Polysaccharide from Angelica sinensis attenuates SNP-induced apoptosis in osteoarthritis chondrocytes by inducing autophagy via ERK1/2 pathway

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Abstract

Objective

Chondrocyte apoptosis plays a vital role in osteoarthritis (OA) progression. *Angelica sinensis* polysaccharide (ASP), a traditional chinese medicine, possesses anti-inflammatory and anti-apoptotic properties in chondrocytes. This study aimed to determine the protective role of ASP on sodium nitroprusside (SNP)-induced chondrocyte apoptosis, and explore the underlying mechanism.

Method

Human primary chondrocytes isolated from articular cartilage of OA patients were treated with SNP alone or in combination with different doses of ASP. Cell viability and apoptosis were assessed, and apoptosis-related proteins including Bcl-2 and Bax were detected. Autophagy levels were evaluated by light chain 3 (LC3)II immunofluorescence staining, mRFP-GFP-LC3 fluorescence localisation and western blot (LC3II, p62, Beclin-1, Atg5). Meanwhile, activation of ERK 1/2 pathway was determined by western blot. The autophagy inhibitors, 3-Methyladenine (3-MA), chloroquine (CQ) and a specific inhibitor of ERK1/2, SCH772984, were used respectively to confirm the autophagic effect of ASP.

Results

The results showed that SNP-induced chondrocyte apoptosis was significantly rescued by ASP, whereas ASP alone promoted chondrocytes proliferation. The anti-apoptotic effect of ASP was related to the enhanced autophagy and depended on the activation of ERK1/2 pathway.

Conclusion

ASP markedly rescued SNP-induced apoptosis by activating ERK1/2-dependent autophagy in chondrocytes, and it made ASP a potential therapeutic supplementation for OA treatment.

Introduction

Osteoarthritis, a progressive and degenerative disease, is characterized by degeneration of articular cartilage and osteophyte formation. Clinical manifestations include joint pain, swelling, joint deformity and limited movement[1]. Many factors, including age, excessive weight bearing, oxidative stress, physiology and biomechanical environment changes in joint, can result in OA[2]. Degeneration of cartilage in OA is mainly due to the dramatically decreased self-repair ability of chondrocytes in a pathological status, presenting as low chondrocyte vitality, abnormally high apoptosis and eventually loss of homeostasis of chondrocyte metabolism[3].

Many studies have reported that various factors could cause chondrocyte apoptosis, such as inflammation, oxidative stress and mechanical stress [4, 5]. Growing evidence highlights that oxidative stress in chondrocytes is related to metabolic disorder and mitochondrial damage, which leads to
massive apoptosis of chondrocytes. A previous study demonstrated that the nitrite levels, a stable end product of nitric oxide (NO) metabolism, are elevated in serum and cartilage in OA samples. SNP, a NO donor compound, induces chondrocyte apoptosis via mitochondrial-dependent signaling[6].

ASP, which is extracted with water as the initial extraction solvent, consists of xylose, galactose, glucose, arabinose, rhamnose, fructose, and glucuronic acid[7-9]. Some studies have reported that ASP exhibits gastrointestinal protective effects, immunomodulatory effects[10], antitumor activity[11, 12], and anti-inflammatory activity[13]. Furthermore, one study has shown the capacity of ASP to protect chondrocytes from H2O2-induced apoptosis via its antioxidant effects[14]. However, the influence of ASP on autophagy is unclear.

Autophagy, literally meaning “self-eating”, is an intracellular catabolic process of delivering cytosol and/or its specific contents to the lysosomes for degradation. The macromolecular constituents are then recycled and utilized by the cells[15]. Basal level autophagy plays an important role in cellular homeostasis through the elimination of damaged organelles and aggregated intracellular proteins[16]. On the other hand, during conditions of cellular stress, such as nutrient deprivation/starvation, hypoxia, pathogen infection, radiation or anticancer drug treatment, the level of autophagy is augmented, resulting in adaptation and cell survival (cytoprotective response)[17]. Once this physiological augmentation of autophagy flux is genetically or chemically blocked, whether during the formation of autophagosome, the fusion of autophagosome and lysosome or the degradation of autolysosome, cell death will generally increase under stress conditions[18, 19]. Cartilage degeneration and cell death caused by autophagy inhibition play a crucial role in the process of OA[20].

