Lycium barbarum polysaccharides exert an antioxidative effect on rat chondrocytes by activating the nuclear factor (erythroid-derived 2)-like 2 signaling pathway

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Abstract

Introduction: Oxidative stress is the main cause of osteoarthritis (OA). Lycium barbarum polysaccharides (LBP) have antioxidant properties. Thus, the potential effect of LBP on H\textsubscript{2}O\textsubscript{2}-stimulated chondrocytes was examined.

Material and methods: The cell viability was detected by CCK-8. The reactive oxygen species (ROS) production and apoptosis rates were determined by flow cytometric analysis. The DNA damage was detected by comet assay. Real-time polymerase chain reaction (qPCR) and Western blot assays were performed to examine the expression of histone 2A family member X (γH2AX), checkpoint kinase 1 (Chk1), poly ADP-ribose polymerase (PARP), cysteinyl aspartate specific proteinase (caspase)-3/8/9, and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and its antioxidant-response element (ARE) dependent factors including heme oxygenase-1 (HO-1) and quinine oxidoreductase-1 (NQO-1).

Results: Compared to the H\textsubscript{2}O\textsubscript{2} group, LBP inhibited the ROS production and DNA damage caused by H\textsubscript{2}O\textsubscript{2} (p < 0.05), respectively. LBP inhibited the mRNA and protein expressions of γH2AX and Chk1 (p < 0.05). Meanwhile, LBP significantly decreased apoptosis (p < 0.05). And LBP inhibited the expression levels of PARP and Caspase-3/8/9 (p < 0.05). Moreover, LBP increased the expression of Nrf2, HO-1 and NQO-1 (p < 0.05). Furthermore, the depletion of Nrf2 that mediated by RNA interference reversed the apoptosis and DNA damage inhibition effect of LBP (p < 0.05).

Conclusions: LBP protected chondrocytes through inhibiting DNA damage and apoptosis caused by H\textsubscript{2}O\textsubscript{2}, in which the Nrf2/ARE signaling pathway played a positive role. It provided an inspiration for clinical application – developing LBP as a therapeutic agent and Nrf2 as a promising candidate.

Key words: Lycium barbarum polysaccharides, antioxidant, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), osteoarthritis.

Introduction

Osteoarthritis (OA), a multifactorial disease, is the most common chronic disorder with increasing prevalence due to the population aging of modern society. Osteoarthritis is often accompanied by inflammation, stiffness and loss of mobility, with common occurrence in hands,
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L. barbarum, whose berries, red in color and sweet in taste, have been used as a traditional herb and food in China for more than 2500 years [14]. The compounds composed of arabinose, glucose, galactose, mannose, xylose and rhamnose [14]. The compounds have been stated to hold a wide array of activities [15], including antioxidant and anti-ageing [16, 17], and anticancer [18, 19]. Their protective effects on the reproductive system [20], nervous system [21] and immune system [22] have already been demonstrated. However, the protective effect of LBP on chondrocytes has not yet been examined in detail and the underlying mechanisms are still indistinct. It is reported that reactive oxygen species (ROS) acted as signaling intermediates of intracellular signaling that participate in cartilage homeostasis maintenance [23, 24]. Excessive ROS exposure would lead to disruption of cartilage homeostasis [25, 26]. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), is a redox sensitive transcription factor. It is claimed that activation of Nrf2 signaling protected against oxidative stress and various forms of inflammation [25, 27]. Nrf2 regulates the expression of heme oxygenase-1 (HO-1) and quinine oxidoreductase-1 (NQO-1) through antioxidant-response elements (AREs) [28]. A recent study showed that Nrf2-knockout mice exhibited more severe cartilage damage than wild-type mice [29]. Based on these findings, we speculated that LBP may protect chondrocytes against OA through the Nrf2/ARE signaling pathway. Therefore, the current study was aimed to explore this hypothesis in vitro.

