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17β-Estradiol Inhibites Tumor Necrosis Factor-α Induced Apoptosis of Human Nucleus Pulposus Cells via the PI3K/Akt Pathway

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Tumor necrosis factor-α (TNF-α) has been widely known to induce degeneration of nucleus pulposus cells (NPCs). 17β-estradiol (17β-E2) has been broadly proven for its function of suppressing cell apoptosis. The aim of this study is to explore whether 17β-E2 protects apoptosis of human NPCs induced by TNF-α via the PI3K/AKT pathway.

Material/Methods: NPCs were divided into four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) and MK2206 (10 um/L, inhibitor of the PI3K/AKT pathway). Flow cytometry was used to measure the apoptotic incidence. Inverted phase-contrast microscopy was used to accomplish the morphological observation for apoptosis of treated cells. Additionally, Cell Counting Kit 8 (CCK-8) assay was used to detected cell proliferation. Western blot and quantitative real-time PCR (qRT-PCR) were applied to explore the expression of pro-caspase-3, caspase-3/p17, cleaved PARP, PARP, Akt, and phospho-Akt (p-Akt).

Results: First, inverted phase-contrast microscopy, CCK-8, and flow cytometry showed that TNF-α induced marked apoptosis, which was abolished by 17β-E2. Furthermore, Western blot and qRT-PCR showed that 17β-E2 protects TNF-α which can induced apoptosis by upregulating p-Akt, whereas Akt was essentially constant. Our data revealed that p-Akt expression peaked at 24 hours in a time-dependent manner (0–48 hours) after treating with TNF-α; and the p-Akt expression generally increased in a time-dependent manner (0–48 hours) after treating with TNF-α and 17β-E2.

Conclusions: 17β-E2 is shown to protect NPCs against TNF-α induced apoptosis by upregulating p-Akt in the PI3K/AKT pathway. 17β-E2 generally increases expression of p-Akt.

MeSH Keywords: Accessory Nerve • Estriol • Tumor Necrosis Factors

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Background

Chronic lower back pain (LBP) is a common disorder among adults that impacts the quality of life. Approximately 85% of people have low back pain during their lives [1,2], and in more than 40% of people with LBP, the LBP is due to intervertebral disc degeneration (IVDD) [3–5]. Reduced cell density and catabolism of the extracellular matrix (ECM) play key roles in IVDD due to disc cell apoptosis [6–8]. Remarkably, aberrant apoptosis and accelerated aging of nucleus pulposus cells (NPCs) are deemed to be the two major cellular processes related to IVDD [9,10]. Once intervertebral disc degeneration has begun, it is very difficult to find a drug-based method to prevent its onset or delay its progression even in the early stage.

Etiologically, inflammatory cytokines including matrix metalloproteinases (MMPs), IL-1β, IL-6, TNF-α, nitric oxide, and prostaglandin E2 seem to be involved in the process of IVDD leading to loss of cells and ECM [11,12]. Among these inflammatory cytokines, TNF-α is known as a pro-inflammatory cytokine for NPCs. During disc degeneration, in addition to infiltrating immune cells, resident NPCs produce high levels of cytokines such as TNF-α [13,14]. Animal studies have suggested that TNF-α may elicit radicular pain, which was associated with long-term sensitization and pathological changes in dorsal root ganglion (DRG) neurons [15]. TNF-α stimulates production of NGF, BDNF, and VEGF, molecules associated with nerve ingrowth and angiogenesis by NPCs [16]. TNF-α upregulates expression of matrix degrading matrix metalloproteinases (MMPs[2]) and two major aggrecanases (ADAMTS-4 and ADAMTS-5) by NPCs [16–18]. PI3K and its target PKB/Akt have emerged as critical signaling molecules that regulate multiple cellular processes, including survival and proliferation in numerous systems [19–21]. Matthew et al. [22] demonstrated that PI3K and Akt could suppress TNF-induced apoptosis in MCF-7cells, while simultaneously increasing NF-κB activity.

Previous studies [23,24] have shown that 17β-E2 could protect rat NPCs against interleukin-1α (IL-1α) and levofloxacin-induced apoptosis. However, it remains unclear whether 17β-E2 protects apoptosis for human NPCs. Our study provides a further exploration focused on whether 17β-E2 inhibits apoptosis of human NPCs induced by TNF-α via the PI3K/AKT pathway.

Material and Methods

Ethics statement

Animal protocols were approved by the Institutional Animal Care and Use Committee of the Third Hospital of Hebei Medical University.

Reagents and antibodies

Reagents and antibodies included: human NPCs (ScienceCell Research Laboratories, USA); Nucleus Pulposus Cell Medium (NPCM) including FBS, Nucleus Pulposus Cell Growth Supplement and penicillin/streptomycin solution (P/S) (Hyclone, Logan, UT, USA); trypsin (Sigma, St. Louis, MO, USA); PBS and DMSO (Solarbio, Beijing, China); Annexin V-FITC/PI kit (BD Pharmingen, San Jose, CA, USA); AQueous MTS Reagent Solution (Promega, Madison, WI, USA); Caspase-3 activity kit (Beyotime, Shanghai, China); Cell Counting kit 8 and 17β-estradiol (Sigma-Alorich (Peprotech, USA); TNF-α (Peprotech, USA); MK2206 (Sigma, St. Louis, MO, USA); first and second antibodies of active caspase-3, PARP, Akt, p-Akt and GAPDH (Proteintech, Wuhan, China).

