Hyperoside ameliorates TNF-α-induced inflammation, ECM degradation and ER stress-mediated apoptosis via the SIRT1/NF-κB and Nrf2/ARE signaling pathways in vitro

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Received March 28, 2022; Accepted June 7, 2022

DOI: 10.3892/mmr.2022.12776

Abstract. Intervertebral disc degeneration (IDD) is the main pathogenesis of numerous cases of chronic neck and back pain, and has become the leading cause of spinal-related disability worldwide. Hyperoside is an active flavonoid glycoside that exhibits anti-inflammation, anti-oxidation and anti-apoptosis effects. The purpose of the present study was to investigate the effect of hyperoside on tumor necrosis factor (TNF)-α-induced IDD progression in human nucleus pulposus cells (HnPcs) and its potential mechanism. The activity and apoptosis of NPCs were detected by Cell Counting Kit-8 and flow cytometry analyses, respectively. The expression of interleukin (IL)-6 and IL-1β were detected with ELISA kits. Western blotting was used to detect the expression levels of proteins. The results showed that hyperoside effectively alleviated TNF-α-induced NPC apoptosis, and hyperoside treatment inhibited the upregulation of inducible nitric oxide synthase, cyclooxygenase-2, IL-1β and IL-6 in TNF-α-stimulated NPCs. Compared with the findings in the TNF-α group, the intervention of hyperoside attenuated the upregulated expression of aggrecan and collagen II, and downregulated the expressions of matrix metalloproteinase (MMP) 3, MMP13 and a disintegrin and metalloproteinase with thrombospondin motifs 5. In addition, hyperoside upregulated sirtuin-1 (SIRT1) and nuclear factor E2-related factor 2 (Nrf2) signaling reversed the protective effect of hyperoside on TNF-α-induced NPCs. In summary, hyperoside ameliorated TNF-α-induced inflammation, extracellular matrix degradation, and endoplasmic reticulum stress-mediated apoptosis, which may be associated with the regulation of the SIRT1/NF-κB and Nrf2/antioxidant responsive element signaling pathways by hyperoside.

Introduction

Intervertebral disc degeneration (IDD) is considered to be the root cause of the occurrence and development of intervertebral disc (IVD) herniation, and its occurrence is affected by a series of factors, including genetic susceptibility, cell senescence, mechanical load, matrix degradation, inflammation and apoptosis (1). With the increasing aging population in the world, IDD has become the leading cause of spinal-related disability worldwide (2). However, the disease cannot be alleviated through drug therapy or surgical treatment in the clinic at present (3). Therefore, there is an urgent need for an effective treatment to alleviate the progression of IDD. IVD is an avascular organ composed of peripheral ring and central nucleus pulposus, of which human nucleus pulposus cells (HNPCs) are responsible for regulating the synthesis and decomposition of extracellular matrix (ECM) components (4). In the pathogenesis of IDD, the reduction in the number of NPCs and the loss of ECM are important features (5). Previous studies have shown that the process of IDD is closely associated with inflammatory reactions (6,7). The nucleus pulposus secretes pro-inflammatory molecules, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-17, of which TNF-α is the most prominent, promoting the degradation of ECM, and leading to cell phenotypic changes and a series of degenerative events (8). In addition, endoplasmic reticulum (ER) stress is also one of the potential factors for the induction of IDD by inducing NPC apoptosis and ECM degradation (9). Thus, finding effective drugs to inhibit ECM degradation, inflammatory response and ER stress-induced apoptosis in HNPCs may be a feasible strategy for the prevention and treatment of IDD.

Hyperoside is an active flavonoid glycoside present in numerous medicinal plants such as *Epimedium*, *Hypericum perforatum* and *Hypericum* (10). Previous studies have shown that hyperoside has a wide range of pharmacological effects, including anti-inflammation (11), anti-oxidation (12) and anti-apoptosis (13). For example, hyperoside plays an...
anti-inflammatory role in sepsis-related cardiac insufficiency (14), acute lung injury (15) and acute liver injury (16). In addition, hyperoside was able to attenuate the IL-1β-induced ECM destruction of chondrocytes (10). However, the role of hyperoside in IDD has not been investigated thus far.