Additional evidence has showed that signaling pathway malfunctions in chondrocytes are involved in aging-related joint diseases such as OA. Extracellular signal-regulated kinase1/2 (ERK1/2) is related to chondrocyte apoptosis, as reported by Shakibaei et al.[21]. However, it was not clear whether there was a relationship between the ERK1/2 signal pathway and autophagy in chondrocytes after treating with ASP and SNP. Thus, our study aimed to identify if there is a link between ASP and autophagy on SNP-stimulated OA chondrocytes in vitro and which signal pathway is involved in it.

**Materials And Methods**

**Reagents**

ASP was purchased from Shanghai Yilin Biotech. Co., Ltd. (Shanghai, China). The purity of ASP is approximately 92%. The component sugars are glucose, galactose, arabinose, rhamnose, mannose, and xylose. The average molecular weight of ASP is 85.0kDa. ASP was dissolved in PBS and diluted with DMEM-F12 for the experiments. Collagenase II (Worthington Biochemical Corp., Lakewood, NJ, USA) was dissolved in DMEM at 2.5 mg/ml to digest articular cartilage. Sodium nitroprusside(SNP) was purchased from Dandong Medical and Pharmaceutical Co., Ltd. (Heilongjiang, China), reconstituted in sterile normal saline at 40 mg/ml and stored at 4°C avoiding light. CQ, 3-MA, P276-00 and SCH772984 were purchased from Selleckchem (Houston, TX, USA).
Isolation and culture of osteoarthritis articular chondrocyte

Cartilage tissue specimens were obtained from OA patients during joint replacement surgery in the Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University. All participants had signed a written informed consent prior to the subjects entering the study. In addition, the study was approved by the Nanjing Medical University Review Board. The information of subjects was performed in Table 1. All the tissues were carefully minced and digested with 2.5 mg/ml collagenase II in serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) for 4-6 hrs at 37°C, filtered through a 70μm cell strainer (BD, Durham, NC, USA), extensively washed with blank DMEM/F12 and finally cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 50μg/mL of ascorbic acid (AA, Sigma), 100 U penicillin, and 100 μg/ml streptomycin (which was referred as the full culture medium for chondrocytes) in a standard cell culture chamber containing 5% CO2. Non-adherent cells were removed after 3 days. Adherent cells were split at a ratio of 1:2 until 90% confluence. Chondrocytes were used from passages 2 to 3 in subsequent experiments.

Characteristic of subjects investigated

| Characteristics                  | OA  |
|----------------------------------|-----|
| Total number of subjects         | 16  |
| Age\(^a\), years                 | 67.4|
| 25th percentile                  | 64  |
| 75th percentile                  | 72  |
| Number of female/male subjects   | 11/5|
| Disease duration\(^a\), years    | 5.7 |
| 25th percentile                  | 3   |
| 75th percentile                  | 8   |
| ICRS grade\(^a\)                 | 2.8 |
| 25th percentile                  | 2   |
| 75\(^{th}\) percentile           | 3   |

\(^a\)Median.

Determination of cell viability and proliferation by MTS assay

Cell viability and proliferation assays were performed using the tetrazolium compound-based CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay (Promega, Madison, WI, USA). OA chondrocytes were seeded at approximately 5000 cells per well in 96-well plates in triplicate for 7 days under regular growth conditions (DMEM/F12 with 10% FBS). After seeding for 24 hrs, ASP was added into the media, and then the MTS assay was performed daily according to the manufacturer's instructions for the subsequent 6 days. Generally speaking, 20μl of MTS solution reagent was pipetted into each well of the 96-well assay plate containing the chondrocytes in 100μl of fresh culture medium. Then the plate was incubated at 37°C for 2 hrs in a humidified, 5% CO2 atmosphere, and the absorbance
at 490nm was recorded using an absorbance microplate reader (Elx808™ Bio-Tek Instruments, Winooski, VT).

