Andrographolide sodium bisulfate-induced apoptosis and autophagy in human proximal tubular endothelial cells is a ROS-mediated pathway

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\textbf{A B S T R A C T}

\textbf{Background and aims:} The nephrotoxic mechanisms of andrographolide sodium bisulfate (ASB) remain largely unknown. This study attempted to explore the mechanism of ASB-induced nephrotoxicity using human proximal tubular endothelial cells (HK-2).

\textbf{Methods:} For this study HK-2 cells were treated with rising concentrations of ASB. Their survival rate was detected using MTT assay and ultrastructure was observed with electron microscopy. l-Lactate dehydrogenase (LDH) assay was followed by examination of mitochondrial membrane potential (MMP). Reactive oxygen species (ROS) was detected using different methods and apoptosis/autophagy related proteins were detected using immunoblotting.

\textbf{Results:} We found that ASB inhibited HK-2 cell proliferation and decreased cell survival rate in a time and dose-dependent manner (\(P < 0.05, P < 0.01\), respectively). With increasing ASB concentration, cell structure was variably damaged and evidence of apoptosis and autophagy were observed. MMP gradually decreased and ROS was induced. The expression of JNK and Beclin-1 increased and activation of the JNK signaling pathway were seen. Apoptosis was induced via the mitochondrial-dependent caspase-3 and caspase-9 pathway, and autophagy related protein Beclin-1 was enhanced by ASB.

\textbf{Conclusion:} The data show that ASB induces high levels of ROS generation in HK-2 cells and activates JNK signaling. Furthermore, ASB induces cell apoptosis via the caspase-dependent mitochondrial pathway, and induces cellular autophagy, in part by enhancing Beclin-1 protein expression.

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1. Introduction

Andrographolide sodium bisulfate (ASB) is a kind of soluble derivative composed of andrographolide and sodium bisulfate and belongs to diterpene lactone compounds. Andrographolide injection (Lianzbizhi injection) is an antibacterial and anti-inflammatory agent and has been widely used in the treatment of various infectious diseases and malignant tumor in China, known as “natural antibiotics” (Hu et al., 2009). During the period of application of the Lianzbizhi injection, adverse events were continuously reported. From 1998 to 2005, 50 cases of Lianzbizhi injection were enrolled in a case report database of the National Adverse Drug Reaction Monitoring Center, and 17 cases of acute renal damage were reported (Monitoring, 2004). The clinical case reports showed that Lianzbizhi injection-induced acute renal damage was characterized by a short onset time after the initial administration. Predominant symptoms included soreness of loins, lumbar and increased levels of creatinine and urea nitrogen (Hu et al., 2009). Nephrotoxicity of ASB remains a serious obstacle in its clinical application, and systematic or comprehensive study of ASB-induced nephrotoxicity is a high priority to guide clinical safety in ASB use.

Reactive oxygen species (ROS) mainly include superoxide anion (O$_2^-$), hydroxyl radical (OH$^-$), and peroxyl radical (RO$^\cdot$), and it is comprehensively involved in intracellular signal transduction. Under normal condition, ROS can stimulate mitosis, induce cellular senescence and regulate apoptosis (D’Autreux and Toledano, 2007). It was previously reported that high ROS levels in the cell was tightly associated with programmed cell death. ROS can injure mitochondria and trigger Caspase-dependent apoptosis (Gobe and Crane, 2010). By contrast, ROS can provoke accumulation of autophagosomes by affecting lysosome function, and can further induce autophagic cell death (Moore, 2008; Scherz-Shouval and Elazar, 2007). These findings indicate that changes in the intracellular oxidation environment are closely associated with both apoptosis and autophagy.

Mitochondria provide energy for vital cellular activities, and are mainly a site for the generation and consumption of the effect of ROS. Hence, mitochondria play a key role in cellular apoptosis (Chen et al., 2007). Aberrant mitochondrial structure and function caused by many types of pathological factors decrease the efficacy of oxidative phosphorylation in mitochondria and increase ROS production. The oxidized unsaturated fatty acids in mitochondrial membranes reduce the membrane mobility of mitochondria. Even the oxidized functional proteins on mitochondrial membranes cause dysfunction of oxidative phosphorylation. Furthermore, more ROS can be generated, and a pernicious circle can result (Ott et al., 2007). A decrease in MMP was one of the earliest events of increased permeability of the mitochondrial inner membrane and which induced apoptotic signal transduction pathways. Increased ROS can block the function of sodium pumps, and promote the retention of intracellular sodium. The mitochondrial swelling would increase the permeability of the mitochondrial membrane, and subsequent decrease in MMP. The Caspase-3 family was activated after release of cytochrome C from the mitochondrial membrane to the cytoplasm, and thus induced cellular apoptosis (Fan et al., 2005).