Sirtuin-1 (SIRT1) is an NAD⁺-dependent deacetylase that functions in a variety of inflammatory and immune responses (17). A previous study suggested that upregulating SIRT1 inhibited the IL-1β-stimulated apoptosis and inflammation of NPCs by activating the PI3K/Akt signaling pathway, and regulated ECM remodeling (18). In addition, SIRT1 inhibited the IL-1β-mediated inflammatory response in HPSCs by regulating the Toll-like receptor (TLR)2/SIRT1/ NF-κB pathway (19). Notably, hyperoside was able to reduce lipopolysaccharide (LPS)-induced inflammation, oxidative stress and apoptosis by upregulating SIRT1 (20). A pervious study showed that hyperoside could attenuate H₂O₂-induced L02 cell damage by activating the nuclear factor E2-related factor 2 (Nrf2)-antioxidant responsive element (ARE) signaling pathway (21). Hou et al (22) reported that hyperoside showed a protective effect on myocardial ischemia-reperfusion injury by inhibiting ER stress and activating the Nrf2 signaling pathway, and activating the Kelch-like ECH-associated protein 1 (Keap1)/Nrf2/ARE signaling pathway was conducive to reducing oxidative stress-IDD degeneration (23). Thus, it was hypothesized that hyperoside may play a protective role in IDD by regulating the SIRT1/NF-κB and Nrf2/ARE signaling pathways.

As aforementioned, the purpose of the present study was to investigate the effects of hyperoside on TNF-α-induced apoptosis of NPCs, ECM degradation and inflammatory response, as well as ER stress, and to assess the underlying mechanism. The present results may provide a new basis for understanding the molecular mechanism of the occurrence and development of IDD, and may suggest the possibility of hyperoside becoming a candidate drug for the treatment of IDD.

Materials and methods

Cell culture. HPSCs were obtained from AccuGen Biotechnology (cat. no. ABI-TC102D). The cells were cultured in DMEM with F12 nutrient mixture (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Hyclone; cytiva), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% CO₂. HPSCs were collected, washed twice with PBS and resuspended in 500 µl with 1X binding buffer at a concentration of 1x10⁶ cells/ml prior to the addition of 5 µl annexin V-FITC. The cells were then gently vortexed and incubated for 20 min at room temperature in the dark. Next, 10 µl of propidium iodide (PI) was added to and incubated for additional 5 min at room temperature in the dark. The stained cells were analyzed using a flow cytometer (BD FACSCalibur; BD Biosciences), and labeled as viable (annexin V and PI negative), early apoptotic (annexin V positive and PI negative) or late apoptotic (annexin V and PI positive). The data were analyzed with FlowJo software (version 10.2; FlowJo LLC).

ELISA. ELISA kits (Beyotime Institute of Biotechnology) were applied to detect the levels of the inflammatory cytokines IL-6 (cat. no. P1326) and IL-1β (cat. no. P1305).