**Edu cell proliferation assay**

Chondrocytes were seeded in 24-well plates and treated with ASP and P276-00, and then cell proliferation rate was detected by Yeuor 594 Edu Imaging Kits (Yeasen Co., Ltd., Shanghai, China) according to the manufacturer’s instructions.

**Cell apoptosis detection by DAPI staining**

Chondrocytes were seeded on sterile glass slides coated with gelatin in medium without AA, and then treated with SNP alone or with ASP for indicated time. Cells were fixed and nuclei were stained with DAPI (Sigma-Aldrich, MO, USA) in the dark for 5 min and the fluorescence (Nikon Eclipse Ti, Japan) was observed.

**Detection of cell apoptosis rate by flow cytometry: Annexin V/PI staining**

2×10^5 chondrocytes were seeded in 6-well plates in medium without AA. Cell apoptosis rates were detected by Annexin V-FITC/PI kit (Vazyme Biotech Co., Ltd., Nanjing, China) according to the manufacturer’s instructions. Generally speaking, the cells were washed with ice-cold PBS and trypsinized. Removing the supernatant after centrifugation, the cells were resuspended in 100 µL binding buffer, and incubated with 5 µL Annexin V-FITC for 10 min at room temperature avoiding direct light. Then 5 µL PI and 400 µL binding buffer were mixed into the flow tube.

The apoptosis ratio was assessed with a flow cytometer (BD, Biosciences, San Jose, CA, USA), and the results were analyzed and assembled by FlowJo software (Tree Star, Inc., USA).

**Immunofluorescence**

2-5×10^4 chondrocytes were seeded on sterile glass slides precoated with gelatin in medium without AA. After the indicated treatment, cells were fixed in 4% paraformaldehyde at 4°C for 15 min and blocked with PBS containing 5% normal goat serum and 0.3% Triton X-100 for 1 hrs at room temperature. Staining of the treated cells with LC3A/B (D3U4C) XP® Rabbit mAb (Alexa Fluor® 488 Conjugate, CST, USA) at 1/100 dilution was performed overnight at 4 °C in PBS containing 1% BSA and 0.3% Triton X-100. Nuclei were counterstained with DAPI in the dark for 5 min and the fluorescence (Nikon Eclipse Ti, Japan) was observed.

**Western blot analysis**

Cultured chondrocytes were lysed with RIPA buffer and boiled. SDS-polyacrylamide gel electrophoresis was conducted on a polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. All antibodies, purchased from Cell Signaling Technology were used to detect the autophagy levels,
apoptosis, proliferation and signaling pathways. Rabbit anti-human β-actin polyclonal antibody was used to detect the actin signal as an internal control and relative expression levels were quantified by running the Quantity One software. Antibodies information were performed in Table2.

**Table2**

Lists of antibodies.

| antibodies                        | manufacturer | Isotypes    | catalog |
|----------------------------------|--------------|-------------|---------|
| Atg5 Antibody                    | CST          | Rabbit IgG  | #2775   |
| Bax Rabbit mAb                   | CST          | Rabbit IgG  | #5023   |
| Beclin-1 Rabbit mAb              | CST          | Rabbit IgG  | #3495   |
| Bcl-2 Antibody                   | CST          | Rabbit IgG  | #4223   |
| CyclinD1 Rabbit mAb              | CST          | Rabbit IgG  | #2978   |
| p21 Rabbit mAb                   | CST          | Rabbit IgG  | #2947   |
| Phospho-p44/42MAPK(ERK1/2) Rabbit mAb | CST          | Rabbit IgG  | #4377   |
| P62 Antibody                     | CST          | Rabbit IgG  | #5114   |
| ERK1/2 Rabbit mAb                | CST          | Rabbit IgG  | #4695   |
| Ras Antibody                     | CST          | Rabbit IgG  | #3965   |
| Raf Antibody                     | CST          | Rabbit IgG  | #9422   |
| p-MEK1/2 Rabbit mAb              | CST          | Rabbit IgG  | #9154   |
| MEK1/2 Mouse mAb                 | CST          | Mouse IgG   | #4694   |
| LC3B Antibody                    | CST          | Rabbit IgG  | #2775   |
| β-Actin Rabbit mAb               | CST          | Rabbit IgG  | #4097   |

