European Review for Medical and Pharmacological Sciences 2021; 25: 5189-5198

The effect of Nrf2 activators tBHQ and 4-octyl itaconate on the nucleus pulposus cell degeneration

W.-X. ZHONG[1], G.-S. ZHANG[2], J. TANG[1]

[1]Department of Orthopedics, Shanghai Xuhui Hospital, Shanghai, China
[2]Department of Orthopedics, Hospital of Xi'an Jiaotong University Health Science Center, Xi'an Jiaotong University, Xi'an, China

Wuxue Zhong and Gensheng Zhang contributed equally to this work

Abstract. – OBJECTIVE: The present study aimed to investigate the impact of two Nrf2 agonists, tBHQ and 4-Octyl Itaconate, on nucleus pulposus (NP) degeneration and explore the underlying mechanism.

PATIENTS AND METHODS: We isolated the NP cells from the disc tissue of disc herniation patients. NP cells were pretreated with an adequate dose of tBHQ, Itaconate, or the mixture of them, and then subjected to the Lipopolysaccharides (LPS) stimulation to induce degeneration. Besides, the Nrf2 gene silenced NP cells were also used as a comparison. Moreover, the LPS-treated NP cells were also cultured in the mix of tBHQ and Itaconate to determine whether the agonists affected reverse degeneration.

RESULTS: LPS treatment suppressed Nrf2 expression and induced the NP cell degeneration with a decrease of cell viability and collagen II expression, an increase of reactive oxygen species (ROS) production, inflammatory cytokine accumulation (IL-1β, TNF-α), and apoptosis (Caspase3, Caspase8). However, tBHQ or Itaconate pretreated NP cells contained a higher level of Nrf2 protein and alleviated the negative effect caused by LPS, which was abolished with the silencing of Nrf2. Additionally, tBHQ showed a better ability to suppress ROS than Itaconate. Meanwhile, Itaconate inhibited a higher amount of IL-1β and TNF-α than tBHQ. Interestingly, when NP cells were pretreated with both tBHQ and Itaconate, the result indicated an excellent anti-ROS and anti-inflammatory peculiarity. Furthermore, when NP cells suffered from LPS first and then treated with the agonist, the anti-ROS and anti-inflammatory effects remained. However, the cell viability, collagen II, and apoptotic degree were not improved.

CONCLUSIONS: Both tBHQ and Itaconate effectively prevent NP cells from degeneration through anti-ROS and anti-inflammation, and the combined use of them may have better effects. But in comparison, their impact on reversing NP cell degeneration has yet to be proven.

Key Words:
Nucleus pulposus cells degeneration, Nrf2 agonist, ROS, Inflammation.

Introduction

Intervertebral disc degeneration (IDD) is the pathological basis of disc herniation, spinal stenosis, and spine diseases[1]. With the development of medical technology, surgical treatment is one of the methods for IDD related diseases, including nucleus pulposus removal, laminectomy, and internal fixation[2]. Though it effectively solves clinical symptoms (chronic pain in the neck and shoulder, lower back, and limb), surgical treatment is only symptomatic treatment. It cannot fundamentally remove the cause of the diseases. Besides, surgical treatment brings nerve root injury, intervertebral space infection, spinal instability, adjacent segment degeneration, postoperative recurrence, and other complications[3,4]. Therefore, exploring the molecular mechanism of IDD can provide an effective strategy for early intervention, delay, or even prevention of IDD related diseases.

The pathology of IDD mainly involves inflammatory infiltration, reduced number and function of nucleus pulposus (NP) cells, change of collagen type, and abnormal metabolism of extracellular matrix (ECM)[5]. These changes are closely related to reactive oxygen species (ROS)[6], a normal aerobic metabolite mainly including hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}), and hydroxyl radical (-OH)[7]. Typically, the generation and removal of ROS are in a dynamic balance. Low concentration of ROS participates as a signaling molecule in normal physiological activities such as cell proliferation and differentiation,

Corresponding Author: Jie Tang, BM; e-mail: Tangwu13@163.com

damage repair, inflammation, and immunity. Under pathological conditions, the excessive production or weak clearance of ROS leads to the cells in an oxidative stress state, causing oxidative damage to DNA and protein lipids, thereby the apoptosis of cells[8]. It has been widely reported that ROS takes part in the pathophysiological inflammatory response and regulates the apoptosis of NP cells by activating JNK, p38MAPK, NF-κB signaling pathways[9,10], providing the pieces of evidence that ROS involved in the occurrence and development of IDD.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an essential anti-oxidant defense factor in the body and is expressed in almost all cell types. When harmful stimulants such as ROS increase, the cytoplasmic Nrf-2 and its inhibitor Keap 1 are depolymerized[11]. After the free Nrf-2 enters the nucleus, it combines with the Maf protein to form a dimer and reacts with anti-oxidant response elements (ARE) to initiate the transcriptional expression of a series of anti-oxidant enzymes[12,13]. The Nrf2-ARE signaling pathway is a vital protective pathway formed by the body against oxidative stress caused by various external causes[14,15].

In the past few years, many natural and synthetic compounds have been proven to activate Nrf2. Some of these compounds have been efficiently used in clinical trials to treat multiple sclerosis[16], like muscular dystrophy, skin cancer, and chronic kidney disease[17,18]. However, clinical research has not yet been carried out in the field of IDD. In this study, we compared the role of two different Nrf2 agonists, tert-butylhydroquinone (tBHQ) and 4-Octyl Itaconate (Itaconate), in the process of NP cells degeneration to provides a theoretical basis for the future treatment of IDD disease.

