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Polysaccharide from angelica sinensis attenuates SNP-Induced Apoptosis in osteoarthritis Chondrocytes by Inducing Autophagy via ERK1/2 Pathway

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Research article

Keywords: Angelica sinensis polysaccharide, Autophagy, Chondrocytes, Osteoarthritis

Posted Date: May 22nd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-29285/v1

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Version of Record: A version of this preprint was published on January 30th, 2021. See the published version at https://doi.org/10.1186/s13075-020-02409-3.

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 dose 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 chondrocyte 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 chondrocyte in a pathological status, presenting as low chondrocyte vitality, abnormally high apoptosis and eventually lose of homeostasis of chondrocyte metabolism[3].

Many studies has 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 leads 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 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]. Cartilage degeneration and cell death caused by autophagy inhibition play a crucial role in the process of OA[18].

Additional evidence has reported that signaling pathway malfunctions in chondrocytes are involved in aging and joint diseases such as OA. Extracellular signal-regulated kinase1/2(ERK)is related to chondrocyte apoptosis, as reported by Shakibaei et al.[19].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 what 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 40mg/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 Changzhou Second People’s Hospital. All participants had signed a written

informed consent prior to the subjects entering the study. In addition, the study was approved by the Ethics Committee of Nanjing Medical University. 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 and finally cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 100 U penicillin, and 100 μg/ml streptomycin in a standard cell culture chamber containing 5% CO_2. Non-adherent cells were removed after 3 days. Adherent cells were split at a ratio of 1:2 until they grew to 90% confluence. Chondrocytes were used from passages 3 to 5 in subsequent experiments.

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 in 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 hours in a humidified, 5% CO2 atmosphere, and the absorbance at 490nm was recorded using an absorbance microplate reader (Elx808™ Bio-Tek Instruments, Winooski, VT)

Cell apoptosis detection by DAPI staining

Chondrocytes were seeded on sterile glass slides coated with gelatin, 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: AnnexinV/PI staining

$2*10^5$ chondrocytes were seeded in 6-well plates. 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

$2\text{-}5\times10^4$ chondrocytes were seeded on sterile glass slides precoated with gelatin. After the indicated treatment, cells were fixed in 4% paraformaldehyde at 4℃ for 15 min and blocked with PBS containing 5% normal goat serum and 0.3% Triton X-100 for 1 h 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 Table1.

Table1

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

Lists of antibodies.

mRFP-GFP-LC3 analysis

Chondrocytes were seeded in precoated slides with a density of 5×10^4 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.

Results

SNP dramatically inhibits chondrocyte viabitily whereas ASP rescues it.

In order to determine the best dosage and time period of SNP application, OA chondrocytes were incubated with three different concerntration 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 time and 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 and continued to decline till 48 hrs. Statistically significant differences in cell viability 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 as used to induce apoptosis for the following studies.

In aim 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 concentration of ASP, 50 and 200 μg/ml, remarkedly 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 D3 to D6 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 wes conducted on Day4. As shown in Figure 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 inbibited(Fig 2e). Collectively, these data suggest that ASP enhances the proliferation of chondrocytes in a p21- and CyclinD1-dependent manner in OA.

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 h and then treated with or without 1 mg/ml SNP for 24 hrs. For the negative control group, the cells were treated with SNP only. As we expected, SNP significantly increased the percentage of apoptotic chondrocytes compared to the control group (P<0.05), on the contrary, pretreatment with ASP significantly reduced the percentage of apoptotic chondrocytes (P<0.05). The DAPI staining results, which intuitively displayed the percentage of apoptoic cells, were consistent with flow cytometry assays (P<0.05) (Fig3 b). 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 varify 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 (P<0.05). On the other hand, ASP-pretreatment significantly increased the expression of Bcl-2 while decreased the expression of Bax (P<0.05) (Fig3 c-e).

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 Beclin-1, Atg5, microtubule-associated protein light chain 3B (LC3II/I) and p62 levels were evaluated by western blot. The results showed that SNP significantly deceased Beclin-1, Atg5 and LC3II expression (P<0.05). On contrary, when pretreated with ASP, increased LC3II, Beclin-1and Atg5 levels were found in chondrocyts(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 4c). Together, these data suggest ASP-induced autophagy rescued SNP-induced apoptosis in chondrocytes.

ERK1/2 signaling pathway is activated in ASP-induced autophagic chondrocytes.