Material and methods

Cell lines and LBP preparation

Rat chondrocytes were purchased from American Type Culture Collection (ATCC, USA). Purified LBP (95% Ultra violet Pure) were purchased from Xian Runxue Biological Technology Co., LTD (Runxue, China), the origin of which is Ningxia wolfberry dried fruit.

Cell treatment

Chondrocytes (1 × 10^5/well) were seeded in a 6-well plate at 37°C in a humidified 5% CO2 atmosphere in Dulbecco’s Modified Eagle Media (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Sangon, China) and 1% pen/strep (Sangon, China). At about 80% confluence, chondrocytes were serum starved overnight and then four treatment groups were used for comparison: 1) normal group, chondrocytes were cultured with 0.1% DMSO; 2) model group, chondrocytes were treated with 0.3 mm H2O2; 3) 0.2 mg/ml LBP treatment groups, chondrocytes were incubated in the medium containing 0.2 mg/ml LBP for 12 h before H2O2 stimulation for 6 h. 4) 0.4 mg/ml LBP treatment groups, chondrocytes were incubated in the medium containing 0.4 mg/ml LBP for 12 h before H2O2 stimulation for 6 h. All the experiments for each group were repeated independently at least 3 times.

Cell transfection with siRNA

At about 80% confluence, cells were transfected with 100 nM siRNA-Nrf2 (SMARTpool) or siRNA Universal Negative Controls (Sigma-Aldrich, USA) using Lipofectamine 2000 (Invitrogen, USA). After 36 h, the cells were treated with LBP before H2O2 stimulation in serum free medium. Western blot was performed to detect the effects of siRNA on Nrf2 expression. Sequence of siRNA-Nrf2 Forward: UCCCGUUUGUAGAUGACAA; Reverse: UUGUCAUGUCCAAACGGGA.

Cell viability assay

Chondrocytes of a 96-well plate (1 × 10^3 cells/well) were serum starved overnight, and then treated with H2O2 (0.1–0.5 μM) in serum free medium for different time periods (6, 12, 24 and 48 h). Cell viability was determined using the CKK-8 kit according to the instructions (Beyotime, China). The absorption at 450 nm was measured by microplate reader (Bio-Rad, USA).

Reactive oxygen species (ROS) measurement

Cells were stained with 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) (Invitrogen, USA) for ROS measurement as described previously [30]. Becton Dickinson (BD) Fluorescence Activating Cell Sorter (FACS) Canto II was used to perform flow cytometric analysis. All are representatives of at least three independent experiments. At least 20,000 events were analyzed.
Measurement of apoptosis

Chondrocytes (1 × 10⁴/well) were cultured in 6-well plates. The Annexin V/PI apoptosis kit (Invitrogen, USA) was adopted to detect the apoptosis. As described in the manufacturer’s instructions, the Annexin-V and PI binding was analyzed using fluorescence-activated cell sorting (BD Canto II, USA).

Total RNA isolation and real time PCR

Total RNA was isolated using RNAiso reagent (Takara, Japan). In brief, the collected cells were lysed with RNAiso Plus, and maintained at room temperature for 5 min. After being centrifuged at 12,000g at 4°C for 5 min, the supernatant was removed and mixed with chloroform (Sinopharm Chemical Regent Co., Ltd, China). After mixing with isopropyl alcohol (SCRC, China) followed by centrifuging, the RNA precipitate was washed with 70% ethanol 3 times. Then the RNA precipitate was dissolved in RNase-free water. Subsequently, according to the manufacturer’s instructions, the cDNA was synthesized using ReverTra Ace (Toyobo, Japan), RNA (1 μg) and oligo dT (Takara, Japan). The mRNA expression was quantified with SYBR Green PCR master mix (Applied Biosystems, USA) and ABI 7500 Real-time PCR system (Applied Biosystems, USA). Relative expression levels were calculated using the 2⁻ΔΔCt method [31]. Primer sequences for real-time PCR were as below:

| Gene    | Forward  | Reverse   |
|---------|----------|-----------|
| Nrf2    | CTGCATTTAGTCATGCTTCTCTC  | TTTAGGGTCGTCTTGGCA  |
| NQO-1  | GCTTTCTCTAATGGGCCAGAAG  | ACATCGACAGGCCCAACTGTTCAA |
| HO-1    | CTGACGAGTGGACGATCTGTGAG  | TGGAAAGGGTCAGGGAAG |
| γH2AX   | GGAAAGCTTGCCAGGGAAGAG  | GACCTTGCTGTATCTGGGTTG |
| Chk1    | AAGCCGGAAGTCAACCAGTA  | AGGGCGGCAAGTCAACCAGTA |
| GAPDH   | GGACATGCAAGGCTGAGAATG  | ATGGTGATGAACGCGCAGTA |

Western blot analysis

Total proteins were extracted using the Proteo-Prep Total Extraction Sample Kit (Sigma-Aldrich, USA). Western blot was performed using the primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies and detection by an enhanced chemiluminescence reagent (Pierce, USA). The primary antibodies used were anti-γH2AX, anti-Nrf2, anti-NQO-1, anti-HO-1, and anti-GAPDH from Santa Cruz Biotechnology (Santa Cruz, USA), anti-caspase-3 and anti-Chk1 from Cell Signaling Technology (CST, USA), and anti-caspase-8 and anti-caspase-9 from Becton Dickson (BD, USA). Secondary antibodies were from Cell Signaling Technology (CST, USA).

Comet assay

The comet assay was carried out following protocols as described previously [32]. Firstly, the slides were coated with 1% normal melting point agarose (Sangon, China). Then, cells were raised in 0.5% low melting point (LMP) agarose (Sangon, China) before being placed on the prepared slides and stored in the freezer for 5 min after being covered by coverslips. Then the cell suspensions on the slides were treated as above. Following the treatments, slides were covered with a third layer of 0.5% LMP agarose and kept in the freezer for 5 min again. After removing the coverslip, the slides were soaked in cold lysing buffer at 4°C overnight. The next day, before electrophoresis, they were flooded with cold fresh electrophoresis buffer for 30 min to allow DNA to denature. Finally, the slides were stained with ethidium bromide.

Statistical analysis

GraphPad software was used for all analyses by one-way ANOVA or t-test for comparison of differences between groups. P < 0.05 indicated statistical significance. Data were presented as the mean ± standard deviation (S.D).

Results

LBP depressed the H₂O₂-induced oxidative stress in rat chondrocytes

The chondrogenic phenotype of the purchased rat cells was first confirmed (Figures 1 A, B). Then, it was observed that the cell viability was inhibited by H₂O₂ in a dose-dependent manner (Figure 1 C). The optimal dose of H₂O₂ was adopted at a concentration of 0.3 mM for the subsequent experiments. Moreover, according to the previous investigations, LBP play multiple roles in biological processes. The results showed that ROS levels were decreased substantially in LBP treatment groups using flow cytometric analysis (Figures 1 D, E). It was suggested that LBP depressed H₂O₂-induced oxidative stress effectively in chondrocytes.

LBP inhibited H₂O₂-induced DNA damage in chondrocytes

DNA damage caused by H₂O₂ was further determined by comet assay in chondrocytes. The results showed that LBP inhibited H₂O₂-induced DNA damage compared to that of the model group (Figure 2 A). Moreover, the mRNA expression of γH2AX and Chk1 was down-regulated in LBP treatment groups compared to that of the model group (Figure 2 B). Although there was no statistical significance in 0.2 mg/ml LBP group; the expression of γH2AX and Chk1 was significantly decreased by 0.2 mg/ml LBP compared to model group. The
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**Figure 1.** A, B – Identity of chondrocytes with toluidine blue staining. A – Image before staining; B – Image after toluidine blue staining. C – CCK8 assay for detection of cytotoxicity of H₂O₂. Control indicates the cells without treatment with H₂O₂; 0.1, 0.2, 0.3, 0.4, 0.5 mM indicate the treatment concentration of H₂O₂. *P < 0.05 vs. control. D – ROS measurement by flow cytometry assay. E – Determination of ROS level. H₂O₂ (0.3 mM H₂O₂); *P < 0.05, **P < 0.01 and ***P < 0.001 vs. control; *P < 0.05 and ***P < 0.001 vs. H₂O₂. Control (normal cells); H₂O₂ (0.3 mM H₂O₂); H₂O₂ + 0.2 (0.2 mg/ml LBP + 0.3 mM H₂O₂); H₂O₂ + 0.4 (0.4 mg/ml LBP + 0.3 mM H₂O₂).
protein expression of γH2AX and Chk1 was significantly down-regulated in LBP treatment groups compared to that of the model group (Figures 2 C, D). Therefore, LBP inhibited H₂O₂-induced DNA damage in chondrocytes.