Patients and Methods

NP Cells Preparation

This research was approved by the Ethics Committee of Shanghai Xuhui Hospital. The NP cells were isolated from the NP tissue donated by the patients undergoing lumbar disc herniation operations in our hospital. Eight patients were participating in the study, and all of them have signed the informed consent before the surgery. We isolated the NP cells following the protocol briefly follows: NP tissue was disintegrated into small pieces and digested with the mixture of 0.25% trypsin and 0.25% type XI collagenase (Sigma-Aldrich, St. Louis, MO, USA) overnight at 37°C. NP cell pellets were filtered from the supernatant of the solution and resuspended in culture medium (DMEM/F12 with 10% fetal bovine serum and 1% penicillin-streptomycin, Gibco, Rockville, MD, USA).

Drug Treatments

We cultured the NP cells in the 24 well plates with a density of 1.5×10^4 per well. To test the optimized concentration of tBHQ, Itaconate (Selleck, Shanghai, China), and the mixture of them, we used ranged concentration of drugs to stimulate NP cells for 24 hours. The highest concentration that did not affect the cell viability was applied in our experiment. Besides, we also used Lipopolysaccharides (LPS, Boehringer, Ingelheim, Germany) to induce NP cell degeneration as the previous describe[19].

Nrf2 Silencing

Small interfering RNA (siRNA) was purchased from Thermo Fisher Scientific (ID: 106668, Waltham, MA, USA). Nrf2-siRNA lentivirus (10 μL virus/mL medium) was added directly to NP cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 48 h, according to the manufacturer's instructions. Nrf2 silencing efficiency in the NP cells was verified by Western blotting.

Western Blotting (WB)

After treatments, total protein of NP cells was isolated with the radioimmunoprecipitation assay (RIPA) method (Beyotime, Shanghai, China), and 45 μg of protein lysates were separated in a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, PVDF blots were incubated with desired primary antibodies: anti-Nrf2 (ab62352, Abcam, Cambridge, MA, USA, diluted in 1:1000), anti-GAPDH (ab9485, Abcam, Cambridge, MA, USA, diluted in 1:3000) and secondary antibody Goat Anti-Rabbit IgG (HRP) (ab7090, Abcam, Cambridge, MA, USA, diluted in 1:1000), which were then detected by enhanced chemiluminescence (ECL) reagents (Beyotime, Shanghai, China). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for the quantification of the blot intensities normalized to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Immunofluorescence (IF)

We determined the collagen II expression in NP cells using IF methods. The F-actin staining was also used to observe the contour of the cells. Briefly, NP cells were fixed and permeabilized before incubation with primary antibody: anti-collagen (ab34712, Abcam, Cambridge, MA, USA, diluted in 1:200) overnight at 4°C. The following day, NP cells were subsequently incubated with Donkey Anti-Rabbit IgG H&L (ab150075, Abcam, Cambridge, MA, USA, diluted in 1:1000), 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China, diluted in 1:1000), and Phalloidin-iFluor 488 Reagent (ab176753, Abcam, Cambridge, MA, USA, diluted in 1:1000) for one h. The staining intensity was measured using the Image-J software (National Institutes of Health, Bethesda, MD, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction Assay (qRT-PCR)

We determined the Caspase 3 and Caspase 8 expression in NP cells using qRT-PCR. Total RNA was isolated by the TRIzol reagents (Toyobo Co, Ltd, Osaka, Japan) and reverse transcribed into complementary deoxyribose nucleic acid (cDNA) according to the manufacturer's instructions. The real-time PCR analysis was performed to analyze 500 ng cDNA from each sample. The mRNA primers are: Caspase 3 Forward (5'-AGAACTGGACTGTGGCATTGAG-3'), Reverse (5'-GCTTGTCGGCATACTGTTTCAG-3'); Caspase 8 Forward (5'-CATCCAGTCACTTTGCCAGA-3'), Reverse (5'-GCATCTGTTTCCCCATGTTT-3'); GAPDH Forward (5'-ACAACTTTGGTATCGTGGAAGG-3'), Reverse (5'-GCCATCACGCCACAGTTTC-3'). We utilized the $2^{-\Delta\Delta Ct}$ method for mRNA quantification by normalization to GAPDH as the reference gene.

Enzyme-Linked Immunosorbent Assay (ELISA)

We determine the IL-1β, TNF-α, and ROS levels in the culture medium with ELISA. Following treatment, we collected the supernatants of the medium and the concentrations of IL-1β, TNF-α, and ROS synthesized by NP cells determined using IL-1β, TNF-α and ROS kits from Abcam (ab214025; ab181421; ab139476, Cambridge, MA, USA) according to the manufacturer's instructions.

Cell Viability Assay

We determined the cell viability by the cell counting kit-8 (CCK-8) assay. NP Cells were seeded at a density of 1×10^4 cells per well in a 96-well plate. Following treatment, the cells were incubated with CCK8 reagent (Beyotime, Shanghai, China) according to the manufacturer's instructions. The intensity of CCK8 product was measured at 550 nm using a microplate reader (Labsystems Multiskan, Helsinki, Finland).

Statistical Analysis

Statistical analysis was performed by Statistical Product and Service Solutions (SPSS) software (version 20.0, Chicago, USA), and data were expressed as the mean ± standard deviation (SD). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

The Optimized Drug Concentration for Cell Culture

To increase the amount of Nrf2 with the agonist as much as possible without affecting the viability of NP cells, we increased the concentration of tBHQ and Itaconate (Figure 1A) from 5 μM to 30 μM in the culture medium and tested the cell viability by CCK8 assay after 24 hours of stimulation. We found the NP cell viability was significantly decreased at the concentration of 30 μM of tBHQ or 20 μM of Itaconate compared to the no-drug group (Figure 1B). Therefore, we used the 25 μM of tBHQ or 15 μM of Itaconate as the maximum safe concentration for cell culture. Additionally, we also wondered about the effect of the mixture of tBHQ and Itaconate on NP cells metabolism. Therefore, the maximum safe concentration of the mixture was also tested. Since the rate of optimized concentration of tBHQ and Itaconate was 5:3, we made the mixture from 5 μM tBHQ + ***3 μM Itaconate, 10 μM*** tBHQ + ***6 μM Itaconate,*** 15 μM tBHQ + ***9 μM Itaconate***, to 20 μM tBHQ + 12 μM Itaconate. From the result of Figure.1C, we found the safe concentration of the mix was 24 μM.