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 autophay. To illuminate the molecular mechanism of anti-apoptosis effect of 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 LC3-II, Atg5, and Beclin-1

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 all of the three compounds(Fig 6a).In addition, ASP significantly increased bright LC3-II puncta compared with SNP group, while bright LC3-II 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 use mRFP-GFP-LC3 autophagic puncta to visualized 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[20]. In addition, synovial cells and cartilage cells in OA produce large amounts of NO[21]. 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[22–24]. 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[25]. One study has reported the role of p21 in potentiating cancer stem cells 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[26]. 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[27], 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[28]. Conversely, the Bax protein increases the permeability of the mitochondrial membrane by forming activated oligomers, promoting Cyt-C release and ultimately inducing apoptosis[29]. Increased Bax expression and decreased Bcl-2 are involved in SNP-induced apoptosis, which was consistent with previous studies. (Fig. 3)

Cetrullo *et al*.[30]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[31]. In addition, NO suppresses cartilage matrix synthesis and enhances degradation[32, 33]. Additionally, oxidative stress can lead to mitochondrial dysfunction, mitochondrial DNA damage, telomere instability, cell senescence, and anabolic dysfunction[34, 35]. 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]. Light chain 3-II has the ability to determine membrane curvature, thus has a role in regulating the size of the autophagosome[36]. 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[37]. 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[38]. 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 flu. 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[39]. Shi *et al*. showed that autophagy levels were significantly inhibited after activation of the p38 signaling pathway in osteoarthritis[40]. In addition, Li X *et al*. reported that the ERK1/2 signaling pathways activation was involved in chondrocyte autophagy, which protected chondrocyte from apoptosis[41]. Moreover, Pathways such as AMPK/mTOR[42], PI3K/AKT[43], and AKT/mTOR[39] 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.

Conclusion

ASP decreases SNP-induced cartilage damage and enhances chondrocyte proliferation in a cyclinD1- and p21- dependent manner. Besides, ASP activates autophagy to protects chondrocytes from apoptosis, via ERK1/2 signal pathway. In addition, inhibitors of autophagy and ERK1/2 pathway significantly abolishes the anti-apoptoic function of ASP against SNP. These findings indicates that ASP might be a promising natural compound for the treatment of OA.

Declarations

Availability of data and materials

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

Abbreviations: OA: Osteoarthritis; ASP: Polysaccharide from *angelica sinensis*; SNP: sodium nitroprusside; NO: nitric oxide; 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; 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

Conflicts of interest:

The authors declare no conflict of interest.

Acknowledgments:

We want to thank all the patients participated in our study. We are also thankful for the support of phase V 333 Project training funds of Jiangsu Province.

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.

Funding

This work was supported by grants from the National Natural Science Foundation of China(81171680 to Y.W.), the project of Jiangsu province (BE2015632 toY.W.), the Natural Science Foundation of the Jiangsu for Youth (BK20180182 to S.N.), the Changzhou Science and Technology Program (CJ20180057 to S.N ; QN201930 to C.L.), and China Postdoctoral Science Foundation (2019M651898 to S.N.).

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 Ethics Committee of Nanjing Medical University.