Our previous studies have found increased levels of blood urea nitrogen (BUN), serum creatinine (Scr), and malondialdehyde (MDA) and decreased levels of superoxide dismutase (SOD) and myeloperoxidase (MPO) in mouse models treated with multiple doses of ASB (Lu et al., 2011). Previous proteomic studies showed that Peroxiredoxin-6 (Prdx6) expression was upregulated, indicating that ASB can aberrantly change the oxidation-reduction system of an organism (Lu et al., 2011).

Previous literatures reported that excessive accumulation of ROS can start Caspase-mediated apoptotic signaling, and can induce cellular autophagy under certain conditions (Chipuk et al., 2006; Scherz-Shouval and Elazar, 2011). ROS is a strong signal for mitogen activated protein kinases (MAPKs), particularly for JNK (Torres and Forman, 2003). JNK can phosphorylate Bcl-2 protein family members and by doing so can promote opening of the permeability transition (PT) of mitochondria. Furthermore, cytochrome C in the mitochondria leaked into the cytoplasm, and promoted cellular apoptosis via Caspase-3 and Caspase-9 (Kharbanda et al., 2000). Additionally, JNK was involved in Beclin-1 (mammalian homologue of Atg6) mediated cellular autophagy (Finkel, 2003).

In the present study, we attempted to see if ASB could induce ROS generation in HK-2 cells, activate the JNK-mediated signaling pathway, and induce apoptosis via the Caspase-dependent mitochondrial pathway. Simultaneously, we also looked at the potential for ASB to induce cellular autophagy by enhancing expression of Beclin-1.

2. Materials and methods

2.1. Cell culture

HK-2 cells were purchased from the Cell Center of the Chinese Academy of Medical Science (Beijing, China), and the cells were previously derived from the American Type Culture Collection (ATCC, USA). HK-2 cells were cultured and passed in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 supplemented with 10% fetal calf serum (FCS), 200 units (U)/mL of penicillin sodium and 200 U/mL of streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO$_2$ in air.

2.2. Experimental groups

ASB presents as a white powder, and its purity is greater than 99%. ASB (lot number: 20100501) was provided by Zhejiang JiuXu Pharmaceutical Co., Ltd. (Zhejiang, China), and was prepared using DMEM/F12 culture medium containing 2% FCS. The cells in the experimental groups were treated with 0 mmol/L, 7.0 mmol/L, 14.0 mmol/L, 29.0 mmol/L and 57.0 mmol/L of ASB. Cells in the control group were treated with an equal volume of DMEM/F12 medium containing 2% FCS.
2.3. MTT assay

HK-2 cell survival rate was detected using the MTT assay after ASB treatment (Ye et al., 2010). At the logarithmic phase of growth, HK-2 cells were conventionally digested and prepared as a single cell suspension, after which the cells were seeded into a 96-well plate at a density of $2 \times 10^5$ cells/mL. The control group was set, and the cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ in air. The medium was discarded when cells had attained 70–80% confluence. Medium containing 0 mmol/L, 7.0 mmol/L, 14.0 mmol/L, 29.0 mmol/L and 57.0 mmol/L of ASB was added into each well in a volume of 100 µL/well. Four replicated wells were set up for each group, and the cells in these four wells were treated with ASB for 8, 16, 24 and 48 h, respectively. After treatment with ASB, 20 µL of MTT reagent (Sigma, Shanghai, China) was added into each well for 4 h. The culture medium was discarded, and 150 µL of dimethyl sulfoxide (DMSO, Sigma) was further added. The crystals of formazan product were then dissolved by oscillating for 10 min. The optical density (OD) value for each well was detected using an enzyme linked immunosorbent assay (ELISA) reader (Bio-tek, Synergy2, USA) at a wavelength of 490 nm. The experiments were performed in triplicate. The cell survival rate was calculated, and the optimal administration time was determined. Cell survival rate (%) = (OD of administration group – OD of blank group)/(OD of control group – OD of blank group) × 100%.