tBHQ and Itaconate Pretreatment Protects Collagen II Synthesis by Nrf2 Activation

To clear the effect of tBHQ, Itaconate, or the mix on the process of NP cells degeneration,

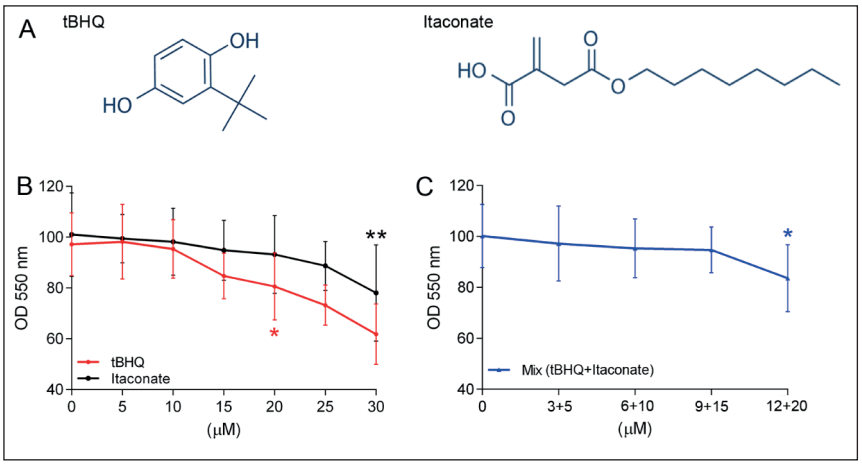

Figure 1. Cell viability after tBHQ and Itaconate treatments. NP cells were cultured with a ranged concentration of tBHQ or Itaconate, or the mix of them for 24 hours. **A,** The chemical formula of tBHQ and Itaconate. **B,** CCK8 assay of NP cells after 24 hours treatment of tBHQ or Itaconate (5 μM to 30 μM). **C,** CCK8 assay of NP cells after 24 hours treatment of mix of tBHQ and Itaconate (from 5+3 ***μM*** to 20+12 ***μM***). Results are expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$ compared to non-treatment group).

we pretreated the NP cells with tBHQ (25 μM), Itaconate (15 μM), or the mix (15 μM tBHQ + ***9 μM Itaconate) for 24 hours. Then, the cells with or without agonist pretreatment were suffered*** from defined media supplemented with a single dose of LPS[20] (30 mg/mL) for three days to induce degeneration. Apart from the normal NP cells, the Nrf2 gene-silenced NP cells were also used to show how the drugs affect NP cells with the deficiency of Nrf2. The cells without any drug treatment were divided as control. The results of the WB analysis of Nrf2 protein indicated that LPS significantly suppressed the Nrf2 expression compared to the control; the NP cells with the pretreatment of tBHQ, Itaconate, or the mix contained a higher level of Nrf2 after LPS stimulation; the efficiency of siRNA transfection was significant that Nrf2 silenced NP cells expressed a shallow dose of Nrf2 undergoing any treatments (Figure 2A, 2B). Furthermore, we analyzed the collagen II expression as the indicator of NP cell degeneration. In Figure 2C, the collagen II expression was shown in the red color, and the entire cell region was presented as green. Compared to the control, LPS treatment reduced the collagen II content, which was alleviated by tBHQ, Itaconate, or the mix pretreatment. Besides, there was no significant difference between tBHQ, Itaconate, and the mix on the collagen II expression. However, when the Nrf2 was silenced, the protection of tBHQ and Itaconate was abolished (Figure 2D). Therefore, these agonists play a positive role in NP cells based on the activation of Nrf2.

tBHQ and Itaconate Pretreatment Suppresses Inflammation, ROS, and Apoptosis During NP Cells Degeneration

In addition to collagen II expression, we also valued the cell viability, ROS generation, inflammation, and apoptotic markers of NP cells to determine the effect of the agonist of Nrf2. As shown in Figure 3A, the CCK8 assay suggested LPS reduced the cell viability compared to the control, and the tBHQ and Itaconate pretreatment did not improve NP cell viability under LPS stimulation. However, we noticed that the mix was functional to increase cell viability. Apart from this, LPS also triggered the ROS production, which was rejected by the pretreatment of tBHQ, Itaconate, and the mix, among of which tBHQ showed the best influence on the anti-ROS function (Figure 3B). For testing the anti-inflammatory effect of tBHQ and Itaconate, we analyzed the IL-1***β*** and TNF-α content in the culture medium after three days of treatment. Compared to the control, LPS highly increased the IL-1***β*** and TNF-α production in NP cells, and tBHQ and Itaconate pretreatment were both useful to suppress the IL-1***β*** and TNF-α expression, which was more significant with Itaconate (Figure 3C). Furthermore, tBHQ and Itaconate were also proved to have a good anti-apoptosis effect reflecting on the suppression of Caspase 3 and Caspase 8 expression (Figure 3D). Collecting from these results, we found that tBHQ showed a better anti-oxidant effect, Itaconate showed a better anti-inflammation effect, and the mix showed an eclectic impact on anti- both