Consent for publication

Not applicable

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Figures

Figure1

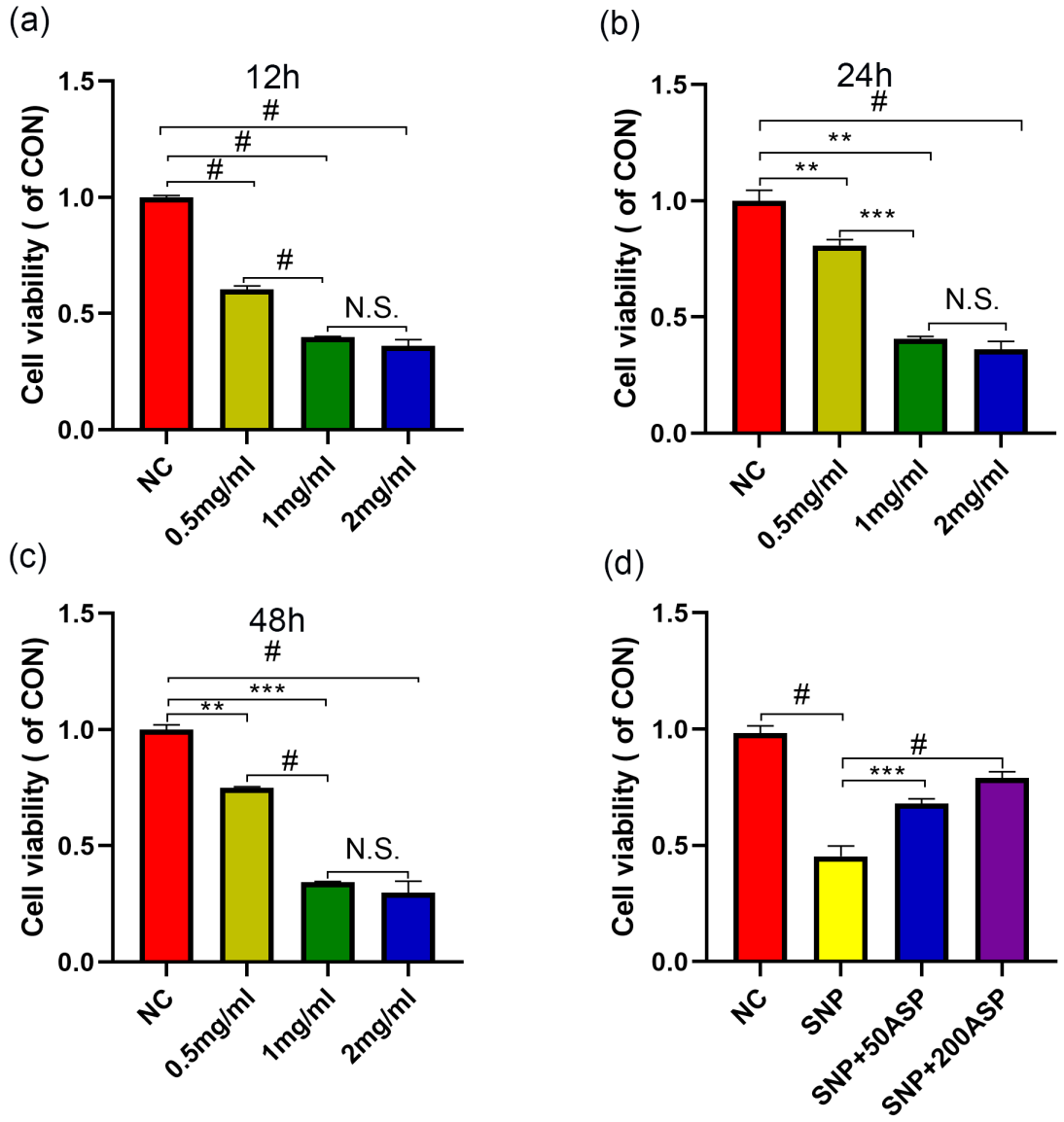

Figure 1

SNP treatment resulted in a reduction of chondrocyte viability compared with the con-trol group in a dose-dependent manner. (a-c). OA chondrocytes were cultured with var-ious concentration of SNP (0.5mg/ml ,1mg/ml, or 2mg/ml) for 12 hrs, 24 hrs, and 48 hrs. (d). OA chondrocytes were previously treated with

different concentrations of ASP (50 μg/ml or 200 μg /ml), then incubated with SNP for 24 hrs. Cell viability was ana-lyzed with MTS. The results were presented as the mean ± SD of three independent experiments. ** p<0.01, *** p<0.001, #p<0.0001, and statistical significance was de-termined by One-way ANOVA.

