Baicalin prevents the apoptosis of endplate chondrocytes by inhibiting the oxidative stress induced by H$_2$O$_2$

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Abstract. Osteoarthritis (OA) is a degenerative disease of articular cartilage. The pathogenesis of OA remains to be fully elucidated, and several studies have found that oxidative stress is important in its pathogenesis. Baicalin is well known and has already been investigated for its role in inhibiting the oxidative stress pathway. Thus, the present study aimed to investigate the role of baicalin on the inhibition of oxidative stress in endplate chondrocytes induced by hydrogen peroxide (H$_2$O$_2$). Following treatment of endplate chondrocytes with different doses of H$_2$O$_2$ with or without baicalin for different incubation durations, a CCK-8 assay and Annexin V/PI staining were used to measure the cell proliferation and apoptotic rates to identify the optimal experimental conditions. Subsequently, for examining the effects and underlying mechanism of baicalin on oxidative stress, the protein expression levels of cleaved-poly (ADP-ribose) polymerase (PARP), B-cell lymphoma-2-associated X protein (Bax) and pro-caspase-3 were analyzed using western blot analysis, intracellular anti-oxidant activities, including those of malondialdehyde (MDA), superoxide dismutase (SOD) and nitric oxide (NO), were quantified, and the levels of endothelial nitric oxide synthase (eNOS) were examined using reverse transcription-polymerase chain reaction analysis. The results revealed that the oxidative stress of endplate chondrocytes induced by 0.5 mM H$_2$O$_2$ for 4 h were the most appropriate conditions for experiments, and pretreatment with 100 µmol/l baicalin for 1 h effectively reversed the effect of H$_2$O$_2$ on the endplate chondrocytes. In addition, Annexin V/PI staining demonstrated that the cell death induced by H$_2$O$_2$ was apoptotic, and baicalin reversed the apoptosis induced by oxidative stress. H$_2$O$_2$-activated PARP cleavage, and the expression of Bax and pro-caspase-3; however, baicalin inhibited the expression of these apoptotic signaling indicators. Baicalin also reduced the levels of MDA, and increased the levels of SOD and NO. Baicalin also significantly elevated the mRNA levels of eNOS in endplate chondrocytes. Therefore, the results of the present study showed that baicalin significantly inhibited the oxidative stress in endplate chondrocytes induced by H$_2$O$_2$, and decreased cell apoptosis.

Introduction

Osteoarthritis (OA) is a degenerative disease of articular cartilage, which is characterized by local and progressive destruction of articular cartilage, and osteophyte formation in the subcartilage edges, and is often accompanied by synovial inflammation. At present, the pathogenesis of articular cartilage injury in OA remains to be fully elucidated. Studies in previous decades have confirmed that oxidative stress is important in the pathogenesis and progression of OA (1,2). Oxidative stress is characterized by excessive reactive oxygen species (ROS) production and reduced cellular antioxidant activity, leading to membrane lipid peroxidation and mitochondrial dysfunction (3-5). If oxidative insult persists, programmed cell death is initiated, which can cause apoptosis of chondrocytes and extracellular matrix degradation (1,2).

Oxidative stress is primarily caused by the excessive accumulation of ROS, which include hydrogen peroxide (H$_2$O$_2$), superoxide anions and hydroxyl radicals. H$_2$O$_2$ is a major component of ROS and is used extensively as an inducer in oxidative stress models (5-9). Therefore, treatment strategies with the potential to prevent oxidative stress induced by H$_2$O$_2$ are considered valuable for patients with OA. Baicalin is a flavonoid glycoside extracted from a type of traditional Chinese medicine, Scutellaria baicalensis, and its chemical structure has been determined (10). Baicalin has been shown to possess antibacterial, anti-inflammatory and anti-apoptotic properties, and is widely used in the treatment of infectious and inflammatory diseases (8,11-14).

The aim of the present study was to investigate the potential protective effects of baicalin treatment on H$_2$O$_2$-induced oxidative stress in endplate chondrocytes in vitro, particularly its effect on cell apoptosis.

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Materials and methods

Isolation and culture of primary rat endplate chondrocytes.

