**Original Article**

**Resveratrol inhibits TNF-α-induced matrix degradation via the p38/MAPK and Akt pathways in human nucleus pulposus cells**

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**Abstract:** Previous studies have proved that resveratrol has beneficial effects on intervertebral disc regeneration. Tumor necrosis factor-α (TNF-α) has been proven to induce matrix degradation, which plays a critical role in intervertebral disc degeneration (IVDD). However, the effect of resveratrol on TNF-α-induced matrix degradation remains unknown. The purpose of this study was to find out whether resveratrol can reverse TNF-α-induced matrix degradation in human NP cells and to further study related mechanism involved in the process. The transcriptional activity, mRNA and protein expression of Collagen II and Aggrecan were measured by luciferase reporter assay, RT-qPCR and western blot. Sulfated glycosaminoglycan (sGAG) level was detected to show the production of proteoglycans in NP extracellular matrix. TNF-α reduced Collagen II and Aggrecan production of human NP cells time-dependently. Resveratrol can rescue TNF-α-induced downregulation of Collagen II and Aggrecan production. MAPK, NF-κB and Akt signal pathways were activated after treatment with TNF-α and resveratrol. P38 inhibitor or Akt inhibitor can abolished the antagonistic effect of resveratrol on TNF-α-induced catabolic response. Our findings provide novel data supporting the protective effect of resveratrol, which may be used as a therapeutic agent in the treatment of IVDD.

**Keywords:** Resveratrol, tumor necrosis factor-α, matrix degradation, nucleus pulposus cell, signal pathway

**Introduction**

Low back pain is a common public health problem worldwide, with a lifetime prevalence of 70-80% [1]. It significantly affects quality of life and burdens the health service to the detriment of all industrialized societies. The causes of back pain are multifactorial, intervertebral disc degeneration (IVDD) is one of the predominant factors that leads to this disabling condition [2-4]. Conservative treatments are currently aiming to alleviate the symptoms, while surgeries involve excising the degenerated motion segment and fusion the adjacent vertebral bodies rather than to solve the underlying biological causes involved in disc degeneration. There is a clear need for the development of novel therapeutic procedures that have the ability to interfere with the biological mechanism of IVDD. As an alternative to the surgical repair or removal of a diseased disc, biologic treatments to promote intervertebral disc (IVD) repair and restore physiologic function have been considered [5-12].

Resveratrol (trans-3,4',5-trihydroxystilbene) was first identified in the roots of white hellebore in 1940, mainly found in the skins of grapes and red wines [13-15] and showed powerful biological functions of anti-inflammatory, antioxidant, cardioprotective, and antitumor properties [16-24]. Resveratrol has also been reported to provide a protective effect on articular cartilage in rabbit models of osteoarthritis and rheumatoid arthritis [25, 26]. More recently, resveratrol has been found to relieve matrix-degrading enzymes upregulation and to facilitate proteoglycan synthesis and accumulation in bovine disc cells [27], as well as to decrease pro-inflammatory cytokines and catabolic enzymes expression in human IVD cells [28]. However, it is not known if resveratrol exerts similar protective effects on TNF-α-induced matrix degradation in human nucleus pulposus (NP) cells.

The purpose of this study was to elucidate whether resveratrol can reverse TNF-α-mediated matrix degradation in human NP cells and
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to further study related mechanism involved in the process.

Material and methods

Human NP cell isolation and culture

IVD specimens were harvested from patients with scoliosis in spine surgery on the basis of acquiring the informed consent of these patients. This procedure was approved by the Animal Ethics Committee of the First Affiliated Hospital of Nanchang University. The NP tissues were digested with proteinase (Calbiochem, CA, USA) and collagenase (Sigma-Aldrich, MO, USA). After filtration, the NP samples were cultured in T25 flasks in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, CA, USA) containing 10% fetal bovine serum (FBS; Invitrogen, CA, USA) and antibiotics. NP cells migrated out after one week, and passaged for use when confluent.

Real-time quantitative reverse transcription-PCR (RT-qPCR)

Total RNA was extracted from NP cells using TRIzol reagent (Invitrogen, CA, USA) and converted to cDNA using ReverTra Ace qPCR RT Kit (TOYOBO, OSAKA, JAPAN). The cDNA and gene-specific primers were added to SYBR qPCR Mix Kit (TOYOBO, OSAKA, JAPAN) and mRNA expression was quantified by using CFX96 Real-time PCR system (Bio-Rad, CA, USA). The sequences of synthesized primers are as follows: Collagen II forward 5’-TTCATGCTAGTCCCTTCT-3’, Collagen II reverse 5’-CTCCATGTTGCAGAAGACTTTCA-3’; Aggrecan forward 5’-ATTGGCACAAAGGTGGATGCTGTC-3’, Aggrecan reverse 5’-TCACCACCCACTCCGGAAGAAGTTT-3’; β-actin forward 5’-AGCCATGTCAGTAGCCATCC-3’, β-actin reverse 5’-CTCTCAGCTGTGGTGTTGAA-3’. Endogenous housekeeping gene β-actin was used to normalize gene expression. ΔΔCT method was performed to calculate the fold change. Each sample was analyzed in duplicate. All primers used were synthesized by Sangon Biotech Co., Ltd (Shanghai, CHINA).