Figure2

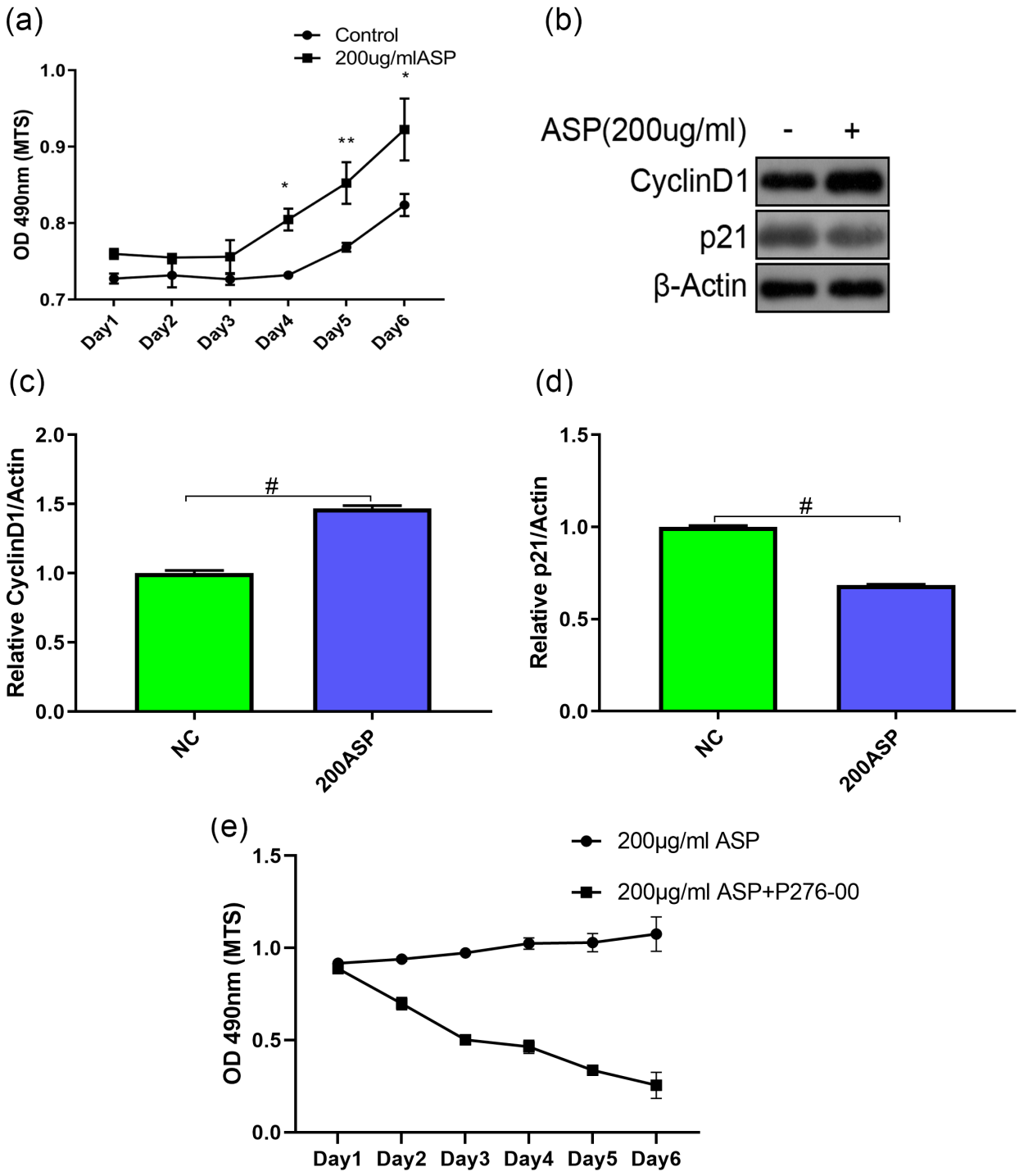

Figure 2

The effect of ASP induced chondrocyte proliferation. (a). Chondrocytes were treated with or without 200 μg /ml ASP for 6 days. The results are presented as the mean ± SD of three independent experiments. (b). Chondrocytes were collected after 4 days' expo-sure to 200 μg /ml ASP. Cyclin D1 and p21 were assessed by western blot. (quantified in c-d). (e). After treatment with the culture medium, 200 μg /ml ASP alone, cocultured with indicated concentration of P276-00 (2.5 μM) for 6 days, respectively. Data were presented as the mean ± SD of three independent experiments. * p <0.05, ** p<0.01, # p<0.0001, and statistical significance was determined by Student's t-test.