**mRFP-GFP-LC3 analysis**

Chondrocytes were seeded in gelatin-precoated slides with a density of $5 \times 10^4$ cells. One day after seeding, cells were infected with mRFP-GFP-LC3-labeled adenovirus (Genechem, Shanghai, China) according to the manufacturer’s instructions. The virus expresses the monomeric RFP-GFP-tagged LC3 (tfLC3) as an autophagic flux reporter comprised of LC3 protein fused with monomeric red fluorescent protein (mRFP) and green fluorescent protein (GFP). The GFP signal would quenched within the lysosome lumen by the acidic and/or proteolytic environment. Yellow puncta which is consist with colocalized GFP
(green) and mRFP (red) fluorescent signals in the cytoplasm indicate early autophagosomes, while the mRFP signals alone (red) represent late autolysosomes.

**Statistical analysis**

Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA, US). Unpaired Student’s *t* test was used for two groups and one-way ANOVA for more than two groups. The symbols *, **, *** and # indicated *p* < 0.05, *p* < 0.01, *p* < 0.001, and *p* < 0.0001 respectively. All quoted *p* values were 2-tailed, and those less than 0.05 were considered statistically significant. All data are from *n* = 3 biological replicates.

**Results**

**SNP dramatically attenuates chondrocyte viability whereas ASP rescues it.**

In order to determine the best dosage and time period of SNP application, OA chondrocytes were incubated with three different concentrations of SNP (0.5, 1, 2 mg/ml respectively) for three different time, and sterile normal saline was added as control. As demonstrated in Fig. 1a-c, the cell viability of chondrocytes was decreased in a dose-dependent manner. Especially when chondrocytes were treated with 1 mg/ml SNP, cell viability was reduced by approximately 50% (*p*<0.01) after 12 hrs. Statistically significant differences in cell viability at 12hrs, 24hrs and 48hrs were not observed between 1 mg/ml and 2 mg/ml group (*p>*0.05), but existed between 0.5 mg/ml and 1 mg/ml group (*p*<0.001). Therefore, 1 mg/ml SNP was used to induce apoptosis for the following studies.

In order to evaluate the protective role of ASP, chondrocytes were pretreated with 50 μg/ml or 200 μg/ml ASP for 2 hrs before 24 hrs incubation with 1 mg/ml SNP. Both concentrations of ASP, 50 and 200 μg/ml, remarkably rescued SNP-induced damage (approximately 30%, *p*<0.001) as Fig 1d showed, which suggested that ASP may protect chondrocytes from SNP induced apoptosis.

**ASP promotes chondrocyte proliferation in a p21 and CyclinD1 dependent manner.**

To explore the effect of ASP on chondrocyte proliferation, we incubated chondrocytes with 200μg /ml ASP for 6 days (ASP was added daily). The results showed that chondrocytes significantly increased from Day3 to Day6 compared to PBS control (Fig 2a). To further examine if ASP-induced chondrocyte proliferation depends on the expression of p21 and cell cycle-related protein (CyclinD1), western blot analysis was conducted on Day4. As shown in Fig 2b-d, ASP significantly decreased p21 (*p*<0.0001) and increased CyclinD1 (*p*<0.0001) protein levels. Additionally, P276-00, a CDK4/CyclinD1 specific inhibitor, was applied in combine with ASP for 6 days, the MTS results showed that ASP-induced chondrocyte proliferation was abolished when CyclinD1 was inhibited (Fig 2e). Furthermore, we also used Edu assay to detect chondrocyte proliferation rate on Day4, the results showed the proliferation rate of chondrocytes in ASP treated groups was increased about 50% compared with control groups. Meanwhile, P27600
significantly inhibited chondrocyte proliferation rate (Fig 2f-g). Collectively, these data suggest that ASP enhances the proliferation of chondrocytes in a p21- and CyclinD1-dependent manner.

