaling pathway.

There were some limitations in the present study. First, only *in vitro* experiments were performed. In addition, only the Nrf2/HO-1 signaling pathway was assessed with regard to the potential mechanism of ANP in this study. It is essential to evaluate related regulators or other signaling pathways, such as the PI3K/Akt and TLR pathways, which may be detected by reverse transcription-quantitative PCR.

In the future, a rat model of IDD may be established via percutaneous needle puncture of the rat tail to further explain the association between ANP and IDD. In addition, future studies should focus on the role of the PI3K/Akt and TLRs signaling pathways in the inhibition of oxidant stress in EPCs.

The present study revealed that in EPCs, ANP may activate Nrf2 nuclear translocation and expression of Nrf2/HO-1, which may inhibit cell apoptosis and oxidant injury induced by $H_2O_2$ under oxidative stress conditions. In conclusion, it was suggested that the Nrf2/HO-1 signaling pathway may represent a target for protection against $H_2O_2$-induced cell apoptosis and oxidant stress. ANP may represent a potential treatment for various diseases related to oxidant injury through its activation of the Nrf2/HO-1 signaling pathway, including IDD.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

FH and QF conceived and designed the study, and reviewed and revised the manuscript. JG and JW performed the experiments and collected the experimental data. LT and YL interpreted the results and contributed to the manuscript preparation. FH and QF confirm the authenticity of all the raw data. All authors agreed to be accountable for the content of the work, and read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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