Figure3

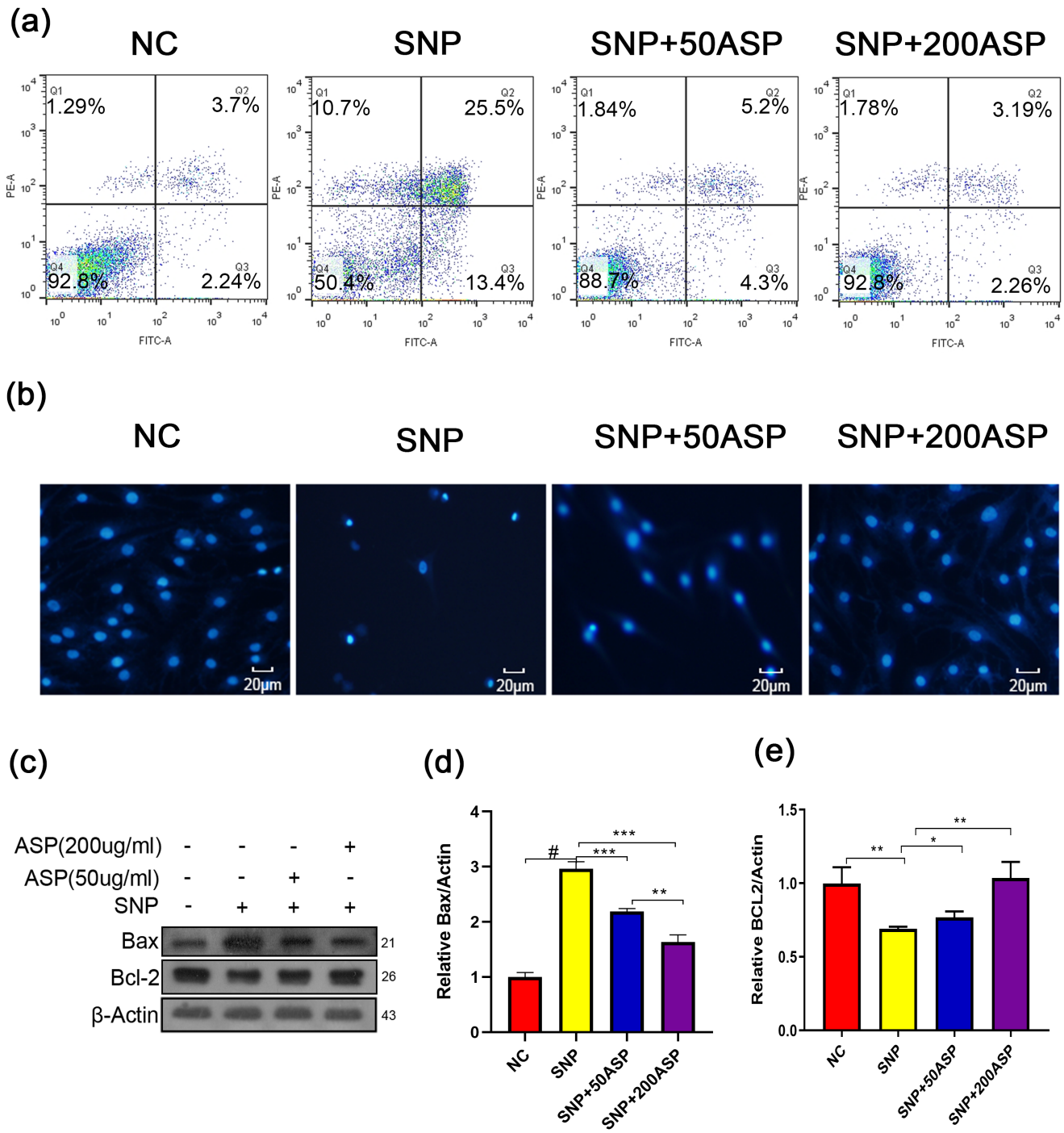

Figure 3

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: Chon-drocytes were cultured in DMEM-F12 for 24 hrs. SNP: Chondrocytes were treated with 1 mg/ml SNP for 24 hrs. SNP+50ASP/SNP+200ASP: Chondrocytes were pre-treated with different concentrations of ASP (50 μg/ml200 μg/ml) for 4hrs then incu-bated with SNP for 24 hrs. Chondrocyte apoptosis was detected by DAPI staining and flow cytometry assays. (c) The level of Bcl2 was measured by western blot. (d-e) Re-sults are presented as the means ± SD of three independent experiments. * $p<0.05$, ** $p<0.01$, ***$p<0.001$, and statistical significance was determined by one-way ANOVA.