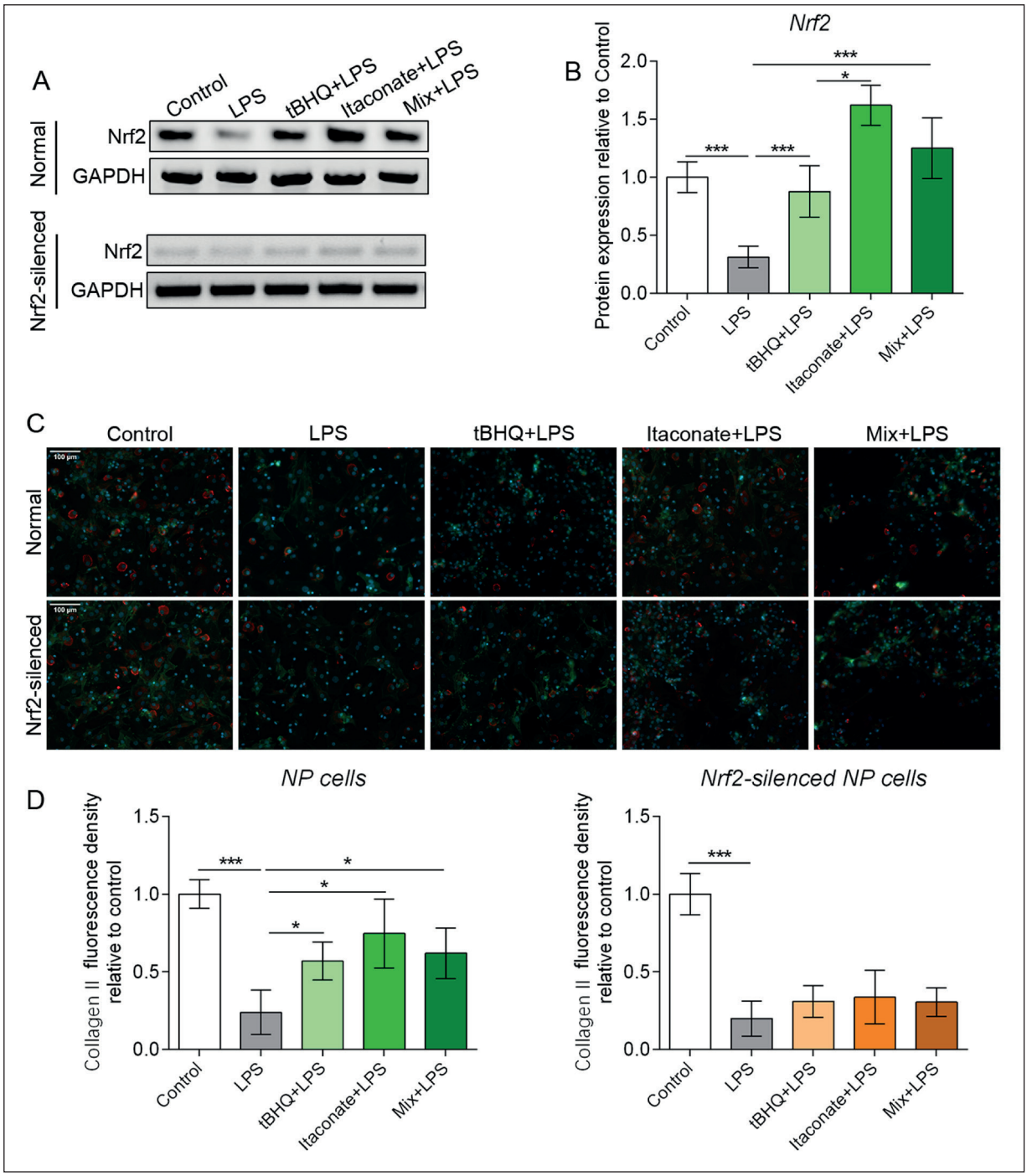

Figure 2. tBHQ and Itaconate pretreatment protects collagen II synthesis by Nrf2 activation. We prepared normal NP cells and Nrf2 gene silenced NP cells. Each cell group was divided into five subgroups. The cells without any drug treatment were set as control; NP cells were subjected to LPS to induce degeneration without or with the pretreatment of tBHQ (15 μM), Itaconate (25 μM), or the mix of them (9+15 μM). **A,** WB analysis for Nrf2 expression of normal and Nrf2-silenced NP cells, and **B,** the quantification of blots measured by Image J software. **C,** IF analysis for collagen II (red) expression and F-actin (green) in each group (magnifications: 200X), and **D,** the quantification of collagen II measured by Image J software. Results are expressed as mean ± SD. (*$p < 0.05$, ***$p < 0.001$).

oxidant and inflammation. Even more noteworthy is that the protection of tBHQ and Itaconate was almost abolished when the Nrf2 gene was silenced. Therefore, it is clear that tBHQ and Itaconate protect NP cells from degeneration via Nrf2 activation.