LBP decreased H₂O₂-induced cell apoptosis in chondrocytes

Subsequently, the effect of LBP on apoptosis of chondrocytes was detected. It was noted that LBP reduced cell apoptosis compared to that of the model group (Figures 3 A, B). PARP and caspase-3/8/9, apoptosis-related genes, were decreased both in the transcription and translated levels in the LBP treatment groups (Figures 3 C–E). It was found that LBP decreased H₂O₂-induced cell apoptosis effectively in chondrocytes.

LBP activated Nrf2/ARE signaling in H₂O₂-stimulated chondrocytes

To uncover the underlying mechanisms, the expression of Nrf2/ARE signaling was examined. The mRNA expression of Nrf2, HO-1 and NQO-1 was rescued significantly in H₂O₂-stimulated chondrocytes (Figure 4 A). Consistently, Nrf2, HO-1 and NQO-1 showed increased protein levels (Figures 4 B, C). Further, the apoptosis inhibition effect of LBP was reversed after knockdown of Nrf2 (Fig-
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**Figure 3.**

- **A** – Flow cytometry analysis for apoptosis.
- **B** – Determination of apoptosis rate. **P < 0.01** and ***p < 0.001 vs. control; **p < 0.01 and ***p < 0.001 vs. H2O2. 
- **C** – Quantitative analysis of PARP and caspase-3/8/9.
- **D, E** – Western blot analysis of PARP and caspase-3/8/9. GAPDH was used as the loading control. **P < 0.01 vs. control; **p < 0.05 and ***p < 0.001 vs. H2O2.

Control (normal cells); H2O2 (0.3 mM H2O2); H2O2 + 0.2 (0.2 mg/ml LBP + 0.3 mM H2O2); H2O2 + 0.4 (0.4 mg/ml LBP + 0.3 mM H2O2)
ures 5 A, B). The mRNA and protein expression levels of Nrf2 were down-regulated after siRNA-Nrf2 transfection (Figures 5 C–E). Moreover, the depletion of Nrf2 induced more serious DNA damage than that of the negative control group (Figures 5 F). Taken together, these results indicated that pretreatment of LBP up-regulated the expression levels of Nrf2, HO-1 and NQO-1 in chondrocytes following H2O2 stimulation in vitro.