**ASP protects chondrocytes by inhibiting SNP-induced apoptosis.**

To study whether the protective effect of ASP on SNP-induced cytotoxicity was mediated by the apoptotic process, we used DAPI staining and flow cytometry assays to assess chondrocyte apoptosis. Chondrocytes were pretreated with 50μg/ml or 200μg/ml ASP for 4 hrs and then treated with or without 1 mg/ml SNP for 24 hrs. For the negative control groups, the cells were treated with SNP only. As we expected, SNP significantly increased the percentage of apoptotic chondrocytes compared to the control groups, on the contrary, pretreatment with ASP significantly reduced the percentage of apoptotic chondrocytes (Fig 3a,c). The DAPI staining results, which intuitively displayed the percentage of apoptotic cells, were consistent with flow cytometry assays (Fig3 b,d). The balance of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax plays an important role in the regulation of mitochondrial integrity and cell survival. To verify whether the mitochondrial-dependent apoptotic pathway was affected by ASP in chondrocytes, the expression levels of Bcl-2 and Bax were detected. Western blot analysis revealed that SNP significantly decreased Bcl-2 while increased Bax expression. On the contrary, ASP-pretreatment significantly increased the expression of Bcl-2 while decreased the expression of Bax (Fig3 e-g).

**ASP protects chondrocytes from apoptosis by inducing autophagy.**

To determine whether the protective effect of ASP on chondrocytes was related to autophagy activation, autophagic protein levels such as microtubule-associated protein light chain 3B (LC3II/I), p62, Atg5 and Beclin-1 were evaluated by western blot. The results showed that SNP significantly deceased Beclin-1, Atg5 and LC3II expression (p <0.05). On the contrary, when pretreated with ASP, increased LC3II, Beclin-1 and Atg5 levels were found in chondrocytes (p <0.05). Moreover, the expression of LC3II, Atg5, and p62 was regulated in a dose-dependent manner (Fig 4a-e). LC3II immunofluorescence staining aligned with western blot results (Fig 4f). Furthermore, SNP increased the expression of p62, whereas decreased levels of p62 were observable after ASP treatment, indicating ASP increased autophagolysosomal degradation (Fig 4a,c). Together, these data suggest ASP-induced autophagy rescued SNP-induced apoptosis in chondrocytes.

**ERK1/2 signaling pathway is activated in ASP-induced autophagy.**

It is well reported that ERK1/2, one of the three main mitogen-activated protein kinase (MAPK) signaling pathway, regulates cell apoptosis and proliferation, and even affects autophagy. To illuminate the molecular mechanism of anti-apoptosis effect by ASP, we assessed the activation of ERK1/2 signaling pathway in ASP- stimulated chondrocytes. As shown in Fig5 a-e, ASP significantly increased the expression of Ras, Raf, phosphorylated MEK1/2 (p-MEK1/2) and phosphorylated ERK1/2 (p-ERK1/2) (p <0.05), accompanied by increased expression of autophagy-related proteins LC3II and p62 degradation in SNP-treated cells, as shown in Fig 6c-e. To further identify whether the ameliorated chondrocyte apoptosis was resulting from ASP-induced autophagy and ERK1/2 signal pathway activation, we used 20
μM CQ (autophagy flux inhibitor), 3-MA (autophagy inhibitor) and SCH772984 (selective ERK1/2 inhibitor) to pretreat chondrocytes. Flow cytometry results showed that the protective effect of ASP on chondrocyte apoptosis was significantly inhibited by 3-MA and SCH772984 treatment (Fig 6a). In addition, ASP significantly increased bright LC3II puncta compared with SNP group, while bright LC3II puncta were decreased by the inhibition of ERK1/2 pathway and 3-MA treatment (Fig 6b). To further evaluate the effect of ASP on autophagy flux, we used mRFP-GFP-LC3 autophagic puncta to visualize the level of autophagy. Enhanced autophagy was observed in ASP treated group whereas inhibited autophagy was detected with ERK1/2 pathway inhibiton (Fig 6f). All these results suggested that ASP-mediated autophagy levels are linked to ERK1/2 signaling pathway in SNP-stimulated chondrocytes.