Cell culture

Cryopreserved human NPCs from ScienceCell Research Laboratories were isolated from nucleus pulposus of human intervertebral disc. NPCM consists of 500 mL of basal medium, 10 mL of FBS, 5 mL of Nucleus Pulposus Cell Growth Supplement, and 5 mL of penicillin/streptomycin solution (P/S). These reagents were mixed together to make the complete human NPC mix before cell recovery. Subsequently, we thawed the vial in a 37°C water bath and returned the cells to 50 mL culture flask containing 15 mL complete HNPC as quickly as possible with minimal handling. Finally, the cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C; culture medium was refreshed the next day to remove residual DMSO and unattached cells. The medium was changed every three days. Once the culture reached 70% confluency, the medium was changed every other day until the culture was approximately 90% confluent.

Morphological observation

Human NPCs were plated according to four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway) in six-well plates at a density of 2×10^5 cells per well and cultured with complete culture medium. Upon reaching 90% confluence, the cells were cultured in serum free DMEM/F12 as four groups. The cells were observed by inverted phase-contrast microscopy (Olympus, Tokyo, Japan) and were photographed by digital camera (Nikon, Japan).

Fluorescence activated cell sorting (FACS) analysis

The Annexin V-FITC/PI apoptosis detection kit (BD Pharmingen, USA) was used to detect apoptotic cells. The detection process was performed according to the manufacturer's protocol.

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Human NPCs were divided into four groups: control, TNF-α (100 ng/mL) TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway). After 24 hours, the cells were washed with PBS and were collected by trypsinization. The cells were washed twice with PBS after centrifugation. From the cell suspension, 1×10^6 cells were re-suspended in 5 mL of 1× binding buffer. These cells were incubated for 15 minutes in the dark along with 5 µL of Annexin V-FITC and 5 µL of PI solution. The samples were analyzed by using a flow cytometer (BD Biosciences, San Jose, CA, USA). The Annexin V-FITC-/PI-Population was used to reflect the live cells, early apoptotic (FITC+/PI-) and necrotic (FITC+/PI+) cells.

Cell Counting Kit 8 (CCK-8) assay

CCK-8 was used to assess cell proliferation. Human NPCs suspension was inoculated in 96-well plates at a density of 2×10^5 cells/well per 100 µL. After 48 hours of treatment, we add 10 µL of the CCK-8 solution to each well of the plate, and the plates were incubated for an additional three hours in a 5% CO_2 incubator at 37 °C. Proliferation of cells was measured by using a microplate reader (Dynatech MR5000, Eggenstein, Germany) at an absorbance of 450 nm.

Caspase-3 activity assay

The first-passage of NPCs were placed in 6-well plates at 2×10^5 cells per well and controls underwent an identical treatment except for the addition of levofloxacin. Caspase-3 activity was detected by using a caspase-3 activity kit (Beyotime, China), which was based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) into the yellow formazan product, p-nitroaniline (pNA). According to the manufacturer's protocol, treated cells were lysed with lysis buffer (100 µL per 2×10^6 cells) for 15 minutes on ice and then were washed with cold HBSS. After incubating the mixture composed of 10 µL of cell lysate, 80 µL of reaction buffer, and 10 µL of 2 mM caspase-3 substrate in 96-well microtiter plates at 37 °C for 4 hours, caspase-3 activity was quantified in the samples with a microplate spectrophotometer (Biotek) at an absorbance of 405 nm. Caspase-3 activity was expressed as the fold change of enzyme activity when compared to that of synchronized cells.

Western blot

Active caspase-3, PARP, Akt, and p-Akt were detected by Western blot with GAPDH as a control. Human NPCs were divided into four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-estradiol (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-estradiol (10 um/L) and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway) for 24 hours, and then the cells were collected. This was accomplished by performing the following steps: (1) Complete lyses of each sample by cell lysis buffer, then centrifugation at 11,000 rpm for 15 minutes. (2) Collected 100 µL of supernatant and add 20 µL lauryl sodium sulfate buffer (6×), then degenerated by boiling water bath for 5 minutes. (3) Separated protein by electrophoretic with 12% SDS-polyacrylamide gelatin under 110 mV, and then transfer samples to nitrocellulose membrane and confined for 2 hour with 200 mA. (4) Added primary antibody of Akt, p-Akt, PARP, active caspase-3 (Bioworld, diluted to 1: 1,000), and GAPDH (Proteintech, diluted to 1: 2,000) at 37°C for one hour. (5) Antibody (anti-rabbit IgG) marked with horse radish peroxidase (HRP) was added at 37°C for one hour. (6) Scan and analyze the result with digital gel imaging (Chemi Imager 4000, Alpha Innotech USA).

Quantitative Real-Time PCR (qRT-PCR)

We performed qRT-PCR to detect expression level of mRNA encoding Akt, p-Akt, PARP, and active caspase-3. TRIzol method (GibcoBRL) was used to isolate total RNA according to the manufacturer's instructions. Total RNA (2 µg) was used as a template to generate cDNA by oligo (dT15) using the GoScript™ Reverse Transcription System (Promega Biotech Co, Ltd., Beijing, China). Primer pairs were used for PCR (synthesized by Invitrogen, Carlsbad, CA, USA) and are shown in Table 1. qRT-PCR was conducted by using the CFX Connect Real-Time PCR Detection System (BioRad Co., Ltd., China) in a final volume of 20 µL which included 10 µL GoTaq®qPCR Master Mix (Promega Biotech Co., Ltd., Beijing, China), 0.4 µL forward primer (10 µM), 0.4 µL reverse primer (10 µM), 2 µL cDNA, and 7.2 µL nuclease-free water. PCR amplification was performed by using the following protocols: 95°C for two minutes, then 40 cycles of 95°C for 15 seconds, and finally 60°C for one minute. 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 transcription levels of GAPDH was served as a loading control.