2.4. LDH release assay

The LDH release assay was used to evaluate the stability of the cell membrane. Cell suspensions were prepared after conventional digestion, and 100 µL cell suspension was seeded into a 96-well plate at a density of $1 \times 10^5$ cells/mL. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ and the medium was discarded at 70–80% confluence. The cells were treated with 0 mmol/L, 7.0 mmol/L, 14.0 mmol/L, 29.0 mmol/L, or 57.0 mmol/L of ASB for 48 h and 1-lactate dehydrogenase (LDH, EC number 1.1.1.27) activity of the supernatant in each group was detected using LDH release kit (CytoTox-ONE™ Homogeneous Membrane Integrity Assay, Promega, Beijing, China), and the background and maximum controls were set according to the manufacturer’s instructions. A microplate reader (Bio-Rad Ltd., Japan) was employed as excitation and emission wavelength was 560 and 590 nm, respectively. Cellular toxicity = 100 × [(fluorescence value of ASB treated group – fluorescence value of 0 mmol/L ASB treated group)/(fluorescence value of maximum control – fluorescence value of background)].

2.5. HK-2 cell ultrastructure

Cells were conventionally digested and prepared as single cell suspensions and seeded into a 25 cm$^2$ culture flask at a density of $2 \times 10^5$ cells/mL. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ in air and the medium was discarded at 70–80% confluence. Cells were treated with 0 mmol/L, 7.0 mmol/L, 14.0 mmol/L, 29.0 mmol/L and 57.0 mmol/L of ASB for 48 h, following which the cells in the different groups were harvested. Cells were washed with pre-chilled phosphate buffered salts solution (PBS) and centrifuged at 670 g for 15 min (Thermo Scientific MR23i, Thermo Fisher, USA). Furthermore, the cells were fixed with pre-chilled 2.5% glutaraldehyde at 4 °C overnight, and the cells were further fixed in 1% osmic acid at 4 °C. Next, cells were sequentially dehydrated with 50%, 70%, 90% and 100% acetone for 15 min each, and the dehydration was performed in triplicate. Next, cells were immersed in acetone/epoxide resin (1:1) for 2 h, and further immersed in pure epoxide resin overnight. The immersed cell aggregates were embedded into the pure epoxide resin at 72 °C for 8 h. Subsequently, 60–90 nm ultrathin sections were obtained using a 200-mesh copper grid, and the sections were stained with uranyl acetate-lead citrate staining complex. Finally, the ultrastructure of the mitochondria was observed using a transmission electron microscope (JEM-1230, Tokyo, Japan).

2.6. Mitochondrial membrane potential detection

An mitochondrial membrane potential (MMP, $\Delta \psi_m$) detection kit from Beyotime Institute of Biotechnology (Shanghai, China) was used to rapidly and sensitively detect MMP changes in the cells, tissues or purified mitochondria using a JC-1 fluorescence probe assay. The decreased MMP can be detected by distinguishing the transformation from red to green fluorescence, and the transformation from red to green fluorescence can be used as an index for the early stages of cellular apoptosis (Ma et al., 2010). Cells in the different groups were treated with 10 µM CCCP for 20 min. These cells were washed with PBS, and centrifuged at 670 × g for 5 min. The resulting cell precipitation was resuspended in 0.5 mL of cell culture medium, and 0.5 mL of JC-1 staining solution was added and mixed. The mixture was incubated at 37 °C for 20 min, and then centrifuged at 670 × g for 4 min at 4 °C, and the supernatant was discarded. The cells were washed twice with 1 × JC-1 staining buffer and centrifuged at 4 °C for 4 min at 670 × g. The cell pellet was retained, and the supernatant was discarded. Cells were resuspended with 1 × JC-1 staining buffer, and analyzed by flow cytometry (FACSAria, BD, USA). The maximum excitation wavelength and emission wavelength of the JC-1 monomer were 514 and 529 nm, respectively. The maximum excitation wavelength and emission wavelength of JC-1 aggregates were 585 and 590 nm, respectively.

2.7. ROS detection in cells

2.7.1. CM-H2 DCFDA staining

Cells were conventionally digested and prepared as cell suspensions, and the cells were seeded into a 6-well plate. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ in air and the medium was discarded at 70–80% confluence. Cells were treated with ASB for 48 h, and the medium was discarded. Cells were washed with PBS, and the oxygen radicals were labeled by adding 10 µmol/L of CM-H2 DCFDA (Invitrogen, California, USA) in a volume of 1 mL/well. Cells were stained at 37 °C for 30 min, and washed with PBS. The cells were observed and photographed at an
excitation wavelength and emission wavelength of 480 and 520 nm, respectively, using a laser scanning confocal microscope (Nikon, Japan).

