Original Article

Protective effect of 17β-estradiol on hydrogen peroxide induced apoptosis of rat nucleus pulposus cells

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Abstract: Background: It has been suggested that intervertebral disc (IVD) cell apoptosis playing a key role in promoting disc degeneration, and Oxidative stress has been proved to induce apoptosis of nucleus pulposus cells (NPCs) in contributing to the process of IVD degeneration. 17β-Estradiol (17β-E2) has been reported for its protective effect on NPCs in our previous studies. However, it is not yet clear whether 17β-E2 has the protective effect on NPCs against apoptosis induced by oxidative stress. Purpose: Based on apoptotic cell model induced by Hydrogen Peroxide (H2O2), the current research was design to explore the effect of 17β-E2 on rat NPCs against apoptosis. Methods: NPCs were isolated from male Sprague-Dawley rats and cultured in complete medium. After two weeks, the NPCs were treated with H2O2 (1, 10, 100, 500 and 1000 μM/L, respectively) for 6 h, and 500 μM/L H2O2 for (1, 3, 6, 12 and 24 h, respectively). Cell counting kit-8 assay was performed to determine cell viability. LDH assay was performed to assess cytotoxicity after different treatments. Apoptotic incidence was analyzed by Fluorescence Activating Cell Sorter (FACS), morphological changes, as well as western blot of active caspase-3. Results: The results showed that H2O2 induced notable apoptosis and over expression of active caspase-3 in a dose- and time-dependent manner. However, the adverse effect caused by H2O2 was obviously reversed by 17β-E2. Besides, cell viability was decreased after treatment with H2O2, which was then increased by the addition of 17β-E2. In particular, the dose-dependent effect of 17β-E2 was remarkable. During the experiments, it was found that all effects resulting from 17β-E2 were eliminated by estrogen receptor antagonist ICI182, 780. Conclusions: These results obtained in this study suggest that 17β-E2 can effectively protect rat NPCs from peroxide-induced apoptosis in a dose-dependent manner, implying the potential of 17β-E2 to prevent IVDD onset or slow its progression in the early stage.

Keywords: 17β-estradiol, apoptosis, oxidative stress, intervertebral disc, nucleus pulposus

Introduction

Lots of spine-related diseases, such as spinal canal stenosis, spondylolisthesis and disc herniation, are caused by intervertebral disc (IVD) degeneration. Results from different studies have shown that 70% of the population experienced low back pain during their life, which generates huge economic losses [1, 2]. Decompression, bone graft and transpedicular screw internal fixation are the limited treatments of the diseases resulting from intervertebral disc degeneration (IVDD) [3]. While we haven’t known the pathological mechanism of IVDD clearly, it is commonly accepted that IVDD is influenced by many factors, including age, genetics, gestation and so on, and among all these factors mechanical stimuli has been recognized as a leading cause in the process of IVDD [4-8]. Excessive apoptosis of intervertebral disc cells as a vital impact in the event of IVDD has been suggested, and there is a growing piece of evidence indicating that the mechanism may be related to oxidative stress [12-14].

There are two species cells in the intervertebral disc. The inner one are the NPCs within a matrix of type II collagen and proteoglycan, and the outer are the annulus fibrosis (AF) cells compose tough fibers which protect and maintain the inner part. Several phenomena such as the reduction of NPCs and the elevation of type II collagen synthesis accompany with IVDD in life
due to the eventual formation of fissures [15]. Obviously, cartilage-special extra-cellular matrix (ECM) components can be produced by the mechanism that excessive cell apoptosis had occurred in NPCs. Then the apoptosis of NPCs triggered a series of events that related to the progress of IVDD. Eventually, the biomechanical structure of intervertebral disc is broken.

The phenomenon that H$_2$O$_2$, which is one of the reactive oxygen species (ROS) working with superoxide anion and hydroxyl radical mediate regulatory events is an essential participant in cell signaling [12]. High levels of ROS causes cell damage through signaling pathway [16]. Researches showed that ROS is produced by various factors such as age, compression, neo-vascularization and injury which lead NPCs to apoptosis and senescence, and eventually accelerate degeneration of the disc [9, 13, 17]. Therefore, the fact that antioxidant resist ROS is a vital way to protect intervertebral disc from degeneration, at the same time, additional benefits have been gained as well [13, 18, 19].