Statistical analysis

All statistical analyses were completed by using SPSS 21.0. Each assay was repeated at least three times independently to acquire reliable quantitative data. The results are presented as the mean ± standard deviation (SD). The means of apoptotic incidences among groups, as well as the absorbance among groups were compared by one-way analysis of variance (ANOVA), accompanied by pairwise comparison by using the SNK-q test. A p value <0.05 was considered statistically significant.

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Table 1. Primer sequences used in RT-qPCR.

Gene	Primer sequence	Length of product (bp)
Akt	F: 5'-TTTATTGGCTACAAGGAACG-3'	213
	R: 5'-AGTCTGAATGGCGGTGGT-3'	
Caspase-3	F: 5'-TGGAATTGATGCGTGATGTT-3'	395
	R: 5'-GTCGGCATACTGTTTCAGCA-3'	
PARP	F: 5'-CCATCGACGTCAACTACGAG-3'	113
	R: 5'-GTGCGTGGTAGCATGAGTGT-3'	
GAPDH	F: 5'- AACTTTGGCATCGTGGAAGGG-3'	2276
	R: 5'-AGGGATGATGTTCTGGGCTGC-3'	

F – forward primer; R – reverse primer.

Results

Inverted phase-contrast microscopy

Human NPCs were divided into four groups and were treated as described earlier. Few apoptotic incidences for NPCs were observed in the control group (Figure 1A). TNF-α (100 ng/mL) induced marked apoptosis (Figure 1B), which was protected by 17β-E2 (10 um/L) (Figure 1C). However, the anti-apoptotic effect of 17β-E2 was inhibited by MK2206 (inhibitor of PI3K/AKT pathway) (Figure 1D).

FACS analysis

Human NPCs were divided into four groups and treated as described earlier. Figure 2A and 2B showed that TNF-α (100 ng/mL) markedly increased the percentage of apoptosis (30.4%), compared with the control group (11.9%) ($p<0.05$). And NPCs pretreated with 17β-E2 reduced ratio of apoptosis (13.4%) induced by TNF-α, however, anti-apoptosis effect of 17β-E2 was inhibited by MK2206 (29.6%) ($p<0.05$), as shown in Figure 2C and 2D.

CCK-8 assay

Cell Counting Kit 8 (CCK-8) assay is a method of cell proliferation activity. Compared to the control group, TNF-α reduced the proliferation activity of NPCs (Figure 3) ($p<0.05$). The addition of 17β-E2 increased the proliferation activity, which was reversed by MK 2206 (Figure 3) ($p<0.05$).

Caspase-3 activity assay

Caspase-3 activity, a crucial mediator of apoptosis, was significantly increased after NPCs were treated with TNF-α (100 ng/mL) for 24 hours, compared to the control group (Figure 4) ($p<0.05$). Meanwhile, Figure 4 also shows that NPCs pretreated with 17β-E2 deceased caspase-3 activity, and MK 2206 restrained the protective effect of 17β-E2 ($p<0.05$).

Western blot

NPCs were divided into four groups and treated as described earlier. As shown in Figure 5A and 5B, TNF-α markedly ($p<0.05$) increased protein levels of both caspase-3/p17 and cleaved PARP, and decreased protein levels of pro-caspase-3 and PARP. The addition of 17β-E2 markedly upregulated protein levels of pro-caspase-3 and PARP; markedly downregulated protein caspase-3/p17 and cleaved PARP, compared with pretreated TNF-α alone ($p<0.05$). The effect of pretreated MK2206 was opposite to the effect of 17β-E2 ($p<0.05$). Figure 5C shows TNF-α downregulated p-Akt ($p<0.05$), while 17β-E2 markedly upregulated protein levels of p-Akt ($p<0.05$) which was reversed by MK 2206 ($p<0.05$). Whereas protein levels of Akt in four groups remained unchanged ($p>0.05$). Figure 5D shows that TNF-α upregulated p-Akt from 0 to 24 hours, while downregulated p-Akt from 24 to 48 hours (all $p<0.05$). However, the addition of 17β-E2 upregulated p-Akt in a time-dependent manner (all $p<0.05$). But protein levels of Akt in a time-dependent manner remained unchanged (all $p>0.05$) (Figure 5E).

qRT-PCR

Human NPCs were treated as described earlier and mRNA expression levels of target genes were detected by qRT-PCR. As shown in Figure 6, TNF-α significantly ($p<0.05$) increased mRNA levels of caspase-3, while reducing mRNA levels of PARP. The addition of 17β-E2 increased mRNA levels of PARP, while reducing mRNA levels of caspase-3, which was reversed by the addition of MK2206. Whereas, mRNA levels of Akt in the four groups remained unchanged ($p>0.05$) (Figure 6, Table 1).