**Discussion**

In this study, we first demonstrated that ASP protects chondrocytes from SNP-induced apoptosis through the activation of autophagy. It has been reported that levels of nitrite, a stable end product of nitric oxide (NO) metabolism, are elevated in serum and synovial fluid samples of OA[22]. In addition, synovial cells and cartilage cells in OA produced large amounts of NO[23]. The negative effects of NO include enhancement of matrix metalloproteinase activity, a reduction in interleukin-1 receptor antagonist synthesis and the promotion of apoptosis, which are closely associated with the occurrence and development of OA[24-26]. Thus, we chose SNP to induce NO-related apoptosis, and discovered that chondrocyte viability declined in a time and dose-dependent manner.

p53, which targets p21, can inhibit cell growth by blocking the cell cycle and induction of cell cycle arrest in the G0-G1 phase when the p53-p21 signaling pathway is activated[27]. One study has reported the role of p21 in potentiating cancer stem cells(CSC) via activation of canonical Wnt signaling due to TCF1/Cyclin D1 upregulation. This results in the promotion of self-renewal and leads to the proliferation of CSC/progenitor cells that fuels tumor growth and metastasis[28]. Our study showed that ASP promotes chondrocyte proliferation via downregulation of p21 and upregulation of CyclinD1 expression, which were consistent with previous studies. (Fig 2)

Since SNP has been reported to induce mitochondrial apoptosis[29], alterations in mitochondrial membrane potential, and associated gene and protein expression levels, were investigated. Decreased mitochondrial membrane potential leads to increased membrane permeability, and mitochondrial membrane permeability may be regulated by the Bcl-2 family[30]. Conversely, the Bax protein increases the permeability of the mitochondrial membrane by forming activated oligomers, promoting Cyt-C release and ultimately inducing apoptosis[31]. In our study, increased Bax expression and decreased Bcl-2 are involved in SNP-induced apoptosis, which was consistent with previous studies. (Fig 3)

Cetrullo et al.[32]demonstrated that oxidative stress inhibits the expression of autophagy-related proteins in chondrocytes and promotes apoptosis. Previous studies have shown OA cartilage produces a larger amount of NO compared with normal cartilage[33]. In addition, NO suppresses cartilage matrix synthesis and enhances degradation[34, 35]. Consequencely, accumulating evidences showed that antioxidants,
like N-acetyl cysteine (NAC) and eicosapentaenoic acid (EPA), can attenuate chondrocytes apoptosis caused by radical oxygen (ROS) and nitrogen (RNS) species in OA or after traumatic cartilage injury\[36, 37\]. Additionally, oxidative stress can lead to mitochondrial dysfunction, mitochondrial DNA damage, telomere instability, cell senescence, and anabolic dysfunction\[38, 39\]. As a result, more and more researchers are focusing on the therapeutic effect of antioxidants on OA. Polyphenols, like carnosol, hydroxytyrosol, curcumin, and genistein have been revealed to have therapeutic potential in arthritis with regard to their antioxidant and anti-inflammatory features\[40\]. Another extensively investigated antioxidant is melatonin. It is reported to play a protective role in OA due to its abilities to regulate apoptosis, ER stress and mitochondrial activity \[41\].