Estrogen is a hormone that produced by both male and female. There are lots of evidence to support that estrogen affects multiple tissues, organs and the characteristics of female [20, 21]. The therapy of estrogen replacement helps postmenopausal women to maintain healthier IVD [22]. Other than that, it is reported that female rats tend to develop disc degeneration after oophorectomy. Supplemental estrogen can stop the development of the ovariectomy-associated IVDD [23]. In general, these studies demonstrate that estrogen is closely associated with IVDD. In addition, 17β-E$_2$ can protect other species cells from apoptosis in human body, including pancreatic βcells, synovial fibroblasts and chondrocytes [24-26]. While 17β-E$_2$ will exert antioxidant effects to protect NPCs from apoptosis induced by ROS, it has not been reported. The purpose of this study is to evaluate whether 17β-E$_2$ has a significant effect in preventing H$_2$O$_2$-induced apoptotic cell death, and discuss the potential advantages of the approach to providing a therapeutic method for the regulation of IVDD.

Materials and methods

Materials

Collagenase type II, trypsin, Hydrogen peroxide (H$_2$O$_2$), 17β-Estradiol (17β-E$_2$), IC182, 780 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12) were obtained from HyClone (HyClone Laboratories, Logan, UT, USA). Hank’s Balanced Salt Solution (HBSS) was bought from Gibco-BRL (Grand Island, NE, USA). The Annexin V/propidium iodide binding kit was obtained from Multi Sciences Biotech, Co., Ltd. (Hangzhou, China) and Cell Counting Kit-8, Hoechst 33258 was purchased from Solarbio (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China). LDH Cytotoxicity Assay Kit was purchased from Beyotime Company (Jiangsu, China). Primary antibodies against caspase-3, Anti-GAPDH and anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Primary disc cell isolation

Male Sprague Dawley rats (weighing 200-220 g) were bought from the Laboratory Animal Center of Hebei Medical University (Hebei, China). The Animal Care and Experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health and were approved by the Animal Ethics Committee of Hebei Medical University. The rats were sacrificed by intravenous administration of 150 mg/kg pentobarbital sodium, and the rat lumbar IVD (L1-L5) collected immediately in a sterile environment. The muscle tissues and ligaments around the IVD were removed. The tissues were then placed into DMEM/F12 and were cut into small pieces (< 1 mm$^3$). To isolate the cells, the IVD tissues in the DMEM/F12 were digested with 0.25% collagenase type II for ~1 h in a water bath at 37°C. Then, the tissue was additionally digested with 0.2% trypsin (including 0.02% EDTA) for ~5 min at 37°C. Following the two-step enzyme digestion, the suspension was filtered through a 70 μm mesh. Subsequently the filtered cells were washed twice with Hank’s Balanced Salt Solution. Finally, the NPCs were added to DMEM/F12 media, supplemented with 15% FBS, 100 U/ml penicillin G, and 100 µg/ml streptomycin is cultured at 37°C in a humidified atmosphere of 95% air and 5% CO$_2$. The medium was changed once every two days. The NPCs were passaged three times prior to being collected for use.

Cell culture and drug treatment

The cells were digested and subcultured into appropriate culture plates and cultured as pre-
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Previously described. When the cell culture became 80-90% confluent, and when the cells confluence in each well reached 80-90%, the medium was replaced with DMEM-high glucose medium without FBS, phenol red, penicillin and streptomycin. This research was divided into three parts. Firstly, to investigate the dose-dependent effect of $H_2O_2$, six experimental groups were established, including one control, five treatment groups. In the five treatment groups, the NPCs were treated with $H_2O_2$ (1, 10, 100, 500 and 1000 μM/L, respectively) for 6 h. Secondly, the time-dependent effect of $H_2O_2$ was explored, we use $H_2O_2$ at the concentration of 500 μM/L to deal with experimental groups for (1, 3, 6, 12 and 24 h, respectively). Finally, in other five treat groups, the NPCs were treated with $H_2O_2$ (500 μM/L) for 6 h with pretreatment of 17β-E$_2$ (0.1, 1, 5, and 10 μM/L 17β-E$_2$, 10 μM/L 17β-E$_2$ combine 10 μM/L ICI182, 780) for 1 h. All the groups were cultured in a humidified atmosphere with 5% CO$_2$ at 37°C.