Western blotting. Cells from each group were collected, and total protein was extracted using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentration was determined using the BCA Protein detection kit (Beyotime Institute of Biotechnology) according to the manufacturer’s protocol. Total protein (30 µg per lane) was separated by 12% SDS-PAGE and transferred into a PVDF membrane. The membranes were blocked in 5% non-fat milk at room temperature for 4 h. Upon washing 3 times with 1X TBS-0.1% Tween 20 for 5 min each, the following primary antibodies (all purchased from Abcam) were added to the membrane and incubated overnight at 4°C: Anti-Bcl-2 (1:1,000; cat. no. Ab32124), anti-Bax (1:1,000; cat. no. Ab32503), anti-glucose-regulated protein (GRP)78 (1:1,000; cat. no. Ab21685), anti-phosphorylated (p)-protein kinase rna ‑like er kinase (PERK) (1:5,000; cat. no. Ab19259), anti-activating transcription factor 6 (ATF6) (1:1,000; cat. no. Ab122897), anti-the C/EBP homologous protein (CHOP) (1:1,000; cat. no. Ab11419), anti-caspase 12 (1:1,000; cat. no. Ab62484), anti-pERK (1:1,000; cat. no. Ab229912), anti-inducible nitric oxide synthase (iNOS) (1:10,000; cat. no. Ab178945), anti-cyclooxygenase (COX)-2 (1:5,000; cat. no. Ab62331), anti-aggrecan (1:1,000; cat. no. Ab37778), anti-collagen I (1:1,000; cat. no. Ab34712), anti-MMP3 (1:1,000; cat. no. Ab52915), anti-MMP13 (1:10,000; cat. no. Ab219620), anti-a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)5 (1:5,000; cat. no. Ab41037), anti-SIRT1 (1:1,000; cat. no. Ab110304), anti-p-NF-κB (1:1,000; cat. no. Ab239882), anti-NF-κB (1:1,000; cat. no. Ab220803), anti-Nrf2 (1:1,000; cat. no. Ab37550), anti-heme oxygenase-1 (HO-1) (1:10,000; cat. no. Ab52947), anti-NAD(P)H quinone dehydrogenase 1 (NQO1) (1:5,000; cat. no. Ab80588) and anti-GAPDH (1:1,000; cat. no. Ab8245). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. Ab150077; 1:5,000) at room temperature for 4 h. Protein bands were visualized using an ECL solution and imaged with a gel imager (C150; Azure Biosystems, Inc.).
The gray value of the protein bands was analyzed with ImageJ (version 1.51; National Institutes of Health), and GAPDH as used as the loading control for normalization.

**Statistical analysis.** All data were analyzed using GraphPad Prism 7 software (GraphPad Software, Inc.). Data are presented as the mean ± SD from ≥3 independent experimental repeats. Statistical differences between 2 groups were compared using an unpaired Student's t‑test, statistical differences among ≥2 groups were compared using one‑way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

Hyperoside enhances TNF‑α‑induced HNPCs viability. The viability of HNPCs was detected by CCK‑8 assay. The results showed that hyperoside (Fig. 1A) produced no obvious damage to the viability of HNPCs at concentrations ≤50 µM, indicating that hyperoside had a good biocompatibility (Fig. 1B). Subsequently, the effect of hyperoside on TNF‑α‑induced HNPCs viability was examined. As revealed in Fig. 1C, cell viability was downregulated by 50% after TNF‑α induction. Compared with the TNF‑α group, hyperoside intervention led to a concentration‑dependent increase in viability of HNPCs.

Hyperoside inhibits TNF‑α‑induced ER stress‑mediated apoptosis in HNPCs. The effect of hyperoside on TNF‑α‑induced apoptosis was detected by flow cytometry. As revealed in Fig. 2A, TNF‑α induced significant apoptosis in HNPCs, and the percentage of late apoptosis reached 8.78%, while treatment with hyperoside reversed cell apoptosis in a concentration‑dependent manner. To further verify the protective effect of hyperoside on ER stress‑induced apoptosis, the expression levels of apoptosis‑related proteins and ER stress proteins were examined by western blotting (Fig. 2B). The results demonstrated that, compared with those in the TNF‑α‑induced group, hyperoside treatment markedly decreased the expression levels of Bcl‑2, whereas the expression levels of Bel‑2 were significantly increased.

Hyperoside reduces TNF‑α‑induced inflammation in HNPCs. ELISA was used to evaluate the effect of hyperoside on TNF‑α‑induced inflammatory factors in HNPCs (Fig. 3A and B). The results demonstrated that, compared with that in the TNF‑α group, hyperoside inhibited the expression of IL‑1β and IL‑6 in a concentration‑dependent manner. Subsequently, the expression level of inflammation‑related proteins was detected (Fig. 3C). The western blot results indicated that hyperoside could also reduce the protein expression of iNOS and COX‑2 in a concentration‑dependent manner compared with that in the TNF‑α group.

Hyperoside regulates the SIRT1/NF‑κB and Nrf2/ARE signaling pathways. To further study the mechanism of hyperoside, western blotting was used to detect the expression of SIRT1/NF‑κB and Nrf2/ARE signaling pathways‑related proteins (Fig. 5). The results revealed that TNF‑α induced the downregulation of SIRT1, Nrf2, HO‑1 and NQO1 proteins, and the upregulation of p‑NF‑κB p65 protein, while hyperoside treatment concentration dependently reversed these effects. The aforementioned results indicated that hyperoside may play its role by regulating the SIRT1/NF‑κB and Nrf2/ARE signaling pathways.