Discussion

In the present study, cell viability was suppressed in H2O2-stimulated chondrocytes in a dose-dependent manner (Figure 1 C). Recently, oxidative stress is considered as major cause of OA pathogenesis [26], which is mainly attributed to the imbalance of redox hemostasis between ROS generation and its removal by antioxidant enzymes [33–37]. LBP is reported to facilitate the alleviation of oxidative stress. Therefore, LBP may be helpful to prevent the cytotoxicity caused by H2O2 in chondrocytes. Interestingly, the pretreatment of LBP in chondrocytes inhibited H2O2-induced ROS (Figures 1 D, E). Moreover, ROS overload would trigger widespread DNA damage in chondrocytes [26]. In line with this report, the H2O2 treatment significantly evoked DNA damage, whereas the pretreatment of LBP mitigated the injury in this study (Figure 2 A). In addition, it is widely recognized that γH2AX and Chk1 are crucial for cell cycle transition in response to DNA damage [38, 39]. The results indicated that the expression of γH2AX and Chk1 was down-regulated in LBP treatment groups compared to that of the model group (Figures 2 B–D). In the meantime, apoptosis generally ensues following DNA damage [33], and the oxidative stress is considered as a high risk factor for it [40]. Thus, apoptosis was further detected in this study. The results revealed that the pretreatment with LBP reduced cell apoptosis caused by H2O2 (Figures 3 A, B). PARP and caspase-3/8/9, apoptosis-related genes [41, 42], were inhibited significantly both in the transcription and translation levels in LBP pretreatment groups (Figures 3 C–E). A recent report in mice bone marrow mononuclear cells supported the apoptosis inhibition effect of LBP [43]. On the other hand, another study showed that LBP inhibited the proliferation
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Figure 5. A – Flow cytometry analysis for apoptosis. B – Determination of apoptosis rate. **P < 0.01 and ***P < 0.001 vs. control. ***P < 0.001 vs. H$_2$O$_2$. C, D – Western blot assay for the effectiveness of SiNrf2. GAPDH was used as the loading control. **P < 0.01 vs. control. E – Quantitative analysis for the effectiveness of SiNrf2. **P < 0.01 vs. control. F – Comet assay for DNA damage. Control (normal cells); H$_2$O$_2$ (0.3 mM H$_2$O$_2$); SiNrf2 (0.4 mg/ml LBP + SiNrf2 + 0.3 mM H$_2$O$_2$); NC (0.4 mg/ml LBP + Sicontrol + 0.3 mM H$_2$O$_2$).
by inducing apoptosis in HeLa cells [43]. These conflicting results may be attributed to the different study model, such as different cell types and distinct concentrations of LBP adopted in these study schedules. Collectively, these results indicated that LBP protected chondrocytes by suppressing ROS generation, DNA damage and apoptosis.

Nrf2, as a redox sensitive transcription factor, is implicated play a role in oxidative stress and various inflammation reactions. Therefore, it is presumable that Nrf2 may be associated with the protective effect of LBP. Excitingly, the pretreatment of LBP rescued the expression of Nrf2 and its downstream targets such as NQO-1 and HO-1, compared to the model group (Figures 4 A–C). Moreover, it was noted that the apoptosis inhibition effect of LBP was reversed obviously by the Nrf2 depletion (Figures 5 A, B). In addition, DNA damage was still severe in the Nrf2 knockdown group, compared to the control (Figure 5 F). It was consistent with a previous report that activation of the Nrf2 signaling pathway mitigated injury and promoted proliferation in neural stem cells in vivo following reoxygenation [44]. Taken together, it can be concluded that LBP likely exerted its antioxidative and protective effects through activating the Nrf2 signaling pathway.

However, there were limitations in the current study. Firstly, the existing research indicates that Nrf2 was modulated by multiple upstream molecules including Keap1 [45], the kinase ERK1/2 [46] and PI3K/AKT/GSK3β signaling [47, 48]. Nevertheless, which upstream regulators regulate Nrf2-mediated chondroprotection, and how, were still not clear in the present study model. Secondly, the results of this study were all from in vitro data, so further investigation in vivo is still needed. Another limitation of the present study was that the chondroprotection effect may not only contribute to LBP absorption. Possibly, it may have relations with the generation of metabolites of LBP due to LBP absorption. Possibly, it may have relations with the generation of metabolites of LBP due to LBP absorption. Possibly, it may have relations with the generation of metabolites of LBP due to LBP absorption.

In conclusion, the present study illustrated that LBP inhibited the ROS production in H2O2-stimulated chondrocytes. Moreover, LBP inhibited DNA damage and cell apoptosis caused by H2O2. Most importantly, it demonstrated that LBP exerted its chondroprotective effect through activating Nrf2/ARE signaling. It also highlighted the potential of LBP as a therapeutic agent and Nrf2 as a promising candidate as novel therapies for the clinical options of osteoarthritis.

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Conflict of interest

The authors declare no conflict of interest.

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