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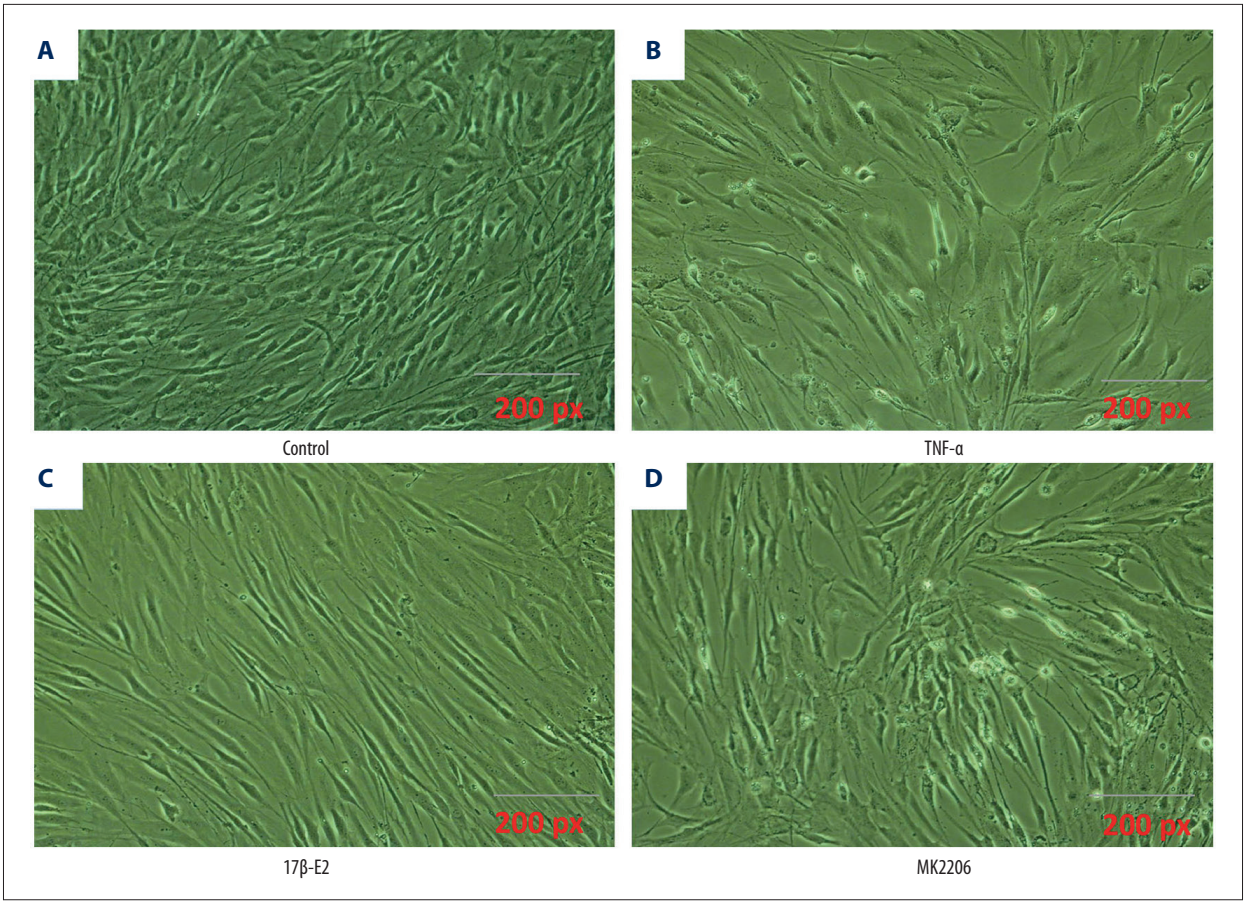

Figure 1. Morphologic changed in apoptotic human NPCs. Human NPCs were divided into four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) for 30 minutes and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway) for 30 minutes. All groups were incubated for 24 hours in serum-free medium without phenol red. Using phase-contrast microscopy, apoptotic cells were characterized by plasma membrane bleeding, cell shrinkage, and nuclei condensing, as indicated by the black arrows. (Scale bar, 200 um). (TNF-α – tumor necrosis factor-α; 17β-E2 – 17β-estradiol).

Discussion

As we know, TNF-α, an inflammatory cytokine, is involved in the process of intervertebral disc degeneration [11–15]. However, 17β-E2 has been proven to have an effect on lowering intervertebral disc degeneration in previous studies [23,24]. PI3K/AKT pathway was shown to be a critical anti-apoptosis signal transduction pathway by increasing p-Akt (active form of Akt), which was specially inhibited by MK 2206. However, the anti-apoptosis mechanism of 17β-E2 for NPCs remained unclear. So we attempted to discover the mechanism: if 17β-E2 could increase the low level p-Akt which was induced by TNF-α, then MK 2206 could inhibit the effect of 17β-E2, and prove that 17β-E2 could inhibit TNF-α induced apoptosis via the PI3K/AKT pathway. And our data exactly provided evidences that 17β-E2 could inhibit TNF-α induced apoptosis in human NPCs by upregulation of the protein level of p-Akt, in addition to upregulation of the protein level of PARP and downregulated of the protein level of caspase-3/p17. When cells were pretreated with MK 2206, inhibitor of the PI3K/AKT pathway, we discovered that the protein level of p-Akt and PARP were reduced; however, the protein level of caspase-3/p17 was increased. Whereas protein levels of Akt in our four groups remained unchanged. All of the aforementioned implied that 17β-E2 could inhibit TNF-α induced apoptosis in human NPCs via the PI3K/AKT pathway. Moreover, TNF-α downregulated the protein level of p-Akt from 0 to 24 hours, while it increasing the protein levels of p-Akt from 24 to 48 hours. However, the addition of 17β-E2 upregulated the protein level of p-Akt in a time-dependent manner.