A total of 40 4-week-old male Sprague-Dawley rats weighing 160-200 g were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and housed at a temperature of 26°C, 40-60% humidity, standard diet and a 14-h light/10-h dark cycle. Rat endplate chondrocytes were obtained following cervical dislocation after anesthesia according to a previous study (15,16). In brief, the region of the lumbar posterior midline was shaved and then covered with sterile drapes. The rats were placed in a prone position on the operating table. Cartilage of the L3-L5 endplates was carefully removed from the vertebrae and placed into a small beaker with high-glucose (4.5 g/l; 25 mM) Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Sterile ophthalmic scissors were used to cut the tissues into 1-mm³ sections, and these were transferred to a 50-ml sterile test tube. The tissues were digested with 0.25% trypsin (Hyclone; GE Healthcare Life Sciences; Logan, UT, USA) at 37°C for 30 min, followed by the addition of 0.2% collagenase (Sigma; Merck KGaA; Darmstadt, Germany) at 37°C for 2 h. Following centrifugation (400 x g for 10 min at room temperature), the supernatant was discarded and the tissue suspension was washed three times with DMEM with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The released cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (both from Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂, and used at passage 4-6 for all experiments. The present study was approved by the Ethics Committee of Shanghai East Hospital Affiliated to Tongji University (Shanghai, China).

Cell viability of endplate chondrocytes treated with H₂O₂.

The endplate chondrocytes were grown in 96-well plates (BD Biosciences, Franklin Lakes, NJ, USA) at a density of ~1x10⁴/ml (200 µl/well) in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. After 24 h, the cells were washed with PBS buffer and incubated in serum-free medium at 37°C for 12 h. The cells were then washed again and incubated with medium containing different concentrations (0.1, 0.5 and 1.0 mM) of H₂O₂ (Lingfeng Chemicals Co., Ltd., Shanghai, China) at 37°C for 4 h, respectively. Untreated cells were referred to as the control group. All the experiments were performed in three independent experiments.

Untreated cells were referred to as the normal control group, and then incubated in medium with various concentrations of H₂O₂ (0.1, 0.5 and 1.0 mM) at 37°C for 4 h. Following incubation, the cells were trypsinized and washed with PBS. Following centrifugation at 100 x g for 5 min at room temperature, the cells were resuspended in 500 µl binding buffer. The suspensions were transferred into 1.5-ml tubes, and 5 µl of Annexin V and 5 µl of PI solution were added. The cells were incubated in the dark at room temperature for 20 min, and flow cytometric analysis was performed using a BD FACSAria II flow cytometer (BD Bioscience). Data were analyzed using BD FACSDiva software version 6.1.3 (BD Bioscience), and Flowjo version 7.6.1 (FlowJo LLC, Ashland, OR, USA). The percentage apoptosis was determined as the percentage of Annexin V-positive/PI-negative cells.

Cell viability of endplate chondrocytes treated with baicalin.

The endplate chondrocytes were incubated with 0.5 mM H₂O₂ at 37°C and 5% CO₂ for 4 h, with baicalin added at different treatment time-points (-1, 0, 1 and 2 h). Untreated cells and cells treated with H₂O₂ only were referred as the control groups. The viability of the endplate chondrocytes was determined using a CCK-8 assay, as described above. In addition to determining the optimal incubation duration, the optimal concentration of baicalin on endplate chondrocytes was investigated. The cells were pretreated with baicalin at various concentrations (50, 100 and 200 µmol/l) for 1 h, and were incubated with 0.5 mM H₂O₂ at 37°C for 4 h, respectively. Untreated cells and cells treated with H₂O₂ only were referred as the control groups. As pretreatment of endplate chondrocytes with 100 µmol/l baicalin was optimal for inhibiting the oxidative stress induced by H₂O₂, the cell viability was compared between the H₂O₂-only group and the groups treated with H₂O₂ at different time-points (4, 8, 12 and 24 h). Untreated cells were referred to as the control group. All the above data are shown as the mean of at least three independent experiments.

Annexin V/PI assay of endplate chondrocytes pretreated with baicalin.

The cells were pretreated with 100 µmol/l for 1 h, and then incubated in medium with 0.5 mM H₂O₂ at 37°C for 4 h. Based on the previously described method, the inhibitory effect of baicalin on the apoptosis of endplate chondrocytes induced by H₂O₂ was measured using an Annexin V/PI assay kit.