2.7.2. Activity of SOD
The activity of SOD was determined using commercially available WST-1 assay kits (SOD Assay Kit-WST, Product Code: S311-08, S311-10, Dojindo Molecular Technologies, China). All procedures complied with the manufacturer’s instructions. The assay was based on its ability to inhibit the oxidation of hydroxylamine by O$_2^-$ produced from the xanthine-xanthineoxiase system. One unit of SOD activity was defined as the amount that reduced the absorbance at 450 nm by 50%. The results of SOD assay were expressed as units per milligram protein (U/mg protein).

2.7.3. GSH consumption
GSH concentration was measured by the method of Tietze (Tietze, 1969) with minor modification. Briefly, cells were washed twice with cold PBS and scraped down by scraper in sodium phosphate buffer 125 mmol/L containing edetic acid 6.3 mmol/L (pH 7.5). After sonication, 20 μL was taken for protein assay, then 1% trichloroacetic acid was added to the lysates, and the mixture was allowed to precipitate for 2 h at 4 °C. After centrifugation at 10,000 × g for 15 min, protein free lysates were obtained. The reaction mixture for determination of GSH content consisted of lysates and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) 6 mmol/L. The absorbance at 405 nm was monitored for 6 min using a microplate reader (Bio-Rad Ltd., Japan). The content of GSH was calculated from the change in the rate of absorbance on the basis of a standard curve.

2.8. Caspase-3 and Caspase-9 activity detection
Caspase-3 and Caspase-9 activity detection kits were obtained from Promega (Fitchburg, USA). HK-2 cells at a logarithmic phase of growth were conventionally digested and prepared as single cell suspensions, and the cells were seeded into a 96-well plate. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ and the medium was discarded at 70–80% confluence. Medium containing 0 mmol/L, 7.0 mmol/L, 14.0 mmol/L, 29.0 mmol/L and 57.0 mmol/L of ASB was added into each well in a volume of 100 μL/well. Four replicate wells were set for each group, and 100 μL of Caspase-3 or Caspase-9 detection reagent was added into each well after 24 h and 48 h of ASB treatment. The reagent was photobiologically incubated with cells at 25 °C for 1 h. The luminescence value was detected using an ELISA reader from BioTek. The luminescence value of Caspase-3 or Caspase-9 found in normal cells at 24 h was used as a basic background/standard value, and the alteration of Caspase-3/-9 found in the cells that were treated with different concentrations of ASB was compared. Finally, the chart was obtained using GraphPad Prism software.

2.9. Western immunoblotting analysis
Cells in the different groups were harvested, and the total proteins were extracted using lysis buffer from Beyotime (20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na$_3$VO$_4$ and leupeptin). Mitochondria and cytoplasmic proteins were extracted using a mitochondria isolation kit from Beyotime. Protein concentrations were detected using a BCA assay (Beyotime, Shanghai, China). Firstly, 30 μg of proteins were analyzed using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and cytochrome C expression in the cytoplasm and mitochondria, JNK, Beclin-1, Bcl-2 and Bax expression in cytoplasm were analyzed. The proteins were transferred onto a nitrocellulose membrane, and the membrane was blocked with 5% skimmed milk in TBST buffer. Rabbit anti-Bcl-2 (N-19, sc-492), goat anti-cytochrome C (C-20, sc-8385), rabbit anti-Bax (N-20, sc-493), rabbit anti-JNK (FL, sc-572), rabbit anti-Actin (H-196, sc-7210), horseradish peroxidase (HRP) labeled goat anti-rabbit and rabbit anti-goat secondary antibodies (Santa Cruz Biotechnology, Delaware, USA) and rabbit anti-Beclin-1 (D40C5, CST, Trask Lane Danvers, USA) were obtained. The membrane was incubated with primary and secondary antibodies at 4 °C and room temperature overnight and for 2 h, respectively. The membrane was developed using an enhanced chemiluminescence (ECL) kit from Santa Cruz Biotechnology. Finally, the images were obtained using Mini-PROTEAN gel imaging system. Actin