Inverted phase-contrast microscopy, in term of morphological structure, was used to identify NPCs that underwent apoptosis following pretreated with TNF-α, which was turned around by 17β-E2. However, the effect of 17β-E2 was suppressed by MK 2206. The results of FACS and CCK-8 were the same as

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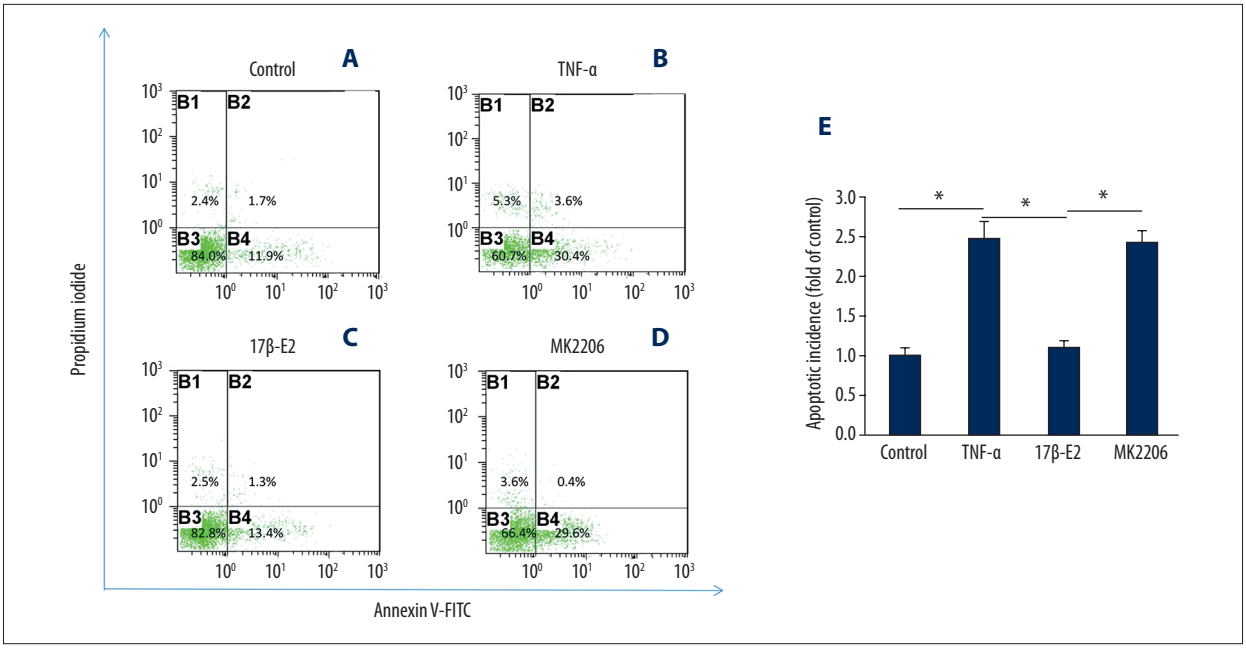

Figure 2. Evaluation of early apoptosis induced by serum withdrawal. Human NPCs were divided into four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) for 30 minutes and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway) for 30 minutes. All groups were incubated for 24 hours in serum-free medium without phenol red. Figure **A–D** are representative graphs obtained by FACS analysis after double staining with annexin V-FITC and propidium iodide. Figure **E** is the early apoptotic incidences of human NPCs. (Mean ±SD; n=3; TNF-α – tumor necrosis factor-α; 17β-E2 – 17β-estradiol; * means *p* value <0.05).

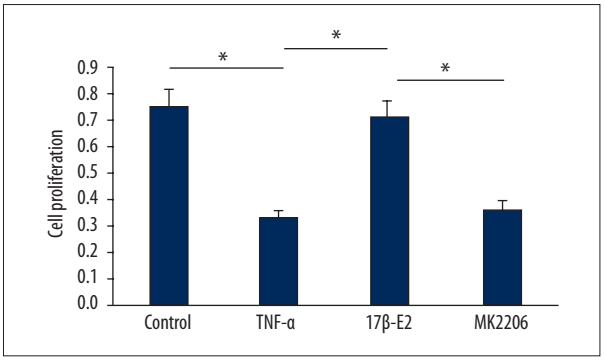

Figure 3. Cell Counting Kit 8 (CCK-8) assay was used to assess cell proliferation activity. Human NPCs were divided into four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) for 30 minutes and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway) for 30 minutes. (Mean ±SD; n=3; TNF-α – tumor necrosis factor-α; 17β-E2 – 17β-estradiol; * means *p* value <0.05).

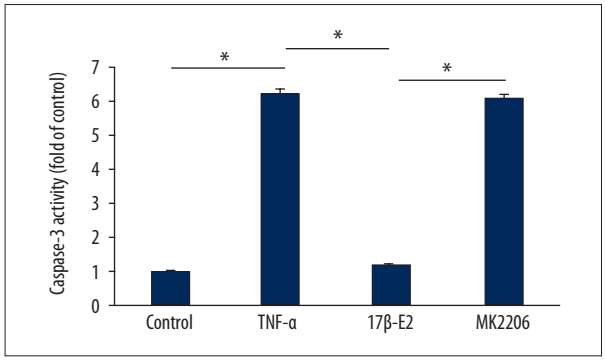

Figure 4. Changes in caspase-3 activity. Human NPCs were divided into four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) for 30 minutes and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway) for 30 minutes. (Mean ±SD; n=3; TNF-α – tumor necrosis factor-α; 17β-E2 – 17β-estradiol; * means *p* value <0.05)

the results of inverted phase-contrast microscopy. It was discovered that the effects of TNF-α on apoptotic cells were prevented by pretreatment with 17β-E2 which were inhibited by MK 2206. The results of the CCK-8 experiment proved that pretreatment with 17β-E2 could increase cell reproductive capacity for NPCs cells. Caspase-3 activity was increased by TNF-α which was suppressed by 17β-E2.