Protein extraction and western blotting

Total cell proteins were extracted using RIPA buffer and measured with a BCA assay (Thermo Scientific). Equal amounts of the proteins were resolved on SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked with 5% nonfat milk in TBST and incubated overnight at 4°C in 5% nonfat milk in TBST with the anti-Collagen II antibody (1:1000, Abcam); anti-Aggrecan antibody (1:1000), anti-GAPDH (1:1000), anti-p65 antibody (1:1000), anti-phospho-p65 antibody (1:1000), anti-p38 antibody (1:1000), anti-phospho-p38 antibody (1:1000), anti-JNK1/2 antibody (1:1000), anti-phospho-JNK1/2 antibody (1:1000), anti-ERK antibody (1:1000), anti-phospho-ERK antibody (1:1000), anti-Akt antibody (1:1000), anti-phospho-Akt antibody (1:1000) all from Cell Signaling. The membranes were washed three times next morning and incubated with anti-rabbit secondary antibody (1:2000, Cell Signaling) for 1 h at room temperature. Immunolabeling was measured using the ECL reagent (Invitrogen, CA, USA). Densitometric analysis was performed using Image J analysis software (Bethesda, MD, USA).

Sulfated glycosaminoglycan (sGAG) detection

Proteoglycans in NP extracellular matrix are made up of sulfated glycosaminoglycan (sGAG) and core protein. sGAG expression was detected using a 1,9-dimethylmethylene blue (DM-MB) method with Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor, CF, UK) to represent the proteoglycans production of NP cells.

Luciferase reporter assay

Cells were plated to 48-well plates at a density of 2×10⁴ cells/well 1 day before transfection. Lipofectamine 2000 (Invitrogen, CA, USA) was used as a transfection reagent. A Collagen II or Aggrecan promoter luciferase construct was utilized to detect the effect of TNF-α and resveratrol on the Collagen II or Aggrecan promoter. NP cells were transfected with 250 ng Collagen II-Luc or Aggrecan-Luc and 250 ng pRL-TK plasmids for 6 h. The next day, cells were harvested and Dual-Luciferase Reporter Assay System (Promega, WI, USA) was used for measurements of firefly and Renilla luciferase activities using a TD-20/20 luminometer (Turner Biosystems, CA, USA).

Statistical analysis

All measurements were performed in triplicate. Data are presented as the mean ± standard error. Differences between groups were assessed by one-way ANOVA with Bonferroni’s post hoc test for multiple comparisons and
Results

TNF-α suppresses collagen II and aggrecan expression in NP cells

To investigate the effects of TNF-α on Collagen II and Aggrecan expression, the cells were treated with TNF-α at different concentrations (10, 25, 50 ng/ml) for various time points (4, 8, 24 h). The results showed time-dependent decreases of Collagen II and Aggrecan mRNA levels (Figure 1A) and sGAG level (Figure 1B) in human NP cells in response to TNF-α. Interestingly, TNF-α also decreased Collagen II and Aggrecan mRNA expression (Figure 1C) and sGAG level (Figure 1D) at different concentrations, but not dose-dependently. Taken together, TNF-α treatment was performed at 25 ng/ml for 24 h in subsequent experiments. The protein productions of Collagen II and Aggrecan were also decreased by TNF-α (25 ng/ml) for 48 h (Figure 1E, 1F).

Student’s T-test. P-values <0.05 was considered statistically significant.
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We then treated NP cells with TNF-α in the presence or absence of resveratrol to explore whether resveratrol rescues the catabolic effect of TNF-α. Resveratrol treatment restored TNF-α-induced down-regulation of Collagen II and Aggrecan transcriptional activity (Figure 2A, 2B), mRNA levels (Figure 2C), and protein

Figure 2. Effect of resveratrol on Collagen II and Aggrecan expression induced by TNF-α. Luciferase reporter assay (A, B), RT-qPCR (C) and sGAG detection (D) show that resveratrol rescued TNF-α induced downregulation of Collagen II and Aggrecan transcriptional activity, mRNA and sGAG levels in NP cells. Western blot (E) and corresponding densitometry (F) reveal that resveratrol abolished TNF-α mediated decreases of Collagen II and Aggrecan protein expression (*P<0.05, #P<0.05).

Resveratrol restores the catabolic effect of TNF-α on NP cells

We then treated NP cells with TNF-α in the presence or absence of resveratrol to explore whether resveratrol rescues the catabolic effect of TNF-α. Resveratrol treatment restored TNF-α induced down-regulation of Collagen II and Aggrecan transcriptional activity (Figure 2A, 2B), mRNA levels (Figure 2C), and protein
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Immunoblot analysis showed the phosphorylation levels of p38, JNK1/2, ERK1/2, NF-κB and Akt were elevated at 30 min after TNF-α treatment, and resveratrol further increased p38 and Akt phosphorylation (Figure 3).