Morphological observation

Cells were sub-cultured in 6-well plates at 2×10$^5$ cells/well in complete culture medium. Following 24 h treatment, cover slips with adherent cells were observed under a fluorescence inverted microscope (Olympus IX50). Three areas of 200×200 pixels in one sample were randomly selected from the image to observe the morphological changes in the apoptotic cells.

Nuclear staining with Hoechst 33258

The NPCs were seeded into a six-well plate and routinely cultured overnight in medium containing 10% fetal calf serum. These cells were then treated as previously described. Subsequently, the cells were fixed with 4% formaldehyde for 15 min at room temperature. The cells were then washed twice with 1X phosphate-buffered saline (PBS) and stained with 10 mg/l Hoechst 33258 for 1 h at room temperature. Finally, the alterations in nuclear morphology were observed under fluorescence microscopy (Nikon TE 2000 U; Nikon, Tokyo, Japan).

Cytotoxicity assay

Release of lactate dehydrogenase (LDH) is an indicator of membrane integrity and hence, cell injury. LDH assay was performed to assess the LDH release level in the culture following treatments as before. The intracellular LDH was determined after lysing the cells by freezing and rapid thawing. The LDH release was measured at an absorbance of 490 nm. The percentage of LDH release was calculated as: (LDH activity in media)/(LDH activity in media + intracellular LDH activity)×100%. Each experiment was done three independent times with good agreement.

Cell viability assay

Cell viability was measured by the conversion of Dojindo’s highly water-soluble tetrazolium salt, WST-8, to a yellow colored water-soluble formazan. The quantity of formazan dye generated by the activity of mitochondrial dehydrogenases in the cells is directly proportional to the cell viability. For the assay, the NPCs (1×10$^4$ cells/well) were incubated in 96-well plates in a humidified atmosphere of 5% CO$_2$ at 37°C. Following treatment, 10 µl CCK-8 solution (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) was added to each well and incubated at 37°C for 2 h. The optical density of each well was measured using a microculture plate reader (Epoch; BioTek, Winooski, VT, USA) at a wavelength of 450 nm.

Apoptosis assay

Cells were sub-cultured in 6-well plates at 2×10$^5$ cells/well with complete culture medium. Following 24 h treatment, cells still attached to the plate and those present in the supernatant were collected together and resuspended in cold binding buffer. The apoptotic incidence was detected using an Annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit. Apoptosis was determined by staining cells with both Annexin V/FITC and propidium iodide (PI), according to the manufacturer’s instructions. Annexin V/FITC was used to quantitatively determine the percentage of cells undergoing apoptosis based upon the loss of membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing phosphatidylserine to the external environment. Cells that were positively stained with Annexin V/FITC and negatively stained for PI were therefore considered to be undergoing...
apoptosis. Cells that were positively stained for both Annexin V/FITC and PI were considered to be undergoing necrosis. The cells were then stained with 5 µl Annexin V/FITC and 10 µl PI, followed by the addition of 500 µl binding buffer for 15 min at room temperature in the dark. The samples were analyzed by flow cytometry (FCM) within 1 h.

**Western blot analysis**

Western blot was carried out to investigate the Expression level of active caspase-3. NPCs following treatment as before were washed with ice cold PBS and harvested in 100 µL of cell Lysis buffer containing 1% protease inhibitor (Solarbio, China). Lysates were centrifuged at 4°C for 5 min at 12,000 rpm and resolved on 12% SDS-polyacrylamide gels (SDS-PAGE). Proteins were transferred by electroblotting to a PVDF membrane (Bio-Rad). The membranes were blocked with 5% non-fat dry milk in TBS (50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 0.1%) and incubated overnight at 4°C in 3% non-fat dry milk in PBST with corresponding primary antibodies. Washing in PBST 3 times for 30 min, the membrane was incubated with a secondary anti-IgG-HRP antibody at room temperature for 1-2 h. Immunolabeling was detected using enhanced chemiluminescence reagent (ECL, Amercontrol Biosciences).