There is a high incidence of LBP in the general population, which is experienced at least once by 80% of people in their

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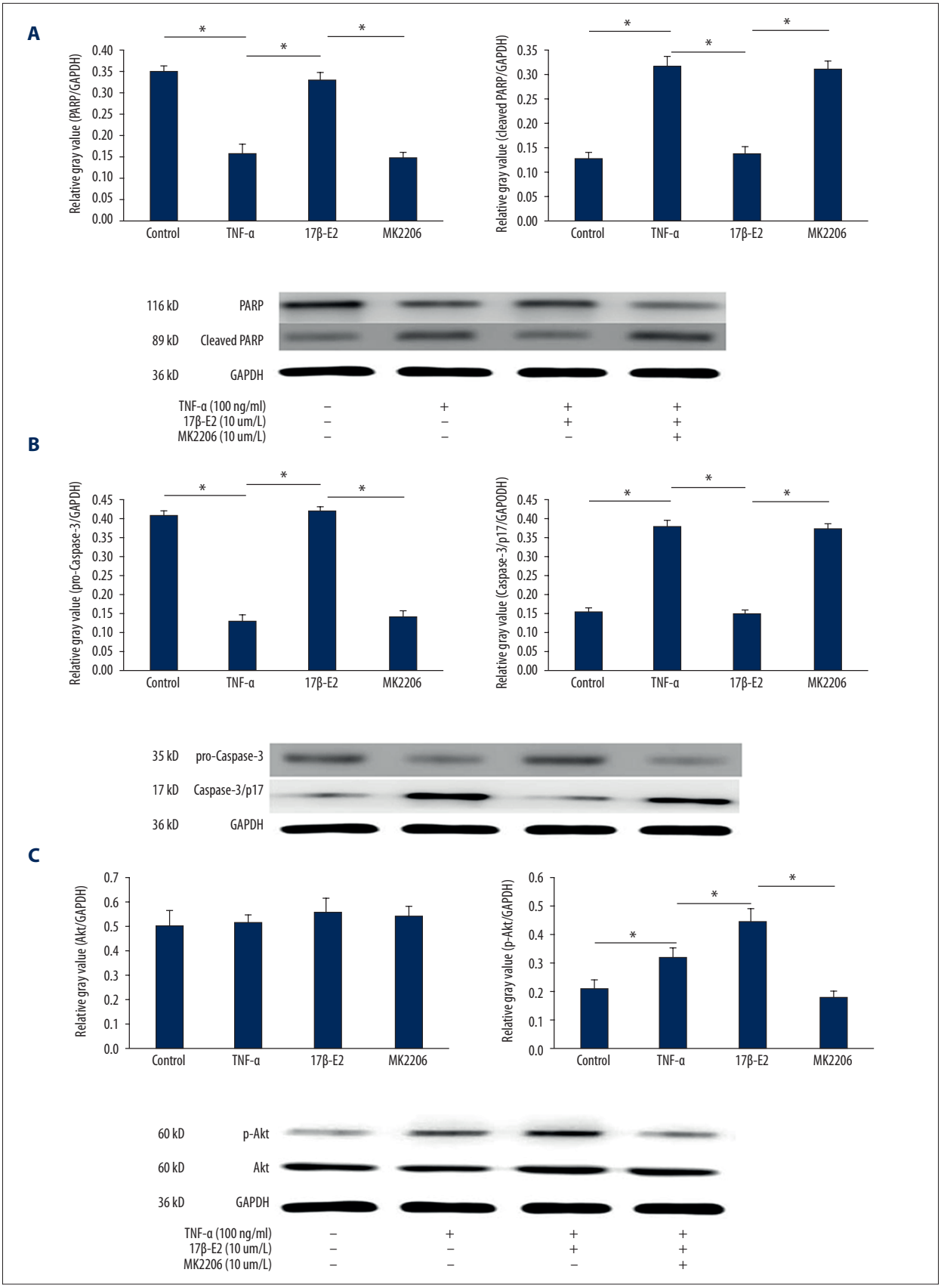

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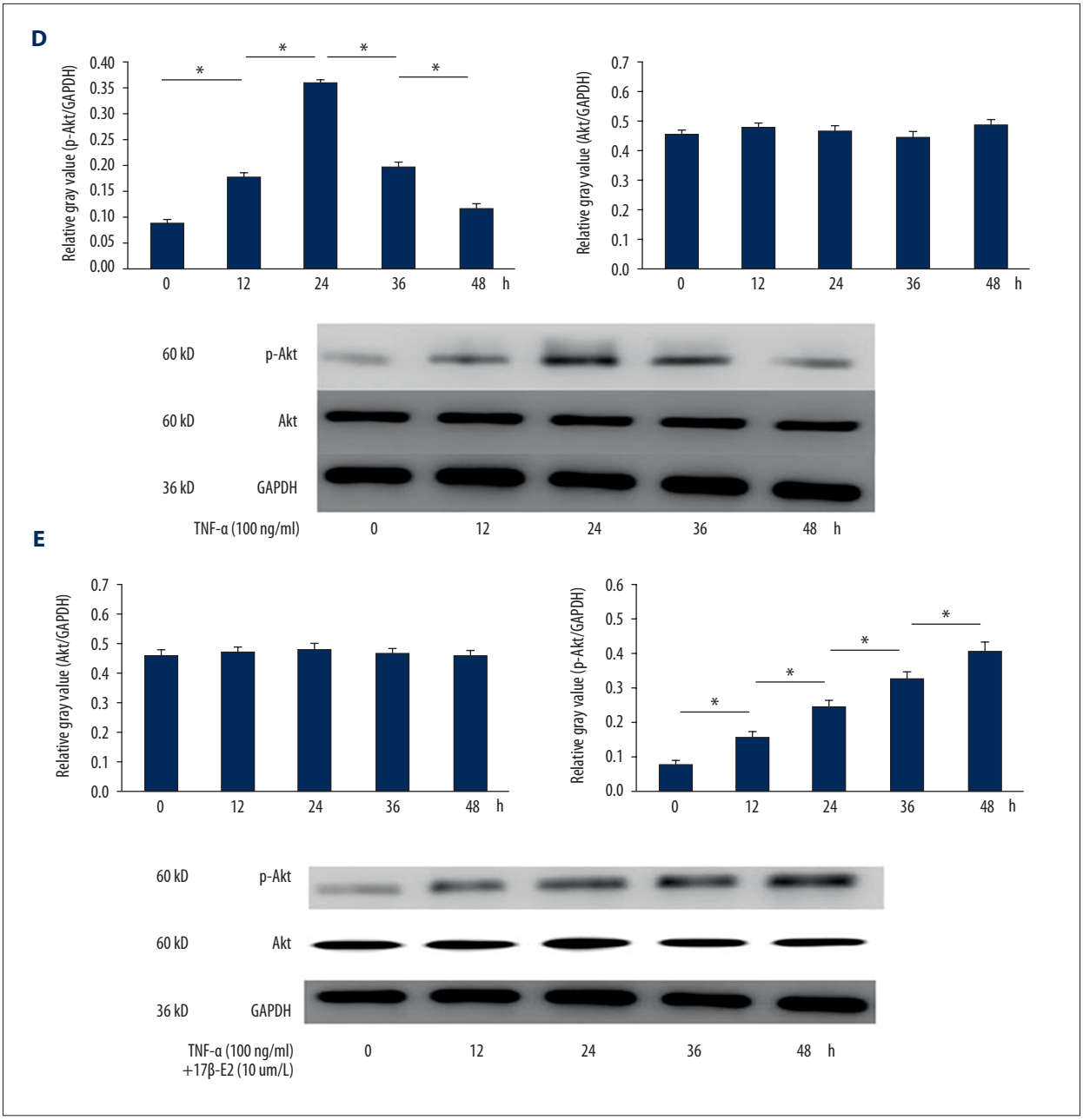