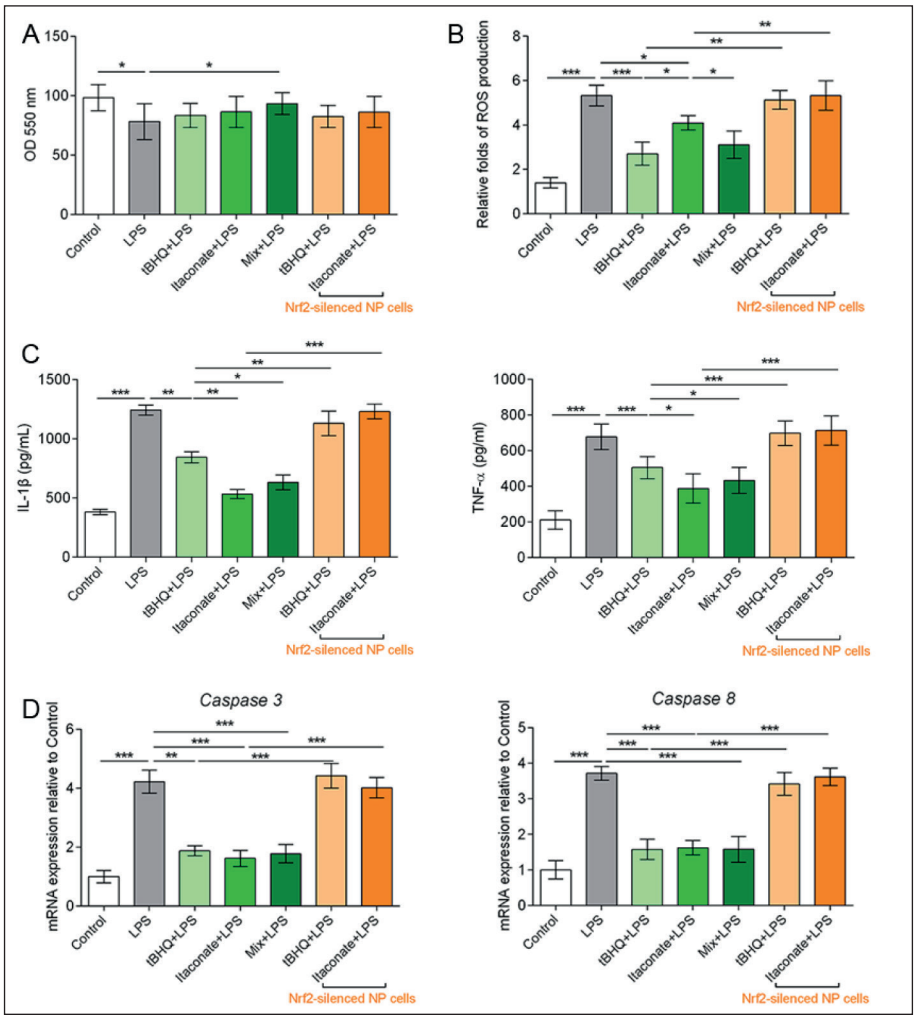

Figure 3. tBHQ and Itaconate pretreatment suppresses inflammation, ROS, and apoptosis during NP cell degeneration. The normal or Nrf2-silenced NP cells were subjected to LPS (3 days) to induce degeneration without or with the pretreatment (24 hours) of tBHQ (15 μM), Itaconate (25 μM), or the mix of them (9+15 μM). **A,** CCK8 assay of NP cells after agonist pretreatment and LPS treatment. **B,** ROS generation, **C,** IL-1β, and **D,** TNF-α content synthesized by NP cells in the supernatant of the medium was tested by ELASA. **D,** Real time-PCR analysis for Caspase3 and Caspase8 expression by normalization to GAPDH. Results are expressed as mean ± SD. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

A Mix of tBHQ and Itaconate is Weak in Reversing NP Cells Degeneration

The pretreatment of Nrf2 agonists can delay the degeneration of NP cells under noxious stimulation, which shows its potentially preventive effect on IDD. However, for degenerated NP cells, does the agonist have a therapeutic effect of reversing degeneration? We used LPS to induce NP cells degeneration and continuously treated the degenerated NP cells with a mix of tBHQ and Itaconate. As shown in Figure 4A and 4B, the combination also increased the Nrf2 expression after LPS treatment. However, the mix did not improve the collagen II synthesis after LPS treatment (Figure 4C, 4D). Additionally, the mix of tBHQ and Itaconate also did not affect the protection of the cell viability compared to the LPS group (Figure 4E). Even though we found the supplement of the mix of tBHQ and Itaconate suppressed the IL-1β and ROS generation after the treatment of LPS (Figure 4F, 4G). Finally, the result of the qRT-PCR of the Caspase 3 also suggested the mix of tBHQ and Itaconate did not reverse the apoptosis of degenerated NP cells (Figure 4H). Therefore, the supplement of tBHQ and Itaconate was not significantly effective in reversing the degeneration of NP cells.

Discussion

Nrf2 is a transcription factor that functions as a sensor for cell damage by triggering cytoprotective gene expression when activated[12]. In the current study, we compared two novel Nrf2 agonists -tBHQ and Itaconate- to determine their effects on LPS-induced NP cell degeneration. There are various opinions on the exact mechanism of IDD, including the decreased vitality of intervertebral disc cells, apoptosis, disturbance of ECM metabolism, a reduced nutritional supply of intervertebral discs, and inflammatory response[21]. Excess ROS can destroy the homeostasis of NP, during of which the original cartilage-like phenotype of NP cells is lost. It is an aerobic metabolite and has a powerful oxidizing ability, which is generally produced in mitochondria, endoplasmic reticulum, plasma membrane, and nucleus[6]. The oxidized respiratory chain on the mitochondrial inner membrane is the primary source of endogenous ROS. If these substances are not removed in a timely and effective manner, lipid peroxidation, DNA, and other protease compounds will occur, which consists of the pathological basis of NP cells damage and degradation of ECM, eventually leading to IDD[22].