Our study suggested that SNP, an oxidative stress inducer, inhibits autophagy levels, which was confirmed by Atg5, Beclin-1, LC3I/II and p62 expression. LC3, an important constituent of autophagosomes, also plays an essential role in the fusion of autophagosomes with lysosomes for the degradation of damaged organelles by lysosomal enzymes\[16\]. LC3II has the ability to determine membrane curvature, thus has a role in regulating the size of the autophagosome\[42\]. Expression level of LC3II could be affected both genetically by autophagy-related genes like Beclin-1 and SQSMT1/p62, or chemically by 3-MA and CQ\[43-46\]. Beclin-1 allows nucleation of the autophagosome and the conversion of LC3B-I to LC3B-II through lipidation by an ubiquitin-like system to form the autophagosome\[43\]. SQSMT1/p62 has a receptor function to recognize ubiquitinated proteins that need to be removed from the cytoplasm during autophagy; its amount is generally considered to inversely correlate with autophagic activity\[44\]. 3-MA inhibits autophagy by blocking autophagosome formation via the inhibition of class III PI3K, whereas CQ block the autophagic flux by decreasing the fusion process of autophagosome and lysosome\[45, 46\]. In our study, we first demonstrated ASP increased autophagy-related protein LC3II, Atg5, Beclin-1 expression, indicating ASP promoted autophagy of chondrocyte. Additionally, we also detected the expression of p62, an autophagy substrate known to recruit ubiquitinated proteins and gets degraded as autophagic flux progress. The results suggest that ASP decreases p62 accumulation induced by SNP and CQ treatment further enhances p62 expression, indicating ASP not only enhanced LC3II expression but also activated autophagic flux. To further explore the correlation between ASP-induced autophagy and SNP-induced apoptosis, we used 3-MA as an autophagy inhibitor to block autophagy initiation. The results suggested that the protective effect of ASP against SNP-induced apoptosis was partly inhibited. All these findings confirm that ASP-induced autophagy plays an important role in preventing SNP-induced apoptosis in chondrocytes.

Autophagy is regulated by multiple signaling pathways in chondrocytes. Inhibition of NF-κB pathway promotes the expression of Atg5, Atg7, and LC3II, and activates autophagy\[47\]. Shi et al. showed that autophagy levels were significantly inhibited after activation of the p38 signaling pathway in osteoarthritis\[48\]. In addition, Li X et al. reported that the ERK1/2 signaling pathways activation was involved in chondrocyte autophagy, which protected chondrocyte from apoptosis\[49\]. Moreover, pathways such as AMPK/mTOR\[50\], PI3K/AKT\[51\], and AKT/mTOR\[52\] were associated with autophagy in chondrocytes. We found that ASP-induced autophagy plays a critical role in the prevention of SNP-induced apoptosis via the ERK1/2 signaling pathway (Fig 5). In keeping with the previous results, we
discovered that ASP induced increased expression of p-ERK1/2 accompanying with a high expression of LC3 in chondrocytes treated with SNP and downregulated the expression of p62 simultaneously. For further proof, the application of SCH772984, an inhibitor of ERK1/2, significantly blocked the ASP-induced autophagy as it decreased the expression of LC3 and restored SNP-induced expression of p62. Taken together, our results clearly demonstrated that the modulation of ERK1/2 plays a key role in the regulation of autophagy in chondrocytes treated with ASP and SNP.

Nonetheless, there are two major limitations in this study that could be addressed in future research. First, this study was based on the primary cultured chondrocytes which were thought to lost their characteristic in vivo in synovium niche. The reality of chondrocytes apoptosis due to OA progression would be more complicated. Secondly, besides of oral intake of ASP, whether there are more efficient ways to treat OA with ASP still needs to be looked into.

Conclusion

ASP decreases SNP-induced cartilage damage and enhances chondrocyte proliferation in a CyclinD1- and p21- dependent manner. Besides, ASP activates autophagy to protect chondrocytes from apoptosis, via ERK1/2 signal pathway. In addition, inhibitors of autophagy and ERK1/2 pathway significantly abolishes the anti-apoptotic function of ASP against SNP. These findings indicate that ASP might be a promising natural compound for the treatment of OA.