Western blot analysis.

The protein expression of endplate chondrocytes was evaluated in the groups of chondrocytes treated with 0.5 mM H₂O₂ only for 4 h, with 0.5 mM H₂O₂ for 4 h+100 µmol/l baicalin pretreatment for 1 h, and in normal control group. For the extraction of proteins, the cells were placed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) for 15 min at 4°C and centrifuged at 1,300 x g for 30 min at 4°C. The protein concentrations were determined using a NanoDrop...
Measurement of oxidative activity. The oxidative activities were measured at 6, 12 and 24 h in the 0.5 mM H$_2$O$_2$, 0.5 mM H$_2$O$_2$+100 µmol/l baicalin pretreatment for 1 h, and normal control groups. The concentrations of malondialdehyde (MDA), superoxide dismutase (SOD) and nitric oxide (NO) were assessed using dedicated kits (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer’s protocols.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The mRNA expression levels of endothelial nitric oxide synthase (eNOS) were determined in the groups treated with 0.5 mM H$_2$O$_2$ only for 4 h, 0.5 mM H$_2$O$_2$ for 4 h+100 µmol/l baicalin pretreatment for 1 h, and normal control group. Total cellular RNA was extracted from the endplate chondrocytes using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). OLIGO was used as the reverse transcription primer, and the total RNA was extracted from the cells of each group as the template, which was then reverse transcribed into cDNA under the RT enzyme. The reaction system included 500 ng RNA template, 5X buffer 2 µl, PrimeScript RT enzyme mix 10.5 µl, 10 µmol/l 50 M OLIGO 0.5 µl and 100 µM random 6 mers 0.5 µl. DEPC water was added up to 10 µl at 37°C for 15 min. PCR amplification was performed in a 20 µl reaction volume. The primer sequences were as follows: eNOS forward, 5′-CCAGCTAGCAGGAGGTTCTGTGGTAT-3′ and reverse, 5′-GTCCTGGAGCATGACGATTT-3′; GAPDH forward, 5′-CGGAGTCAGCCAGCTTGTGGTAT-3′ and reverse, 5′-ACGCTTCCTTTGTTGCTG-3′. Triplicate reactions were run with a volume of 20 µl, containing 2 µl cDNA, 10 µl 2X SYBR-Green mix, 6 µl ddH$_2$O, 1 µl PCR forward primer (10 µM) and 1 µl PCR reverse primer (10 µM).

Following an initial denaturation at 95°C for 5 min, the PCR conditions were as follows: 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The 2$^{-ΔΔCt}$ (quantification cycle) method (17) was used to calculate the mRNA expression levels of eNOS in each sample. Data were normalized to the GAPDH content of the same sample.

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analysis was performed using Student’s t-test and one-way analysis of variance using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

$H_2$O$_2$ induces apoptotic cell death of endplate chondrocytes. To characterize the effects of $H_2$O$_2$ on the induction of cell death of endplate chondrocytes, cell viability and apoptotic rates were detected. As $H_2$O$_2$ concentration increased, the cell viability gradually decreased (Fig. 1A; P<0.05), and apoptotic cell death was significantly increased (Fig. 1B-F; P<0.05). On the basis of these experiments on cell viability and the Annexin V/PI staining assay, 0.5 mM H$_2$O$_2$ was selected as a model dose for the following experiments.

Baicalin inhibits apoptotic cell death of endplate chondrocytes induced by $H_2$O$_2$. As shown in Fig. 2A, cell viability was highest when endplate chondrocytes were pretreated with baicalin for 1 h (P<0.05). Subsequently, the cell viability gradually decreased when the endplate chondrocytes were treated with baicalin at or following the start of incubation with $H_2$O$_2$. Without the coexistence of $H_2$O$_2$, no significant differences in cell viability were observed among the group pretreated with 50 µmol/l baicalin, the group pretreated with 100 µmol/l baicalin and the control group (P>0.05). However, when the pretreatment concentration of Baicalin reached 200 µmol/l, the cell viability of endplate chondrocytes was significantly lower, compared with that in the control (P<0.05, Fig. 2B). Furthermore, in the cells incubated with 0.5 mM H$_2$O$_2$ for 4 h with baicalin pretreatment for 1 h, the viability of cells in the group pretreated with 100 µmol/l baicalin was significantly higher, compared with that in the other two groups (P<0.05), which was considered the optimal dose for application in the subsequent experiments (Fig. 2B). As shown in Fig. 2C, compared with the group treated with $H_2$O$_2$, alone, cell death was significantly decreased in the group treated with $H_2$O$_2$+baicalin at 4, 8, 12 and 24 h (P<0.05), which suggested that baicalin may inhibit the cell apoptosis induced by $H_2$O$_2$. The results of the Annexin V/PI staining assay (Fig. 2D-F) also showed that baicalin inhibited the cell apoptosis induced by $H_2$O$_2$.