Figure 5. Protein level of pro-caspase-3, caspase-3/17p, PARP, cleaved PARP, Akt, and p-Akt. Human NPCs were divided into four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) for 30 minutes and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway) for 30 minutes. Protein level of Akt and p-Akt were observed in a time-dependent manner for pretreated TNF-α and pretreated TNF-α and 17β-E2, respectively. Figure **A** represents protein level of PARP and cleaved PARP; Figure **B** represents protein level of pro-caspase-3 and caspase-3/p17; Figure **C** represents protein levels of Akt and p-Akt; Figure **D** represents protein levels of Akt and p-Akt in time-dependence pretreated with TNF-α (100 ng/mL); Figure **E** represents protein levels of Akt and p-Akt in time-dependence pretreatment with TNF-α (100 ng/mL) and 17β-E2 (10 um/L). (Mean ±SD; n=3; TNF-α – tumor necrosis factor-α; 17β-E2 – 17β-estradiol; * means *p* value <0.05).

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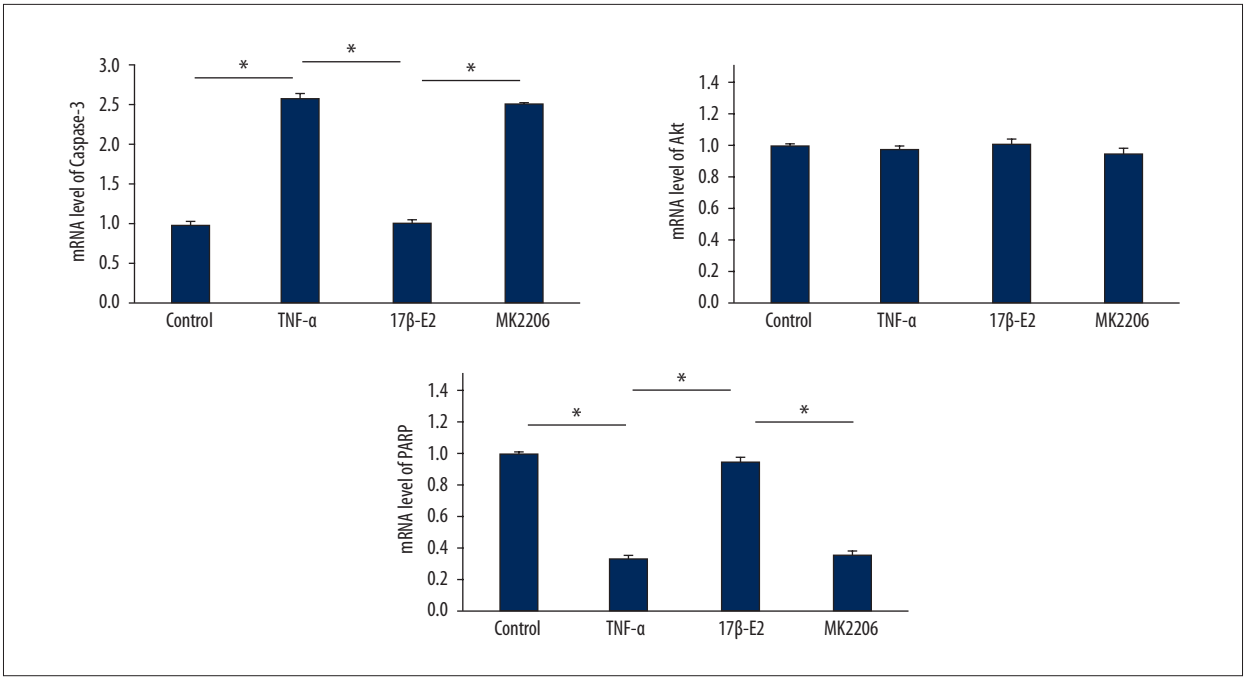