**Real-time quantitative RT-PCR (q-PCR)**

Q-PCR was carried out to detect expression level of mRNA encoding caspase-3. Trizol method (GibcoBRL) was used to isolate Total RNA according to the manufacturer's introduction. Total RNA was measured fluorometrically using the CyQuant-Cell Proliferation Assay Kit (Molecular Probes). cDNA synthesis was performed using the ThermoScript®RT-PCR System (Invitrogen, Carlsbad, CA, USA). For semi-quantification of the genes of interest, we utilized the DyNamo SYBR Gren 2-step qRT-PCR Kit (Finnzymes) in a total volume of 20 μL, performing real-time PCR reaction in an M×300P cycler. Amplicons of caspase-3 was amplified with primers listed in Table 1. Standard curves were run in each optimized assay which produced a linear plot of threshold cycle (Ct) against log (dilution). The amount of target was quantified based on the concentration of the standard curve and was presented as relative Ct value. The quantity of target was normalised against the quantity of GAPDH.

**Statistical analysis**

Values are presented as the mean ± standard deviation. Statistical analyses were performed using the SPSS 13.0 statistical software program (SPSS, Inc., Chicago, IL, USA). The means of apoptotic incidences among groups, as well as the absorbances among groups were compared by one-way analysis of variance, followed by pairwise comparison using the Student-Newman-Keuls-q test. All statistical tests were two-sided and P < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Morphological changes of apoptotic NPCs induced by H₂O₂ and the protective effects of 17β-E₂**

Using a fluorescence inverted microscope, apoptotic cells exhibited plasma membrane blebbing, cell shrinkage and nuclei condensation. The effect of H₂O₂-induced apoptosis with a dose- and time-dependent manner (Figure 1A and 1B). Few apoptotic cells were observed in the control group. As compared with the control group, treatment with H₂O₂ (500 μM/L) induced more apoptotic cells. Pretreatment with 17β-E₂ (10 μM/L) resulted in a decrease in the number of apoptotic cells less than those treated with H₂O₂ (500 μM/L), estrogen receptor antagonist ICI182, 780 (10 μM/L) could revert the protective effects of 17β-E₂ (10 μM/L). There was no significant difference between the H₂O₂ (500 μM/L) and H₂O₂ (500 μM/L) + 17β-E₂ (10 μM/L) + ICI182, 780 (10 μM/L) groups (Figure 1C).

**Effect of 17β-E₂ on nucleic morphology in H₂O₂-treated NPCs**

Subsequent to culture with H₂O₂ or the H₂O₂/17β-E₂ combination, morphological changes in the NPCs were observed by Hoechst

**Table 1. Sequence of primers used for qPCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3</td>
<td>5’GCTCGCAATGGTACCGATGT3’ (sense)</td>
</tr>
<tr>
<td></td>
<td>5’TTCACGATTAAGGTCAATTT3’ (antisense)</td>
</tr>
</tbody>
</table>
Protective effect of estrogen

Figure 1. Protective effect of 17β-E₂ on H₂O₂-induced apoptosis in NPCs. Morphological and nuclei changes in rat NPCs. Phase contrast microscopic images of NPCs stimulated with different treatments and representative photomicrographs of NPCs stained with Hoechst 33258. Apoptotic cells presented shrinkage and vacuole. Apoptotic cells nuclei were characterized as condense or fragment. The effect of H₂O₂ with a dose- and time-dependent manner (A and B). 17β-E₂ protect NPCs from H₂O₂-induced apoptosis in a concentration-dependent manner (C). Scale bar is 20 μm; 17β-E₂, 17β-estradiol; H₂O₂, Hydrogen Peroxide; ICI182, 780, estrogen receptor antagonist.