Baicalin inhibits the activation of the apoptotic cell death pathway induced by $H_2$O$_2$. Classic apoptotic cell death is induced through a pathway, which involves the cleavage of PARP and pro-caspase-3, and the activation of Bax. As shown in Fig. 3A and B, the protein expression of cleaved PARP, Bax and pro-caspase-3 proteins were determined following treatment of the endplate chondrocytes with 0.5 mM H$_2$O$_2$ for 4 h, which were significantly higher, compared with that in the normal control (P<0.05). By contrast, baicalin pretreatment downregulated the protein expression levels of cleaved PARP, Bax and pro-caspase-3 (P<0.05). The quantified the results of western blot analysis are shown in Fig. 3B.

Baicalin decreases the oxidative activity in endplate chondrocytes induced by $H_2$O$_2$. As a biomarker of oxidative stress,
the levels of MDA in endplate chondrocytes were increased by H$_2$O$_2$ treatment at 6, 12 and 24 h (P<0.05), and this was reversed by baicalin at all time-points (P<0.05; Fig. 4A). As an enzyme, which regulates oxidative stress, the levels of SOD were significantly decreased by exposure to H$_2$O$_2$ (P<0.05), however, pretreatment with baicalin for 1 h effectively elevated levels of SOD in endplate chondrocytes at different time-points (4, 8, 12 and 24 h) with H$_2$O$_2$. Data are presented as the mean ± standard error of the mean. *P<0.05 H$_2$O$_2$ vs. control group, baicalin+H$_2$O$_2$ vs. control groups. Inhibitory effects of baicalin on the apoptosis of endplate chondrocytes induced by H$_2$O$_2$ were determined in the (D) control, (E) H$_2$O$_2$, (F) baicalin+H$_2$O$_2$ groups. The results of the Annexin V/PI staining assay showed that baicalin inhibited the cell apoptosis induced by H$_2$O$_2$: H$_2$O$_2$, hydrogen peroxide.

Figure 2. Effects of baicalin on endplate chondrocytes. (A) Effects of baicalin treatment on H$_2$O$_2$-induced loss of endplate chondrocytes at different time-points (-1, 0, 1 and 2 h). (B) Effects of 1 h baicalin treatment on endplate chondrocytes at various concentrations (50, 100 and 200 µmol/l) with or without H$_2$O$_2$. (C) Effects of 1 h baicalin treatment on endplate chondrocytes at different time-points (4, 8, 12 and 24 h) with H$_2$O$_2$. Data are presented as the mean ± standard error of the mean. *P<0.05 H$_2$O$_2$ vs. control group, baicalin+H$_2$O$_2$ vs. control groups. Inhibitory effects of baicalin on the apoptosis of endplate chondrocytes induced by H$_2$O$_2$ were determined in the (D) control, (E) H$_2$O$_2$, (F) baicalin+H$_2$O$_2$ groups. The results of the Annexin V/PI staining assay showed that baicalin inhibited the cell apoptosis induced by H$_2$O$_2$: H$_2$O$_2$, hydrogen peroxide.
levels of eNOS, an enzyme, which functions in catalyzing the release of NO, were also investigated. The mRNA expression of eNOS was downregulated by H$_2$O$_2$, and this was reversed by baicalin (P<0.05; Fig. 5).