Fig. 1 – Changes in HK-2 cell survival rates after the cells were time-dependently treated with different concentrations of ASB (data described as mean ± SD, n = 3). (A) Chemical structure of andrographolide sodium bisulfate; (B) Alterations seen in HK-2 cell survival rates after the cells were time-dependently treated with different concentrations of ASB. *Data values were compared with 0 mmol/L of ASB, P < 0.01; # and ## represents the comparisons with 24 h treatment, P < 0.05 and P < 0.01.
Fig. 2 – Effect of ASB on the rate of LDH release from HK-2 cells (data described as mean ± SD, n = 3). HK-2 cells were treated with 0, 7, 14, 29 and 59 mmol/L of ASB for 48 h and cytotoxicity was expressed as LDH leakage from the cell to culture medium. **P<0.01, as compared with the control group.

was used as a loading control, and protein expression was quantified by optical density obtained using ImageJ software program.

2.10. Statistical analysis

Values were expressed as the means ± SD. Data was using one-way ANOVA with GraphPad 5 software program, P<0.05 was considered significant.

3. Results

3.1. Effect of ASB on HK-2 cell survival rate

Cell survival rate gradually decreased with the duration of administration time and increase in drug concentration. Compared with the 0mmol/L group, the survival rate in the 7.0 mmol/L group was 98.01% after 48 h of treatment (P>0.05). Compared with the normal group, the survival rates in the 14.0 mmol/L, 29.0 mmol/L and 57.0 mmol/L groups gradually decreased (P<0.05 and P<0.01 respectively). Compared with 8 h, 16 h and 24 h of treatment, a more obvious decrease in cell survival rate was observed at 48 h (Fig. 1).

3.2. Effect of ASB on integrity of HK-2 cell membrane

The integrity of HK-2 cell membrane gradually decreased after 48 h treatment of ASB, and LDH leakage from the cell to culture medium increased in a dose-dependent manner (Fig. 2). Compared with 0 mmol/L group, significant differences were observed in the 7.0 mmol/L, 14.0 mmol/L, 29.0 mmol/L and 57.0 mmol/L groups (all P value <0.01).

3.3. Effect of ASB on HK-2 cell ultrastructure

As shown in Fig. 3, the ultrastructure of HK-2 cells was observed using an electron microscope after 48 h of ASB treatment at different concentrations. Cells in the control group presented intact organelles, tight connection of cells and clear cell microvilli. Cells in the 7.0 mmol/L group showed

Fig. 3 – Effect of ASB on the ultrastructure of HK-2 cells. Electron micrographs show chromatin margination and autophagy induced by ASB. (A–D) HK-2 cells were treated with 0, 7, 14 and 29 mmol/L of ASB for 48 h, respectively (bar = 0.5 μm). The arrows in the electron micrograph represent autophagosomes.
In the 14.0 mmol/L group, the chromatin was further aggregated, and a few autophagic vacuoles were generated (arrowhead indicated). In the 29.0 mmol/L group, the number of lysosomes increased, and a large quantity of autophagic vacuoles were generated. Cells treated with higher concentration of ASB underwent significant cell damage and autophagy was not observed in 57.0 mmol/L group (data not shown).

3.4. Effect of ASB on mitochondrial membrane potential in HK-2 cells

With the increase in drug concentration, the ratio of red fluorescence density to green fluorescence density gradually decreased, and the mitochondria were depolarized and their membrane potential was decreased (Fig. 4).
3.5. **Effect of ASB on ROS generation in HK-2 cells**

With the elevation of ASB levels, ROS levels increased as detected using three different methods. Fig. 5A–D represents the results of CM-H2DCFDA staining which show that the fluorescence intensity was enhanced with the increase of ASB. Moreover, cell number was gradually reduced in a dose dependent manner. In the 5.0 mmol/L group, probes cannot be labeled, and the fluorescence signal cannot be collected (data not shown). SOD enzyme activity in ASB treated cells was detected and the data revealed that SOD activity was reduced by ASB (Fig. 5E). Furthermore, GSH consumption assay also reflected ROS level in HK-2 cells. As presented in Fig. 5F, GSH concentration significantly decreased with the elevated ASB.