33258 staining. As presented in Figure 1A and 1B, in the control group, NPC nuclei was round and stained homogeneously with Hoechst 33258, in H₂O₂-treated NPCs, a considerable proportion of cells displayed characteristics of apoptosis with condensed and fragmented nuclei, the ratio of apoptosis cells was amplify with dose and time. Treatment with 0.1, 1, 5 and 10 μM/L 17β-E₂ led to a significant reduction in the number of apoptotic cells with fragmented nuclei (Figure 1C). These results suggest that 17β-E₂ is able to inhibit the H₂O₂-induced nucleic morphological changes in NPCs, with dose-dependent manner.

Effect of 17β-E₂ on cell cytotoxicity in H₂O₂-treated IVD

LDH assay was carried out to determine the cellular integrity following H₂O₂ and 17β-E₂ treat-
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Figure 2. Measurement of LDH release. After the exposure of NPCs with H$_2$O$_2$, 17β-E$_2$ and ICI182, 780. The release of LDH was measured at 490 nm. Results are presented as percentage of LDH release. Histogram for statistical analysis shows the LDH release level (fold of control) in the different treatment groups, with values expressed as mean ± SD (n = 5). *P < 0.05; #P > 0.05 17β-E$_2$, 17β-estradiol; H$_2$O$_2$, Hydrogen Peroxide; ICI182, 780, estrogen receptor antagonist; LDH, lactate dehydrogenase.

Effect of 17β-E$_2$ on cell viability in H$_2$O$_2$-treated NPCs

The NPC viability and metabolic activity were analyzed by CCK-8 assay. The results indicated that NPCs which treated with H$_2$O$_2$ were able to reduce the number of metabolically active cells and viability in a time- and dose-dependent manner (Figure 3A and 3B). A significant reduction in cell viability was observed at 6 h following 500 μM/L H$_2$O$_2$ exposure. However, when NPCs were pre-treated with 17β-E$_2$ (0.1, 1, 5, or 10 μM/L) for 1 h and then exposed to H$_2$O$_2$ for 6 h, cell viability was improved in a dose-dependent manner. The most significant increase was observed in the 10 μM/L 17β-E$_2$-treated group compared with cell viability following 500 μM/L H$_2$O$_2$ treatment (Figure 3C). Estrogen receptor antagonist ICI182, 780 could revert the protective effects of 17β-E$_2$ (Figure 3D).
Protective effect of estrogen

It was further investigated whether 17β-E₂ could inhibit 
H₂O₂-induced apoptosis in cells and whether 17β-E₂ (0.1,
1, 5, and 10 μM/L) pretreatment could decrease apoptosis 
in a concentration-dependent manner. The level of 
apoptotic cells was determined by double staining with 
Annexin V/FITC and PI. The apoptotic ratio of cells was 
calculated as a percentage of apoptotic cells/total cells. 
Annexin V/FITC and PI labeled cells were quantified by FCM,
thus allowing for discrimination between viable/intact ce-
lls (Annexin V-PI-), early apoptotic (Annexin V+PI-) and late 
apoptotic or necrotic cells (Annexin V+PI+). As is shown 
in Figure 4A and 4B, the per-
centage of apoptosis incre-
as in cells following treat-
ment with H₂O₂ with dose and time. Cells pretreated with 
17β-E₂ showed a reduced rate of apoptosis. To further illustrate 
the potential contribution of 17β-E₂, estrogen rece-
ptor antagonist ICI182, 780, was used. When cells were 
incubated with both 17β-E₂ and ICI182, 780, the protec-
tive effects of 17β-E₂ were reduced (Figure 4C). To inves-
tigate the association be-
tween the concentration of 
17β-E₂ and the protective effects by 17β-E₂, four differ-

Figure 3. Cell viability assay. 
Cell viability was analyzed using 
a CCK-8 assay. The NPCs were 
stimulated with different treat-
ments. The results are expressed 
as mean ± SD (n = 5). *P < 0.05; 
#P > 0.05. 17β-E₂, 17β-estradiol; 
ICI182, 780, estrogen receptor 
antagonist, H₂O₂, Hydrogen Per-
oxide, CCK-8, Cell Counting Kit-8.
Protective effect of estrogen

A

Annexin V-FITC

B

Annexin V-FITC
Protective effect of estrogen

![Graph showing the protective effect of estrogen over time.](image)