Figure4

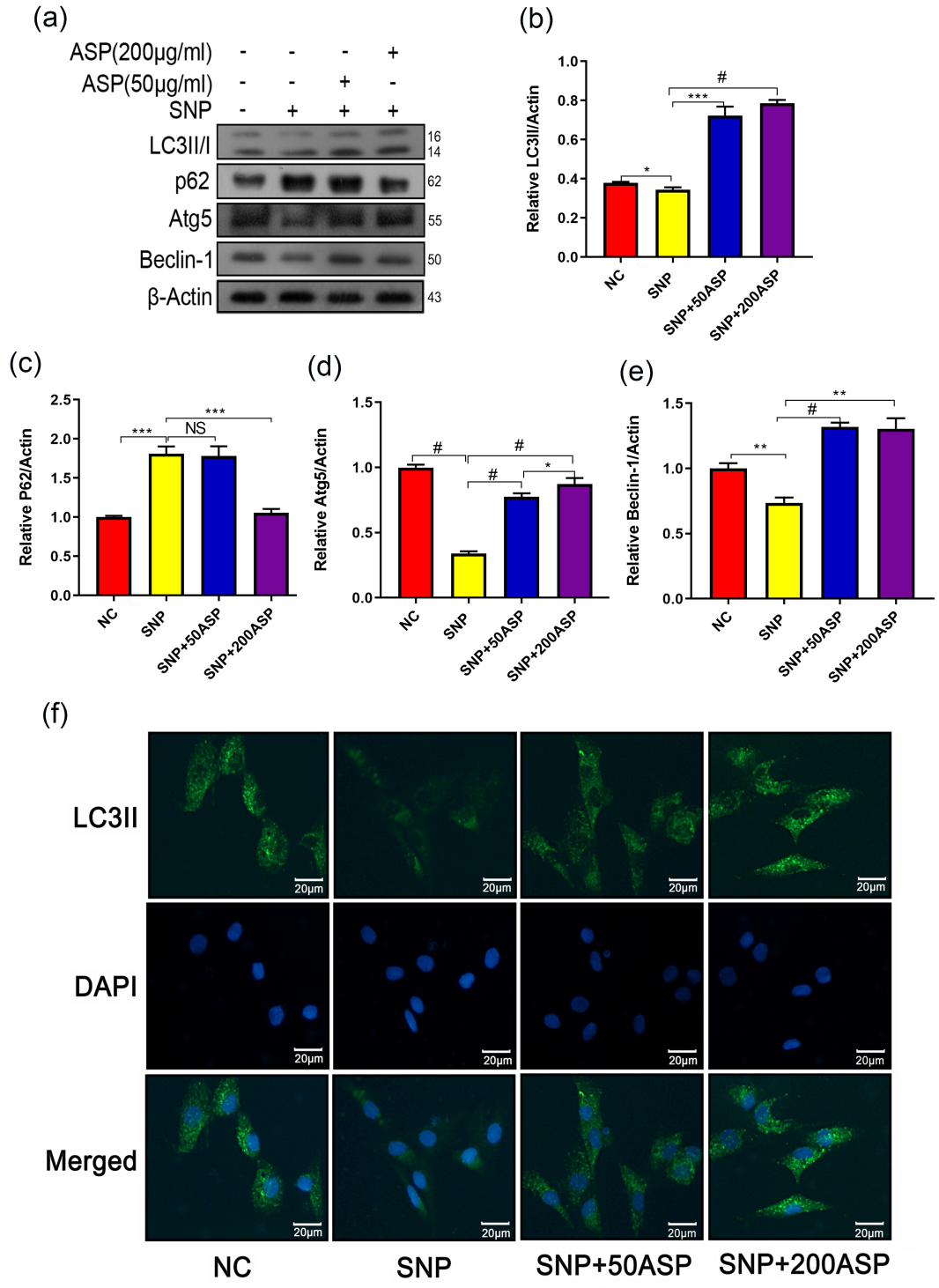

Figure 4

ASP induced autophagy in chondrocytes stimulated with SNP (1 mg/ml). (a). Autoph-agy-related proteins (LC3II/I, p62, Beclin-1, Atg5) were detected by western blot. (b-e). The quantitative results are presented as the means ± SD of three independent experi-ments. (f).LC3-II immunostaining. Significantly increased green bright puncta showed the formation of the autophagosomes. * p<0.05, ** p<0.01, ***p<0.001, # p<0.0001, and statistical significance was determined by by one-way ANOVA.