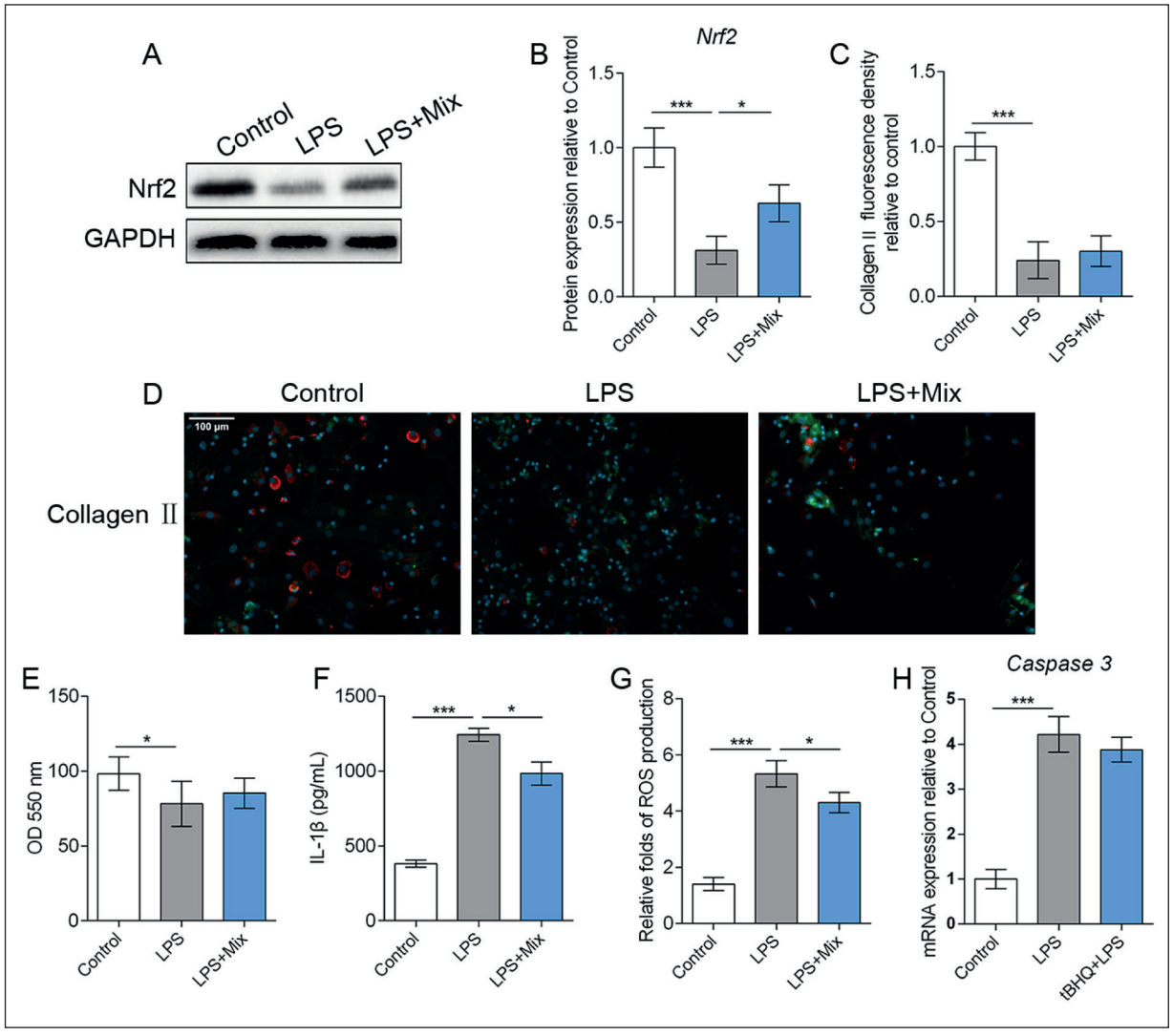

Figure 4. The effect of a mix of tBHQ and Itaconate in reversing NP cell degeneration. NP cells were pretreated with LPS (3 days) to induce degeneration and subjected to the mix of tBHQ and Itaconate (9+15 μM) for another 3 days. **A,** WB analysis for Nrf2 expression and **B,** the quantification of blots measured by Image J software. **C, D,** IF analysis for collagen II (red) expression and F-actin (green) (magnifications: 200X), and the quantification of collagen II measured by Image J software. **E,** CCK8 assay. **F, G,** ELISA assay for the IL-1β and ROS production. **H,** Real time-PCR analysis for Caspase3 expression by normalization to GAPDH. Results are expressed as mean ± SD. (*$p < 0.05$, ***$p < 0.001$).

Furthermore, excessive inflammatory cytokines correlate with IDD progression. Studies have shown that several critical pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, are significantly increased in the disc of IDD patients[23]. They negatively affect the collagen II generation from NP cells decreases, which accelerates the imbalance of ECM. Besides, inflammatory factors can induce various matrix-degrading enzymes in the cell, such as MMPs and ADAMTs, thereby aggravating IDD. The oxidative stress response and the inflammatory response are often accompanied by mutual cause and effect, leading to apoptosis of the NP cells[24]. In contrast, the inhibition of pro-inflammatory cytokines could significantly delay the IDD process. Resveratrol is widely reported to play a role in preventing IDD via suppressing ROS, TNF-α, and IL-1β[25-27]. Furthermore, cortistatin is newly found to protects against IDD by inhibiting mitochondrial ROS and inflammasome activation caused by TNF-α. Additionally, activation of Nrf2 is perceived to suppress ROS production efficiently and meanwhile decrease the generation of inflammatory cytokines[28]. Depend on the suppression of ROS and inflammation, medicines, like Moracin[29], Luteoloside[30], and Kinsenoside[31], are verified to ameliorate the progression of IDD through Nrf2 activation functionally.

What we focused on in this study has been confirmed to activate Nrf2 in various cells. tBHQ widely exists in foods, including oils, crackers, and cereals, and is a well-characterized Nrf2 activator through preventing Nrf2 repression by Keap1[32]. Li et al[33] used tBHQ to protect hepatocytes against saturated fatty acids-induced lipotoxicity through Nrf2 activation. tBHQ also alleviates chronic pulmonary toxicity by reducing oxidative stress and endoplasmic reticulum stress[34]. Besides, Itaconate is elucidated to have an outstanding therapeutic value for the treatment of systemic lupus erythematosus through inhibiting the inflammation by disrupting Keap1-Nrf2 pathway[35]. However, no evidence was provided that tBHQ and Itaconate can be used to interrupt IDD. In our experiment, we first tested the safe concentration of cell culture with tBHQ and Itaconate, and we wanted to use as much as possible, with no harm to NP cells. LPS is efficient to induce NP cells degeneration by increasing the ROS production and triggering inflammation response. After pretreated with tBHQ and Itaconate, NP cells presented a strong defense to the negative effect of LPS; collagen II expression was increased, the cell viability was improved, ROS production decreased, IL-1β and TNF-α were suppressed, and the apoptosis was eventually prevented. Indeed, these protective manners were abolished when the Nrf2 gene was silenced. Therefore, these two agonists of Nrf2 are confirmed to protect NP cell degeneration effectively.