- **Time:** Control, 1h, 3h, 6h, 12h, 24h
- **Apoptosis rate (%)**
- **Variables:** 17β-estradiol (E2), ICI 182,780, H2O2

![Histogram showing the effect of different treatments on apoptosis rate.](image)
Protective effect of estrogen

Figure 4. Annexin V-FITC/PI staining assay. Evaluation of apoptotic incidence. Representative graphs obtained by flow cytometry analysis following double staining with Annexin V/FITC and propidium iodide. The apoptotic incidences of Intervertebral Disc Cells (IVD) cultured with H$_2$O$_2$ and stimulated with ICI182, 780 or various concentrations of 17β-E$_2$. As show in (A, B), H$_2$O$_2$ induced IVD cells apoptosis with a dose- and time-dependent manner. 17β-E$_2$ could inhibit H$_2$O$_2$-induced apoptosis in a concentration-dependent manner (C). Histogram for statistical analysis shows the LDH release level (fold of control) in different treatment groups, mean ± SD (n = 5). *P < 0.05, **P > 0.05. FITC, fluorescein isothiocyanate; H$_2$O$_2$, Hydrogen Peroxide; ICI182, 780, estrogen receptor antagonist; 17β-E$_2$, 17β-estradiol.

Effect of 17β-E$_2$ on the expression levels of caspase-3 in H$_2$O$_2$-treated NPCs

To investigate the effects of 17β-E$_2$ on the expression of caspase-3 in H$_2$O$_2$-treated NPCs, Western blot was carried out. The result indicated that H$_2$O$_2$ were capable of upregulating the protein level of caspase-3 in NPCs in a concentration- and time-dependent manner, 500 μM/L H$_2$O$_2$ significantly upregulated caspase-3 compared with that of the control group (Figure 5A and 5B). Intriguingly, treatment with 17β-E$_2$ was able to inhibit this in a dose-dependent manner. Addition of estrogen receptor antagonist ICI182, 780 resulted in elimination of the protective effects of 17β-E$_2$. As presented in Figure 5C.

Effect of 17β-E$_2$ on the mRNA encoding caspase-3 levels of in H$_2$O$_2$-treated NPCs

In order to investigate the mRNA encoding caspase-3 levels, we performed qPCR on NPCs treated with H$_2$O$_2$, 17β-E$_2$, and ICI182, 780. As shown in Figure 6, 17β-E$_2$ increased the mRNA encoding caspase-3 in a concentration- and time-dependent manner significantly (Figure 6A and 6B). 17β-E$_2$ is able to inhibit the mRNA encoding caspase-3 level induced by H$_2$O$_2$ in NPCs, with dose-dependent manner (Figure 6C). Estrogen receptor antagonist ICI182, 780 could revert the protective effects of 17β-E$_2$ (Figure 6D) (P < 0.05).

Discussion

In recent years, many studies have shown that various factors such as age [13], high glucose [27, 28] and mechanical stimulus [9] can induce apoptosis in NPCs via oxidative stress. Previous studies have revealed that apoptosis of NPCs can be induced by H$_2$O$_2$ [19, 12]. So we have sound reasons to believe that Reactive oxygen species (ROS) is a key intermediate in the procession of the apoptosis in NPCs leading to IVDD. In present study, we found that H$_2$O$_2$ can induce the apoptosis of NPCs in dose- and time-dependent manner. We chose a concentration of 500 μM/L for 6 h to contribute model for this study.