EX527 (a SIRT1 inhibitor) and ML385 (a Nrf2 inhibitor) reverse the protective effect of hyperoside on TNF‑α‑induced HNPCs. The SIRT1 inhibitor EX527 and the Nrf2 inhibitor
EX527 were employed to verify the mechanism of the protective effect of hyperoside on TNF-α-induced HnPcs. CCK-8 assay (Fig. 6A) and flow cytometry (Fig. 6B) were performed to detect cell viability and apoptosis, respectively. The results demonstrated that both EX527 and ML385 could partially reverse the protective effect of hyperoside on TNF-α-induced viability of HnPcs and promote apoptosis. The expression levels of apoptosis-related proteins (CHOP, Bax, caspase 12 and Bcl-2) and ER stress proteins (GRP78, p-PERK and ATF6) further verified that EX527 and ML385 reversed the inhibitory effect of hyperoside on TNF-α-induced apoptosis mediated by ER stress to a certain extent (Fig. 6C). Next, the effects of EX527 and ML385 on the expression of intracellular inflammatory factors were observed, and treatment with the inhibitor exacerbated the expression of intracellular inflammatory factors (IL-1β and IL-6) (Fig. 7A and B) and inflammation-related proteins (iNOS and COX-2) (Fig. 7C) compared with the findings in the TNF-α + hyperoside group. Finally, the expression of ECM degradation-related proteins (aggrecan, collagen II, MMP3, MMP13 and ADAMTS5) in HNPCs was detected, and similar results were obtained (Fig. 7D), suggesting that hyperoside may improve TNF-α-induced inflammation, ECM degradation and ER stress-mediated apoptosis through the SIRT1/NF-κB and Nrf2/ARE signaling pathways.
Figure 3. Hyperoside reduces TNF-α-induced inflammation in human nucleus pulposus cells. (A and B) The expression level of inflammatory cytokines (A) IL-1β and (B) IL-6 were detected by ELISA. (C) Western blotting was performed to detect the expression levels of COX-2 and iNOS. ***P<0.001 vs. control; ##P<0.01 and ###P<0.001 vs. TNF-α. COX, cyclooxygenase; iNOS, inducible nitric oxide synthase.

Figure 4. Hyperoside inhibits TNF-α-induced degradation of ECM in human nucleus pulposus cells. The expression of ECM degradation related proteins (Aggrecan, Collagen II, MMP3, MMP13 and ADAMTS5) was detected by western blot analysis. ***P<0.001 vs. control; *P<0.05, **P<0.01 and ***P<0.001 vs. TNF-α. ECM, extracellular matrix; MMP, matrix metalloproteinase.
Discussion

IDD is one of the main causes of low back pain, which seriously affects the life and health of patients (26). The development of IDD is characterized by cellular and biochemical changes in the microenvironment of the IVD, resulting in progressive functional and structural impairment (27). The main pathological characteristics of IDD include production of pro-inflammatory mediators, loss of ECM, cell senescence and cell death (1,28,29). These changes further lead to the disruption of normal disc function. Inflammation is considered to be the main factor leading to IDD (7). Shamji et al (30) showed that the expression levels of macrophage products such as il-4, il-6, il-12 and interferon γ in herniated IVD tissue were significantly increased. Second, the degradation of ECM, resulting in the loss of type II collagen and nucleus pulposus (nP) proteoglycans, is also one of the characteristics of IDD (31). There is abundant evidence that TNF-α could stimulate the expression of a variety of MMPs and ADAMTS5, leading to the degradation of aggregates and collagen (8,32). In addition, the ER is responsible for lipid biosynthesis, calcium storage and protein folding (33). Previous studies have shown that persistent ER stress could induce programmed cell death, particularly apoptosis (34,35). A recent study has also reported that cholesterol induces IDD by activating ER stress in NP cells (36). In summary, controlling the inflammatory response, ER stress-induced apoptosis and the degradation of ECM is considered to be a potential and feasible strategy for the treatment of patients with IDD.