Figure5

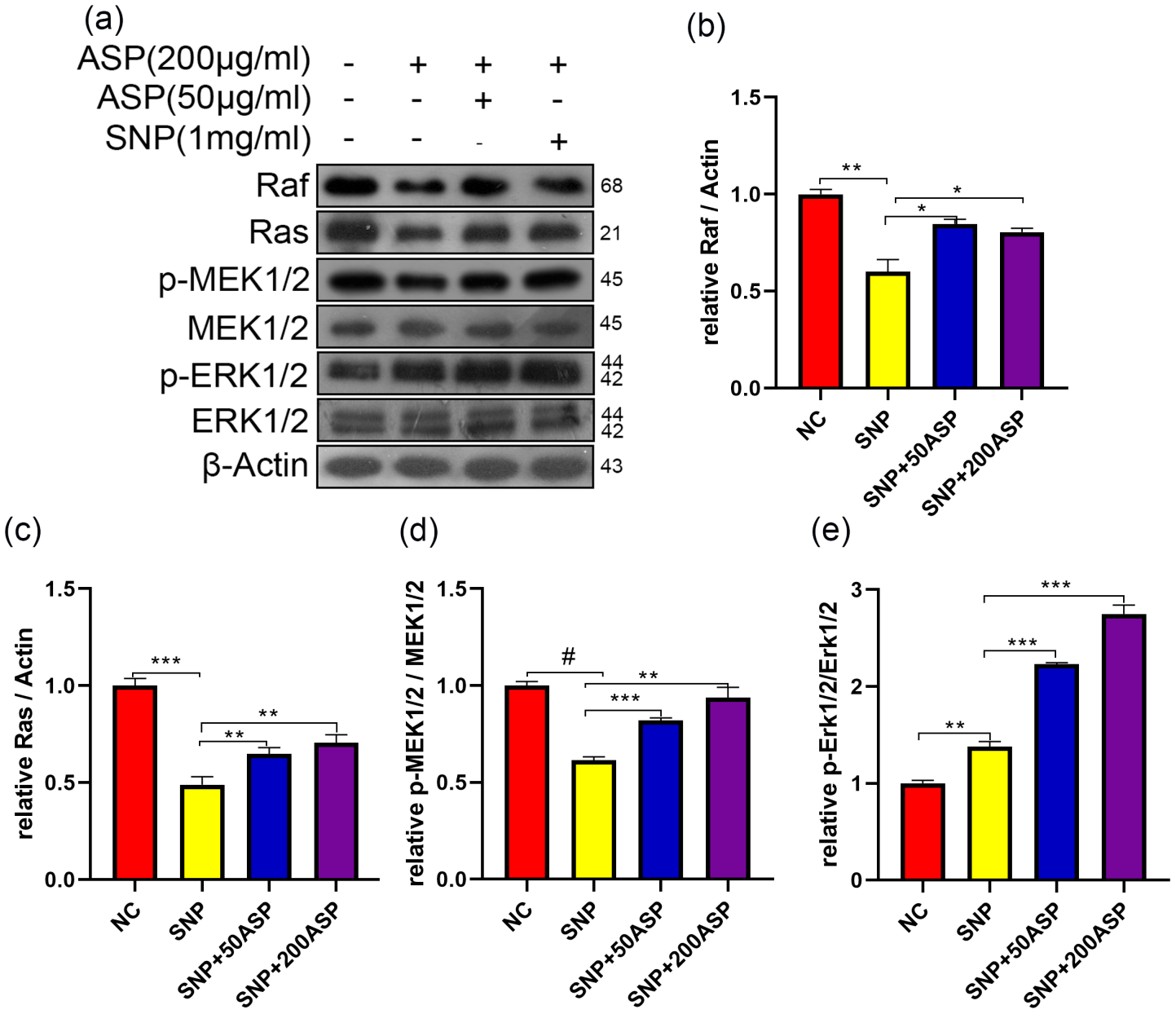

Figure 5

ASP activated ERK1/2 signaling pathway in chondrocytes stimulated with SNP. Chon-drocytes were pretreated with ASP for 4 hrs then stimulated with SNP (1 mg/ml) for 24 hrs. (a-b). The level of Raf, Ras, p-MEK1/2 and p-ERK1/2 in chondrocytes after stim-ulation were measured by western blotting. (c-f). Quantitative analysis of Raf, Ras, p-MEK1/2 and p-ERK1/2 in chondrocytes. The data are presented as the mean ± SD of three independent experiments. # $p<0.0001$, and statistical significance was determined by by one-way ANOVA.