17β-E$_2$, as one of the molecule of estrogen, has been used as a contraceptive and principal constituents of hormone replacement therapy formulations in postmenopausal women [29]. A large amount of recent studies are focused on the anti-oxidation effects of E$_2$. In this research, NPCs exposed to H$_2$O$_2$ underwent a significant increase in cellular apoptosis and exhibited the distinct morphological features of apoptosis. We observed that pretreatment of NPCs with 17β-E$_2$ before exposure to H$_2$O$_2$ resulted in a remarkable decrease in the percentage of apoptotic cells. This study supports our hypothesis that 17β-E$_2$ can exert antioxidant effects to protect NPCs from apoptosis induced by H$_2$O$_2$ to slow down IVDD. Estrogen has remarkable effects on the reproductive system, neurotransmitter release, bone structure, cognitive function and blood vessel [30, 31]. It is indispensable to take the importance of estrogen which has prominent implications into consideration. The protective effects of estrogen have been investigated widely, including prevent the increase of LVEDP by increasing the activity of nitric oxide synthase in the heart, lead to an increase of antioxidant capacity in total serum that result in an improvement of the antioxidant status in women, attenuate hyperoxia-induced apoptosis in astrocytes, and have an anti-apoptotic function in skeletal muscle cells [32-35]. However, whether 17β-E$_2$ can protect NPCs from Oxidative stress-induced apoptosis has not been confirmed. As far as we have known, the present study is the first one to demonstrate that 17β-E$_2$ can prevent the apoptosis induced by H$_2$O$_2$ in NPCs.
Other studies have shown that phenol red, a pH indicator that has been widely used in growth medium, exhibits minor oestrogenic activity that is similar to the effect of steroid hormones [36]. Wsierska-Gadek et al [37] showed that phenol red promotes the cell proliferation and cell cycle progression of human cells expressing the estrogen receptor strongly when added it into the culture medium. In order to eliminate the effects of phenol red, medium without phenol red was used. The results of the present study indicated that 17β-E² has the ability of protecting NPCs from apoptosis via anti-oxidation pathway, and pretreatment of NPCs with the estrogen receptor antagonist ICI182, 780 could impair the protective effects of 17β-E². Higher concentrations of 17β-E² (0.1-10 µmol/l) exerted a stronger protective effect. Sum up, these results support our conclusion that 17β-E² can protect NPCs from apoptosis induced by H₂O₂.

Apoptosis of disc cells can be triggered by various stimuli such as excessive ROS, tumor necrosis factor alpha, serum deprivation, cyclic stretch and compression through different apoptotic cascade pathways. ROS, participating in
Protective effect of estrogen

Figure 6. The expression level of caspase-3 detected by qPCR. The mRNA level was determined by Real-time quantitative R-T RNA. NPCs were stimulated by different treatments. H$_2$O$_2$ significantly increased the mRNA expression of caspase-3 in a concentration- and time-dependent manner (A, B). 17β-E$_2$ is able to inhibit the mRNA encoding caspase-3 level induced by H$_2$O$_2$ in NPCs, with dose-dependent manner (C). Estrogen receptor antagonist ICI182, 780 could revert the protective effects of 17β-E$_2$ (D) (Mean ± SD; n = 5, *P < 0.05, #P > 0.05) 17β-E$_2$, 17β-estradiol; H$_2$O$_2$, Hydrogen Peroxide; ICI182, 780, estrogen receptor antagonist.

The regulation of various cellular functions, are one of the most important intracellular signaling systems. DNA, lipids, proteins, and degrade the ECM were damaged by Excessive ROS [38]. Although the precise mechanism of cell apoptosis is not clear enough, caspase-3 as a central executioner plays a vital role in the caspase apoptotic cascade pathway. Upregulation of the expression and activity of caspase-3 has been observed in different cellular apoptotic models, and specific inhibitors can successfully decrease this. Caspase-3 can be a therapeutic target for slowing down the processes of disc degeneration. Our findings in the apoptotic model are consistent with this theory. H$_2$O$_2$ significantly enhanced the protein expression level of caspase-3, compared with the control group, resulting in marked apoptosis as demonstrated by hoechst 33258 staining and FCM analysis. The results support our conclusion that caspase-3 takes part in the process of apoptosis induced by Oxidative stress, and estrogen can exert an antioxidative effect in the process of apoptosis.

There are several limitations in our study. Firstly, only four con-
centrations of 17β-E₂ were selected in our search, and more concentrations should be required to further explorations of the dose-response effects of 17β-E₂ in the cellular apoptosis. Secondly, caspase-3 is the only protein we have detected, and additional studies are necessary to further elucidation of the signaling mechanisms which mediate the anti-apoptotic action of 17β-E₂ in NPCs. Thirdly, the NPCs of rat were the only type of cell explored in this study, which cannot adequately represent human NPCs, and more studies should be remained to investigate close to clinical.

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Disclosure of conflict of interest
None.

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