Effect of MAPK, NF-κB and Akt signal pathways in resveratrol induced anti-catabolic role

To investigate whether MAPK, NF-κB and Akt signal pathways were involved in resveratrol-induced anti-catabolic effect towards TNF-α, we treated the NP cells with TNF-α and resveratrol in the presence or absence of p38 inhibitor (SB203580, 10 µM), JNK1/2 inhibitor (SP600125, 25 µM), ERK1/2 inhibitor (PD98059, 20 µM), NF-κB inhibitor (SM7368, 10 µM) and Akt inhibitor (triciribine, 20 µM). Compared to the group of TNF-α plus resveratrol, the Collagen II and Aggrecan transcriptional activity (Figure 4A, 4B), mRNA levels (Figure 4C), protein expression (Figure 4E, 4F) and sGAG secretion (Figure 4D) in the group of TNF-α and resveratrol plus p38 inhibitor or Akt inhibitor were significantly decreased; while inhibition of JNK1/2, ERK1/2 or NF-κB showed no significant change. These results suggest that the inhibition of p38 or Akt signaling abolished the anti-catabolic effect of resveratrol against TNF-α.

Discussion

The IVD is a major joint of the spine which allows for motion and posture. It is composed of a gelatinous NP, fibrous annulus fibrosus (AF) and sandwiched between two cartilaginous endplates. The degeneration of the IVD is associated with back pain incidence and severity, and is purported to be one of the major reasons for hospital visits. The causes of degeneration are multifactorial and include both environmental and genetic factors. During degeneration, there are biochemical and cellular changes that occur in the disc including the loss of cells, proteoglycans and water, which impacts on the biomechanics and function of the disc.

While the etiology of disc degeneration is not clear, however, one major characteristic in the degeneration is inflammation. Many studies have indicated that degenerated IVDS are in a chronic inflammatory state, and that inflammatory factors can induce degenerative changes in IVD. Human studies have suggested that
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Increased expression of multiple pro-inflammatory cytokines is associated with back pain pertaining to IVDD. Examination of surgically removed degenerated disc fragments have shown expression of IL-1, IL-8, TNF-α, IL-10 and PGE2 [29, 30]. Repeated physical injury in rat IVDs can induce persistent inflammation with expression of various pro-inflammatory cytokines that are associated with IVDD [31].

Resveratrol has been demonstrated to process antitumor, anti-aging, anti-inflammatory and neuroprotective activities [32]. Csaki C et al. [33] and Shakibaei M et al. [34] have shown the potential application of resveratrol for the treatment of osteoarthritis. An animal study demonstrated that resveratrol could reduce cartilage degradation and loss of matrix proteoglycans on cartilage tissue [25]. Resveratrol has also been reported to decrease transcript and protein production of major pro-inflammatory cytokines and matrix catabolic enzymes, and shown analgetic potential to reduce NP-mediated pain in an in vivo animal model of painful radiculopathy [28]. A study performed by Li et al. [27] showed that resveratrol facilitated proteoglycan synthesis and reversed IL-1β-mediated proteoglycan loss in bovine IVD cells.

This study illustrates the potent anabolic effects of resveratrol on human IVD homeostasis. Treatment with TNF-α reduced Collagen II and Aggrecan at the transcriptional and protein levels, decreased sGAG accumulation in human NP cells. In addition, resveratrol reversed the catabolic effects of TNF-α that have been implicated in IVDD.

NF-κB is a key transcription factor that regulates various physiological processes including inflammatory responses and apoptosis, which also plays a critical role in the process of disc degeneration [35, 36]. The MAPK signaling pathways have been shown key role in the transduction of extracellular signals to cell responses that may include cell growth, proliferation, differentiation, development, inflammatory responses, apoptosis and invasion in mammalian cells [37, 38]. The PI3K/Akt signaling pathway is important for cell growth, differentiation and survival [39]. In the present study, it was found that the phosphorylation levels of p38, JNK1/2, ERK1/2, NF-κB and Akt were increased at 30 min after TNF-α treatment, and resveratrol further increased p38 and Akt phosphorylation. In addition, inhibitor experiments were performed to determine whether resveratrol can rescue the catabolic effect of TNF-α via MAPK, NF-κB and Akt signal pathways. The results indicated that the inhibition of p38 or Akt signaling can abolish the protective effect of resveratrol against TNF-α.

Conclusions

Our results shed light on the inflammatory response of multiple downstream regulatory molecules after stimulation with resveratrol in human NP cells, which are regulated through p38 and Akt signaling pathways. These data provide us with a better understanding of the anti-inflammatory, antioxidant, and antiproliferative and anabolic effects of resveratrol. In addition, as the mechanisms behind the suppressed inflammatory response following exposure to resveratrol for long time periods are not known, which still requires further investigation to address. Such study results may provide novel data supporting the protective effect of resveratrol, which may be used as a therapeutic agent in the treatment of IVDD.

Disclosure of conflict of interest

None.

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