Abbreviations

OA: Osteoarthritis; ASP: Polysaccharide from angelica sinensis; SNP: sodium nitroprusside; NO: nitric oxide; AA: ascorbic acid; LC3: microtubule-associated protein 1 light chain 3; SQSTM1/p62: sequestosome 1; Beclin-1: autophagy regulated protein; ERK1/2: extracellular signal-regulated kinase1/2; SCH772984: inhibitor of ERK1/2 signal pathway; RIPA: radioimmunoprecipitation assay buffer; DMEM: Dulbecco's modified Eagle's medium; PVDF: polyvinylidene fluoride; CSC: cancer stem cells; CyclinD1: cell cycle regulatory proteins D1; MAPK: mitogen-activated protein kinase; Raf: raf kinase, effector of Ras; Cyt-c: cytochrome c oxidase; Atg5: autophagy-related 5; Atg7: autophagy-related 7; Bax: BCL2-associated X protein; Bcl-2: BCL2 apoptosis regulator; 3-MA: 3-Methyladenine; CQ: chloroquine; mTOR: mechanistic target of rapamycin kinase; AKT: serine/threonine kinase

Declarations

Availability of data and materials

The datasets used in the present study are available from the corresponding authors on reasonable request.

Conflicts of interest:
The authors declare no conflict of interest.

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**Authors’ contributions**

Yuji Wang and Andre J. Van Wijnen contributed to the conception and design of the study. Chao Xu and Su Ni performed the main experiments. Shijie Jiang, Chao Zhuang and Ruixia Zhu contributed to the drafting of the article. Gongyin Zhao completed the acquisition or preparation of clinical samples. Chao Xu, Su Ni, Chenkai Li, and Liangliang Wang contributed to the analysis and interpretation of the data. Yuji Wang contributed to the critical revision and provided important intellectual feedbacks. All the authors read and approved the final manuscript.

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**Ethics approval and consent to participate**

All participants had signed a written informed consent prior to the subjects entering the study. The study was approved by the Nanjing Medical University Review Board and the file number is [2017] KY035-01.

**Consent for publication**

Not applicable

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**Figures**

**Figure 1**

(a) 12h  
(b) 24h  
(c) 48h  
(d)
Figure 1

SNP treatment resulted in a reduction of chondrocyte viability compared with the control group in a dose-dependent manner. (a-c). OA chondrocytes were cultured with various concentration of SNP (0.5 mg/ml, 1 mg/ml, or 2 mg/ml) for 12 hrs, 24 hrs, and 48 hrs. (d). OA chondrocytes were pretreated with different concentrations of ASP (50 μg/ml or 200 μg/ml), then incubated with SNP for 24 hrs. Cell viability was analyzed with MTS. The results were presented as the mean ± SD of three independent experiments (n=3). ** p<0.01, *** p<0.001, #p<0.0001, and statistical significance was determined by One-way ANOVA.
Figure 3

The protective effect of ASP on chondrocyte apoptosis. Chondrocytes were pretreated with ASP for 4 hrs then stimulated with SNP (1 mg/ml) for 24 hrs. (a-b). NC: Chondrocytes were cultured in medium without AA for 24 hrs. SNP: Chondrocytes were treated with 1 mg/ml SNP for 24 hrs. SNP+50ASP/SNP+200ASP: Chondrocytes were pretreated with different concentrations of ASP (50 µg/ml/200 µg/ml) for 4hrs then incubated with SNP for 24 hrs. Chondrocyte apoptosis was detected by DAPI staining and flow cytometry.
assays (quantified in c-d). (e). The level of Bcl2 and Bax were measured by western blot (quantified in f-g). Results are presented as the means ± SD of three independent experiments (n=3). * p<0.05, ** p<0.01, ***p<0.001, # p<0.0001 and statistical significance was determined by one-way ANOVA.