**Discussion**

In the present study, a model of 0.5 mM H$_2$O$_2$-induced oxidative stress was successfully induced in endplate chondrocytes, which was characterized by reduced cell viability, increased intracellular ROS and reduced intracellular antioxidant activity. These pathophysiological processes led to apoptosis by activation of the intrinsic apoptotic pathway. As a flavonoid glycoside extracted from a type of traditional Chinese medicine, *Scutellaria baicalensis*, baicalin has been reported to significantly attenuate oxidative injury, and partially prevent apoptosis during the oxidative stress reaction (8,10-14,18). For example, Lin et al (8,11) found that baicalin ameliorated H$_2$O$_2$-induced cytotoxicity in a human renal proximal tubular epithelial cell line (HK-2 cells), and showed effects against renal ischemia-reperfusion injury through the inhibition of inflammation and apoptosis. Therefore, the present study investigated the potential protective effects of baicalin treatment on H$_2$O$_2$-induced oxidative stress in endplate chondrocytes in vitro.

Initially, different time-points of induction with baicalin incubation under H$_2$O$_2$ stimulation were investigated, which showed that 1 h pretreatment with baicalin exhibited optimal protective effects. Secondly, it was observed that the potential protective effects of baicalin pretreatment against
oxidative stress-related injury in endplate chondrocytes were dose-dependent. However, baicalin significantly decreased cell viability when its concentration reached 200 µmol/l. Therefore, to achieve optimal protective effects, 100 µmol/l baicalin was selected as the optimal concentration for inhibiting oxidative stress in endplate chondrocytes induced by H2O2.

In the present study, the protective effects of baicalin on oxidative stress of endplate chondrocytes induced by H2O2 were confirmed through the analysis of cell viability. The cell viability of endplate chondrocytes stimulated by H2O2 was significantly lower, compared with that in than the normal control, as detected by the CCK-8 assay. Notably, a significant increase in cell viability was observed following baicalin pretreatment for 1 h at the various time-points. Baicalin was found to inhibit the activation of the apoptotic cell death pathway triggered by H2O2. Classic apoptotic cell death is induced through a pathway involving the cleavage of PARP and pro-caspase-3, and the activation of Bax (19–21). The abnormally high expression levels of cleaved PARP, Bax and pro-caspase-3 induced by H2O2 were significantly reversed by pretreatment with baicalin. In addition, baicalin suppressed oxidative activity in the endplate chondrocytes induced by H2O2, via effectively reducing levels of MDA, increasing levels of SOD, and elevating NO activities (5,9,22), eNOS, an enzyme which activates the expression of NO (23–25), was also shown to be increased following pretreatment with baicalin, which suggested that baicalin also inhibited the apoptosis through upregulating the expression of eNOS.

However, the mechanism of baicalin-mediated protection against oxidative stress remains to be fully elucidated. Chen et al (18) reported that baicalin inhibited oxidative-stress-induced apoptosis via modulating the activation of extracellular signal-regulated kinases and inducing the gene expression of heme oxygenase-1 in rat C6 glioma cells. Lin et al (8) reported that the effect of baicalin was through the inhibition of endoplasmic reticulum stress and the activation of nuclear factor erythroid 2-related factor 2 signaling, which were important during baicalin-mediated protection. The targets of baicalin also remain to be fully elucidated. A previous study indicated that baicalin activates AMP-activated protein kinase (AMPK) through the Ca2+/calmodulin-dependent protein kinase β (CaMKKβ)-dependent pathway in HeLa and A549 cells (26). AMPK is important in cell physiology and also affects the cell response to oxidative stress. However, at present, no direct association between the renal protective effects of baicalin and activation of the Ca2+/CaMKKβ-AMPK pathway has been reported. Peroxisome proliferator-activated receptor-γ (PPARγ) has also been suggested as a target of baicalin (27). Baicalin was found to activate PPARγ and suppress down-stream nuclear factor-kB-mediated inflammation in aged rat kidneys. In addition, studies have suggested other potential targets, including the proteasome (28), macrophages (29) and notch signaling (30). These possibilities were not examined in the present study, and warrant further investigation.

In conclusion, the results of the present study suggested that baicalin pretreatment protected endplate chondrocytes from the oxidative stress-related damage induced by H2O2. The role of baicalin was predominantly based on inhibiting the production of ROS, increasing intracellular antioxidants and attenuating apoptosis. The results of the present study require verification in future in vivo investigations, however, the results provide further insight into the potential benefits of baicalin for patients with oxidative stress-related diseases, including OA.

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References