Figure 6. qRT-PCR analysis. The mRNA level of active caspase-3, PARP, Akt, and p-Akt. Human NPCs were divided into four groups: control, TNF-α (100 ng/mL), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L), TNF-α (100 ng/mL) with pretreated 17β-E2 (10 um/L) for 30 minutes and MK2206 (10 um/L, inhibitor of PI3K/AKT pathway) for 30 minutes. (Mean ±SD; n=3; TNF-α – tumor necrosis factor-α; 17β-E2 – 17β-estradiol; * means *p* value <0.05).

lifetime [25]. LBP is usually association with IVDD due to apoptosis of NPCs. Apoptosis is a common event in many physiological processes including physiological apoptosis and aberrant apoptosis, and it leads to tissue structure damage [26–28], which often is pathogenic. Previous articles showed that numerous factors could cause the aberrant apoptosis of NPCs [29,30], including cytotoxic [31], chronic unpredictable stress [32], mechanical loading, altered biomechanics, and extracellular matrix degradation [33] all which induced apoptosis of IVD cells *in vivo* or *in vitro*. Both Yang et al. [34] and Wei et al. [35] found that IL-1β could induce rat apoptosis of NPCs. TNF-α is one type of inflammatory cytokines which is secreted by monocytes and macrophages, and plays an important role in inflammatory response, cells proliferation, and apoptosis [36,37]. TNF-α is also a critical inflammatory cytokines causing IVDD [38,39]. Andrade et al. [38] found that when compared with first time lumbar disc herniation (LDH) patients, recurrent LDH patients had higher levels of TNF-α, and they discovered that TNF-α and its receptors (TNFR) were correlated with the severity of pre- and post-operative leg pain in LDH patients; conclusions which our study supports.

Our results showed that TNF-α not only induced apoptosis for human NPCs in cytomorphology, but also reduced proliferation activity, increased caspase-3 activity, and apoptosis incidence. Besides at the molecular level, compared to the control group, TNF-α significantly ($p<0.05$) increased protein levels of both caspase-3 and p-Akt and decreased protein levels of PARP, which implied TNF-α induced apoptosis for human NPCs. Previous studies reported that the PI3K/Akt pathway was involved in cell migration and invasion [40–42]. Akt is the critical components of signal transduction following PI3K activation [43]. We therefore questioned whether the PI3K/Akt was associated with the TNF-α induced invasion of NPCs in a time-dependent manner. Following TNF-α treatment, NPCs cells showed a striking effect on p-Akt, which reached its peak at 24 hour after the TNF-α (100 ng/mL) application and tended to reduce from 24 to 48 hours. However, total Akt level remained unaltered for 48 hours. The overall trend was consistent with reports from points of Lü [44], but the peak of p-Akt occurred at 2 hours after treatment with TNF-α (10 ng/mL). The possible reasons to explain these differences are differences in concentration for TNF-α and study cells.

The PI3K/Akt pathway, mostly studied in the context of inhibiting tumors, plays an important role in regulating cell growth, survival, and apoptosis [45]. The PI3K/Akt signaling pathway components are frequently altered in human cancers. Evidence has shown that the PI3K/Akt signaling pathway is involved in the integrin-mediated inhibition of apoptosis [46,47]. Akt and p-Akt (which is an activation form of Akt), are expressed in a wide variety of tumor cells. Activated p-Akt promotes cell proliferation and inhibits cell apoptosis by regulating downstream proteins including BAD, GSK-3β, and NF-κB [48]. In our

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previous studies [23,24,34], 17β-E2 was found to have a protective effect on rat NPCs via the upregulation of type II collagen (COL2a1) and aggrecan, and downregulation of MMP-3 and MMP-13 [49]. In the present study, we further confirmed the effect of 17β-E2 against TNF-α induced apoptosis for human NPCs. A preliminary exploration has been performed to detect the involvement of the PI3K/Akt pathway in signal transduction arising in the anti-apoptotic process of 17β-E2. It was noted that 17β-E2 upregulated the level of p-Akt, which could be inhibited by MK 2206. We also explored the effect of 17β-E2 on the PI3K/Akt pathway in a time-dependent manner (0–48 hours) and found that the protein expression of p-Akt generally increased from 0 hours to 48 hours and total Akt level remained unaltered for 48 hours, implying the protective effect of 17β-E2 was positively correlated with the time of action. The critical role of 17β-E2 in inhibiting apoptosis by upregulating the PI3K/Akt pathway has been emphasized once again.

It is well-known that 17β-E2 can have an anti-apoptosis effect on NPCs, as shown by Yang et al. [24]. Levofloxacin could increase the effect of serum deprivation on anoikis by downregulating COL2 for rat NPCs *in vitro* via the Bax/Bcl-2/caspase-3 pathway. Wang et al. [23] found different concentrations of 17β-E2 against interleukin-1β induced apoptosis for rat annulus fibrosus cells. Reference ranges for 17β-E2 are age- and gender-specific, as well as biologically specific, including stage of puberty/adolescence, stage of menstrual cycle, and stage of menopause [50]. We must consider the possible side effects of 17β-E2, which is always associated with breast cancer. Hence, the density range of 17β-E2 for preventing NPCs from apoptosis and lowering the risk of breast cancer needs to be further studied.

This work still has some limitations. First, even though we chose human NPCs as subject cells, it was still a study *in vitro*. Therefore, we need to assess the anti-apoptosis mechanism of signal transduction of 17β-E2 *in vivo* in further studies. Second, we tried to explore the appropriate concentration range of 17β-E2 that protected the NPCs and reduced the occurrence of cancer. Third, human NPCs were present in monolayers, which cannot fully represent human NPCs.

Conclusions

17β-E2 could protect against TNF-α induced apoptosis via the PI3K/AKT pathway for human NPCs by upregulating p-Akt and PARP, and downregulating caspase-3.

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