Figure6

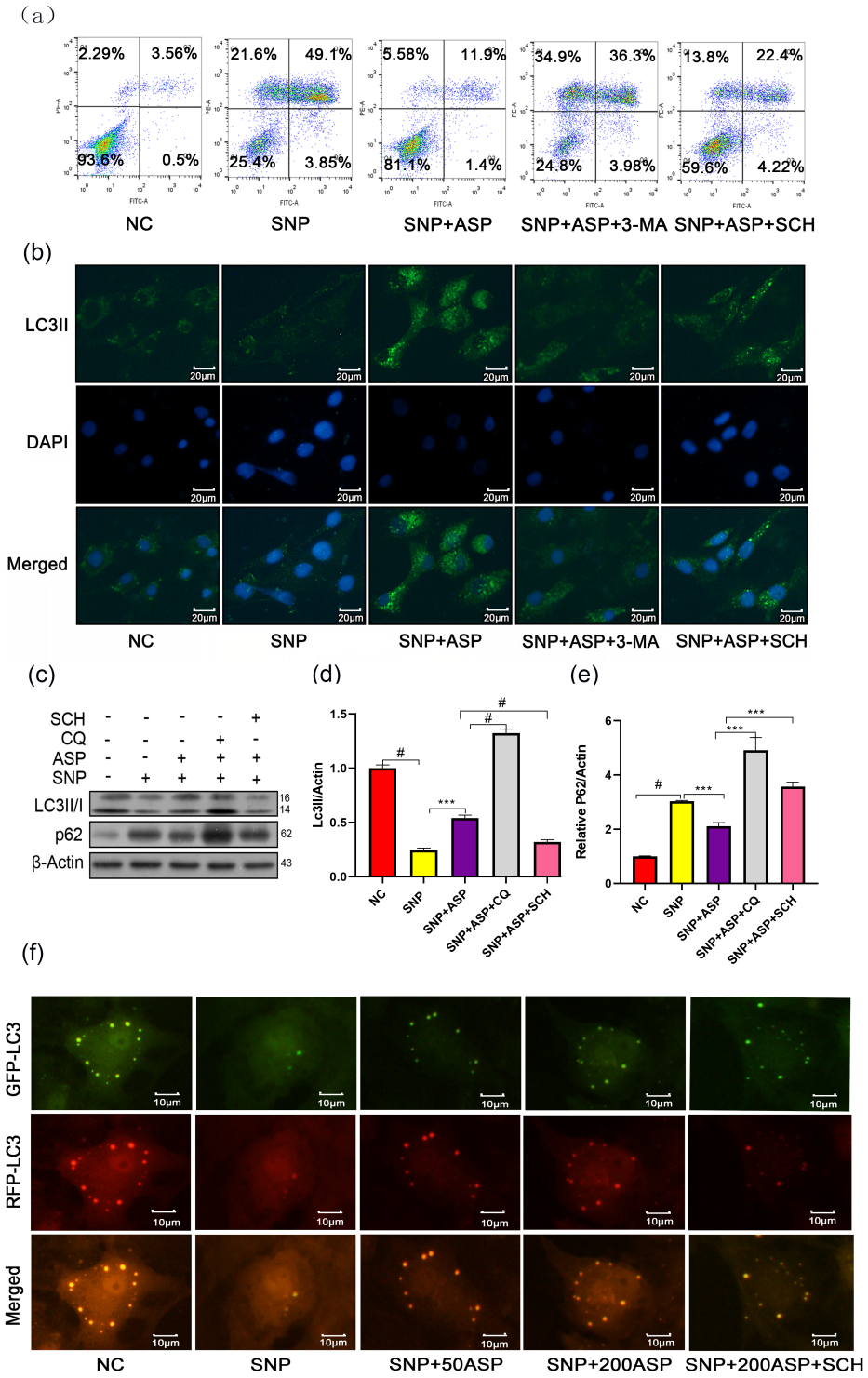

Figure 6

ASP diminished chondrocyte apoptosis via activation of autophagy. Chondrocytes were pretreated with 3-MA (10 μM), CQ (20 μM), SCH772984 (10 nM) for 4 hrs before ASP treatment followed by SNP (1 mg/ml) for 24 hrs. (a).Chondrocyte apoptosis was measured by flow cytometry. (b).The formation of autophagosome was detected by LC3-II immunostaining. (c). Levels of LC3II/I and p62 in chondrocytes after stimula-tion were measured by western blotting. (d). Quantitative analysis of LC3II in chon-drocytes.

(e). Quantitative analysis of p62 in chondrocytes. (f). Representative micros-copy images of tfLC3 puncta in chondrocytes stained for autophagosomes (green) and autolysosomes (red) for colocalization as an indicator of autophagy. Autophagy in-creased in the ASP treated groups in a dose-dependent manner. The data are presented as the mean ± SD of three independent experiments. ***P<0.001 #P<0.0001, and statistical significance was determined by by one-way ANOVA.