Hyperoside, as one of the main components of Traditional Chinese Medicine (Huangkui capsule, which a patented drug), has multiple biological effects, including anti-inflammatory, antiviral, antioxidant and anticancer effects (37,38). Previous studies have confirmed that hyperoside has an anti-apoptotic effect in hamster lung fibroblast (V79-4) (39) and PC12 (40) cells. In addition, hyperoside protects myocardium from ischemia-reperfusion injury by inhibiting ER stress and activating the Nrf2 signaling pathway (41). Hyperoside can upregulate pituitary adenylate-cyclase-activating polypeptide to inhibit the activation of NOD-, LRR- and pyrin domain-containing protein 3 inflammasomes, thus effectively inhibiting N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neuro-inflammation (42). Therefore, the present study focused on the effects of hyperoside on TNF-α-induced HNPCs inflammation, ECM degradation and ER stress. Consistent with the protective effect of hyperoside previously reported (41), in the present study, hyperoside could concentration-dependently inhibit the TNF-α-induced, ER stress-mediated apoptosis of HNPCs, and reduce TNF-α-induced inflammation and ECM degradation.

Regarding the protective role of hyperoside in an in vitro model of IDD, hyperoside was previously reported to reduce LPS-induced inflammation, oxidative stress and apoptosis by upregulating SIRT1, which activated Wnt/β-catenin (20). SIRT1 is the most important and most widely studied member of the sirtuin family, and plays a role in inflammatory, oxidative stress and immune responses (43,44). SIRT1 inhibits IL-1β-mediated NPCs inflammation by regulating the Toll-like receptor 2/SIRT1/NF-κB signaling pathway (19). In addition, Jiang et al (45) reported that hyperoside is considered an Nrf2 inducer, reducing the damage of N-acetyl-para-amino-phenol to liver by reducing the production of reactive oxygen species.
Activation of the Keap1/Nrf2/ARE signaling pathway helps to reduce oxidative stress-induced disc degeneration (23). Shao et al (46) found that quercetin inhibited the expression of senescence-associated secreted phenotype factor via the Nrf2/NF-κB axis, and improved the progress of IDD. In addition, tea polyphenols could reduce oxidative stress-induced disc degeneration by regulating the Keap1/nrf2/are signaling pathway (47). Similarly, the present study showed that hyperoside treatment relieved TNF-α-induced downregulation of SIRT1, Nrf2, HO-1 and NQO1 protein expressions and upregulation of p-NF-κB p65 protein expression in a concentration-dependent manner, and the SIRT1 inhibitor EX527 as well as the Nrf2 inhibitor ML385 reversed the protective effect of hyperoside on TNF-α-induced HNPCs. These results suggested that hyperoside may play a role in IDD by regulating the SIRT1/NF-κB and Nrf2/ARE signaling pathways.
Although the present study was the first to confirm the inhibitory effect of hyperoside on TNF-α-induced inflammation and ECM degradation of human nucleus pulposus cells, effects of hyperoside on other aspects related to IDD, such as cell senescence (48) and oxidative stress (7), were not observed. Furthermore, the present research results...
are only supported by in vitro experiments, and further verification in vivo will be the focus of our next study. Of note, the protective mechanism of hyperoside may not only be associated with the regulation of the SIRT1/NF-κB and Nrf2/ARE signaling pathways, but other pathways and the optimal concentration of hyperoside need to be further investigated.

In summary, to the best of our knowledge, the present study is the first one to report that hyperoside improves TNF-α-induced inflammation, ECM degradation and ER stress-mediated apoptosis, indicating that it may play a protective role in IDD, which is associated with the regulation of the SIRT1/NF-κB and Nrf2/ARE signaling pathways.

Acknowledgements
Not applicable.

Funding

The present study was supported by Wuhuan Municipal Health Commission (grant no. WZ15B09).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TX and RP designed the study. JY, LM and PL performed the experiments. TX and LM revised the manuscript. JY, LM and PL collected and analyzed the data. RJ, TX and JY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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