Interestingly, based on ensuring NP cell activity, tBHQ and Itaconate show different advantages in anti-inflammatory and anti-ROS properties. After we maximized the dose of drugs, tBHQ suppressed the ROS production more significantly than Itaconate, and Itaconate pretreated NP cell presented a more potent ability in anti-IL-1β and TNF-α**.** However, no difference was observed in the measurement of cell viability and apoptosis. We then wondered whether the combination of tBHQ and Itaconate could optimize the effect of both anti-inflammation and anti-ROS. As except, we also used the maximum dose of the mixture of them and found the mix showed not only good anti-inflammatory effect but also had strong anti-oxidant properties. Moreover, the mix also improved the cell viability, which was superior to a single pretreatment of tBHQ or Itaconate. Though tBHQ and Itaconate are both Nrf2 agonists, they present different advantages in preventing NP cell degeneration. To continuously explore whether the application of the mix plays a role in reverse NP cell degeneration, we used the LPS to cause degeneration and then subjected the cells into the medium containing the drugs. The mixture also activated the Nrf2 expression. However, the collagen II expression, cell viability, and apoptosis were not improved. Meanwhile, the inflammation and ROS were still suppressed by the stimuli of the mix of tBHQ and Itaconate. In contrast to its preventive effect, the therapeutic effect of the mix is not so noticeable. It perhaps has a more significant result when the stimulation time is extended.

Conclusions

Collectively, our present results suggest pretreatment with tBHQ or Itaconate both prevents NP cells degeneration by activating Nrf2. In comparison, tBHQ and Itaconate show much more potent anti-oxidant and anti-inflammation capacity, respectively. Besides, the mix of tBHQ and Itaconate takes the advantages of each. Additionally, our results show that earlier use of the mix of tBHQ and Itaconate has a better effect on preventing NP cell degeneration. Taken together, the no-

velty of this study uncovers the impacts of tBHQ and Itaconate in activating Nrf2, which would be a promising therapy in preventing IDD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Patil P, Niedernhofer LJ, Robbins PD, Lee J, Sowa G, Vo N. Cellular senescence in intervertebral disc aging and degeneration. Curr Mol Biol Rep 2018; 4: 180-190.
2) Oh CH, Kim DY, Ji GY, Kim YJ, Yoon SH, Hyun D, Kim EY, Park H, Park HC. Cervical arthroplasty for moderate to severe disc degeneration: clinical and radiological assessments after a minimum follow-up of 18 months--Pfirrmann grade and cervical arthroplasty. Yonsei Med J 2014; 55: 1072-1079.
3) Smith LJ, Nerurkar NL, Choi KS, Harfe BD, Elliott DM. Degeneration and regeneration of the intervertebral disc: lessons from development. Dis Model Mech 2011; 4: 31-41.
4) Collin EC, Carroll O, Kilcoyne M, Peroglio M, See E, Hendig D, Alini M, Grad S, Pandit A. Ageing affects chondroitin sulfates and their synthetic enzymes in the intervertebral disc. Signal Transduct Target Ther 2017; 2: 17049.
5) Wang F, Cai F, Shi R, Wang XH, Wu XT. Aging and age related stresses: a senescence mechanism of intervertebral disc degeneration. Osteoarthritis Cartilage 2016; 24: 398-408.
6) Feng C, Yang M, Lan M, Liu C, Zhang Y, Huang B, Liu H, Zhou Y. ROS: crucial intermediators in the pathogenesis of intervertebral disc degeneration. Oxid Med Cell Longev 2017; 2017: 5601593.
7) Suzuki S, Fujita N, Hosogane N, Watanabe K, Ishii K, Toyama Y, Takubo K, Horiuchi K, Miyamoto T, Nakamura M, Matsumoto M. Excessive reactive oxygen species are therapeutic targets for intervertebral disc degeneration. Arthritis Res Ther 2015; 17: 316.
8) Che H, Li J, Li Y, Ma C, Liu H, Qin J, Dong J, Zhang Z, Xian CJ, Miao D, Wang L, Ren Y. p16 deficiency attenuates intervertebral disc degeneration by adjusting oxidative stress and nucleus pulposus cell cycle. Elife 2020; 9: e52570.
9) Chen ZB, Yu YB, Wa QB, Zhou JW, He M, Cen Y. The role of quinazoline in ameliorating intervertebral disc degeneration by inhibiting oxidative stress and anti-inflammation via NF-kappaB/MAPKs signaling pathway. Eur Rev Med Pharmacol Sci 2020; 24: 2077-2086.
10) Mavrogonatou E, Angelopoulou MT, Kletsas D. The catabolic effect of TNFalpha on bovine nucleus pulposus intervertebral disc cells and the restraining role of glucosamine sulfate in the TNFalpha-mediated up-regulation of MMP-3. J Orthop Res 2014; 32: 1701-1707.
11) Bellezza I, Giambanco I, Minelli A, Donato R. Nrf2-Keap1 signaling in oxidative and reductive stress. Biochim Biophys Acta Mol Cell Res 2018; 1865: 721-733.
12) Tonelli C, Chio I, Tuveson DA. Transcriptional regulation by Nrf2. Antioxid Redox Signal 2018; 29: 1727-1745.
13) Niture SK, Khatri R, Jaiswal AK. Regulation of Nrf2-an update. Free Radic Biol Med 2014; 66: 36-44.
14) Yamamoto M, Kensler TW, Motohashi H. The KEAP1-NRF2 system: a Thiol-based sensor-effector apparatus for maintaining redox homeostasis. Physiol Rev 2018; 98: 1169-1203.
15) Kobayashi EH, Suzuki T, Funayama R, Nagashima T, Hayashi M, Sekine H, Tanaka N, Moriguchi T, Motohashi H, Nakayama K, Yamamoto M. Nrf2 suppresses macrophage inflammatory response by blocking pro-inflammatory cytokine transcription. Nat Commun 2016; 7: 11624.
16) Robledinos-Anton N, Fernandez-Gines R, Manda G, Cuadrado A. Activators and inhibitors of NRF2: a review of their potential for clinical development. Oxid Med Cell Longev 2019; 2019: 9372182.
17) Liu Q, Gao Y, Ci X. Role of Nrf2 and its activators in respiratory diseases. Oxid Med Cell Longev 2019; 2019: 7090534.
18) Sivandzade F, Prasad S, Bhalerao A, Cucullo L. NRF2 and NF-B interplay in cerebrovascular and neurodegenerative disorders: molecular mechanisms and possible therapeutic approaches. Redox Biol 2019; 21: 101059.
19) Liu Y, Lin J, Wu X, Guo X, Sun H, Yu B, Shen J, Bai J, Chen Z, Yang H, Geng D, Mao H. Aspirin-mediated attenuation of intervertebral disc degeneration by ameliorating reactive oxygen species in vivo and in vitro. Oxid Med Cell Longev 2019; 2019: 7189854.
20) Iwata M, Ochi H, Asou Y, Haro H, Aikawa T, Harada Y, Nezu Y, Yogo T, Tagawa M, Hara Y. Variations in gene and protein expression in canine chondrodystrophic nucleus pulposus cells following long-term three-dimensional culture. PLoS One 2013; 8: e63120.
21) Bowles RD, Setton LA. Biomaterials for intervertebral disc regeneration and repair. Biomaterials 2017; 129: 54-67.
22) Nasto LA, Robinson AR, Ngo K, Clauson CL, Dong Q, St CC, Sowa G, Pola E, Robbins PD, Kang J, Niedernhofer LJ, Wipf P, Vo NV. Mitochondrial-derived reactive oxygen species (ROS) play a causal role in aging-related intervertebral disc degeneration. J Orthop Res 2013; 31: 1150-1157.
23) Molinos M, Almeida CR, Caldeira J, Cunha C, Goncalves RM, Barbosa MA. Inflammation in intervertebral disc degeneration and regeneration. J R Soc Interface 2015; 12: 20141191.
24) Ishibashi H, Tonomura H, Ikeda T, Nagae M, Sakata M, Fujiwara H, Tanida T, Mastuda K, Kawata M, Kubo T. Hepatocyte growth factor/c-

met promotes proliferation, suppresses apoptosis, and improves matrix metabolism in rabbit nucleus pulposus cells in vitro. J Orthop Res 2016; 34: 709-716.

25) Wang XH, Zhu L, Hong X, Wang YT, Wang F, Bao JP, Xie XH, Liu L, Wu XT. Resveratrol attenuated TNF-alpha-induced MMP-3 expression in human nucleus pulposus cells by activating autophagy via AMPK/SIRT1 signaling pathway. Exp Biol Med (Maywood) 2016; 241: 848-853.

26) Jiang Y, Xie Z, Yu J, Fu L. Resveratrol inhibits IL-1beta-mediated nucleus pulposus cell apoptosis through regulating the PI3K/Akt pathway. Biosci Rep 2019; 39: BSR20190043.

27) Wang W, Li P, Xu J, Wu X, Guo Z, Fan L, Song R, Wang J, Wei L, Teng H. Resveratrol attenuates high glucose-induced nucleus pulposus cell apoptosis and senescence through activating the ROS-mediated PI3K/Akt pathway. Biosci Rep 2018; 38: BSR20171454.

28) Tang Z, Hu B, Zang F, Wang J, Zhang X, Chen H. Nrf2 drives oxidative stress-induced autophagy in nucleus pulposus cells via a Keap1/Nrf2/p62 feedback loop to protect intervertebral disc from degeneration. Cell Death Dis 2019; 10: 510.

29) Gu R, Huang Z, Liu H, Qing Q, Zhuan Z, Yang L, Su Z, Huang W. Moracin attenuates LPS-induced inflammation in nucleus pulposus cells via Nrf2/HO-1 and NF-kappaB/TGF-beta pathway. Biosci Rep 2019; 39: BSR20191673.

30) Lin J, Chen J, Zhang Z, Xu T, Shao Z, Wang X, Ding Y, Tian N, Jin H, Sheng S, Gao W, Lin Y, Zhang X, Wang X. Luteoloside inhibits IL-1beta-induced apoptosis and catabolism in nucleus pulposus cells and ameliorates intervertebral disk degeneration. Front Pharmacol 2019; 10: 868.

31) Wang Y, Zuo R, Wang Z, Luo L, Wu J, Zhang C, Liu M, Shi C, Zhou Y. Kinsenoside ameliorates intervertebral disc degeneration through the activation of AKT-ERK1/2-Nrf2 signaling pathway. Aging (Albany NY) 2019; 11: 7961-7977.

32) Boss AP, Freeborn RA, Duriancik DM, Kennedy RC, Gardner EM, Rockwell CE. The Nrf2 activator tBHQ inhibits the activation of primary murine natural killer cells. Food Chem Toxicol 2018; 121: 231-236.

33) Li S, Li J, Shen C, Zhang X, Sun S, Cho M, Sun C, Song Z. tert-Butylhydroquinone (tBHQ) protects hepatocytes against lipotoxicity via inducing autophagy independently of Nrf2 activation. Biochim Biophys Acta 2014; 1841: 22-33.

34) Wang Y, Gu YH, Liu M, Bai Y, Liang LY, Wang HL. TBHQ alleviated endoplasmic reticulum stress-apoptosis and oxidative stress by PERK-Nrf2 crosstalk in methamphetamine-induced chronic pulmonary toxicity. Oxid Med Cell Longev 2017; 2017: 4310475.

35) Tang C, Wang X, Xie Y, Cai X, Yu N, Hu Y, Zheng Z. 4-Octyl Itaconate activates Nrf2 signaling to inhibit pro-inflammatory cytokine production in peripheral blood mononuclear cells of systemic lupus erythematosus patients. Cell Physiol Biochem 2018; 51: 979-990.