3.6. **Effect of ASB on Caspase-3 and Caspase-9 activities in HK-2 cells**

Caspase-9 activation was seen earlier than Caspase-3. Caspase-9 activity was significantly increased in line with increasing concentrations after 24 h of treatment. However, Caspase-9 activity decreased after ASB treatment at a concentration of 57.0 mmol/L suggesting that Caspase-9 activity reached a peak value. Further the drug concentration influenced the integrity of the cell structure. After 48 h of treatment, 29 mmol/L of ASB decreased Caspase-9 activity, suggesting that cellular activity was markedly influenced by the duration of drug administration (Fig. 6B). After 24 h of administration, Caspase-3 activity slowly increased dose-dependently with the ASB concentration. Caspase-3 activity depended on Caspase-9 activation, and reached a peak value at a concentration of 57 mmol/L. Caspase-3 activity markedly increased with duration of ASB administration, and its activity peaked at an ASB concentration of 29 mmol/L. Furthermore, Caspase-3 activity decreased at a concentration of 57 mmol/L, suggesting that cell integrity and activity was critically affected (Fig. 6A).

3.7. **Change of Cyto C, Bcl-2 and Bax expression in HK-2 cell cytoplasm and mitochondria**

Compared with the normal control group, the band density of Bcl-2 significantly decreased with an increase in ASB levels. By contrast, Bax expression was augmented in a dose-dependent manner. Mitochondrial cytochrome C decreased and by contrast increased in the cytoplasm, suggesting that cytochrome C had translocated from the mitochondria to the cytoplasm (Fig. 7).

3.8. **Change of JNK and Beclin-1 expression in HK-2 cells**

Compared with the normal group, JNK expression first increased and then decreased as ASB concentrations...
increased. However, Beclin-1 expression was augmented dose-dependently. ASB activated JNK and Beclin-1 expression simultaneously. Moreover, JNK expression was presumably inhibited with the increase of autophagic protein expression (Fig. 8).

4. Discussion

Andrographolide sodium bisulfate (ASB) is a kind of soluble derivative of andrographolide, which is the major bioactive compound in andrographis paniculata. The present study investigated the autophagic and apoptotic effects of ASB in HK-2 cells. The results indicated that ASB induced autophagy and apoptosis in HK-2 cells, which were consistent with the changes in autophagic and apoptotic markers.

Fig. 7 – Effect of ASB on Cytochrome C, Bcl-2 and Bax expression in HK-2 cells. HK-2 cells treated with 0, 7, 14, 29 and 57 mmol/L ASB for 48 h were used for western blotting to detect the levels of Bcl-2, Bax and Cyto C were detected respectively in cytoplasm and mitochondria. Actin was used as a loading control. *P < 0.05, **P < 0.01 vs. respective control cells. Data is described as mean ± SD, n = 3.
compound isolated from *Andrographis paniculata* (Chuan-Xin-Lian in Chinese), a medicinal herb that is widely used in China and other parts of Asia for the treatment of upper respiratory tract infections (Roxas and Jurenka, 2007). We now know that andrographolide exerts a wide range of therapeutic actions, including immunosuppressant (Burgos et al., 2005; Iruretagoyena et al., 2006), anti-thrombotic (Li et al., 2009), anti-inflammatory (Abu-Ghefreh et al., 2009; Bao et al., 2009; Wang et al., 2007), anti-neoplastic (Varma et al., 2011), anti-viral (Chen et al., 2009), anti-bacterial (Jiang et al., 2009), anti-diabetic (Zhang et al., 2009), antioxidative stress (Akowuah et al., 2009), anti-pyretic (Suebsasana et al., 2009) and anti-oedematogenic and anti-nociceptive (Lin et al., 2009; Sulaiman et al., 2010) activities.

Acute renal damage reported in clinical application of ASB was related to ROS and cell damage as described before. Our study confirmed cell toxicity of ASB in human proximal tubular endothelial HK-2 cells. MTT assay showed that cell viability decreased in a dose and time dependent manner and LDH release assay revealed that LDH leakage level was also induced by ASB but was not proportionate to the cell damage level measured by MTT assay, showing that most of the cells maintained the integrity of cell membrane.

Oxidative stress can be defined as the imbalance between cellular oxidant species production and antioxidant capability. Toxic ROS can cause cellular damage by oxidizing nucleic acids, proteins, and membrane lipids (Griendling et al., 2000). We detected in this study the ROS production using H2DCFDA staining and antioxidants including SOD activity and GSH concentration. The data commonly confirmed the role of ROS in ASB induced cell damage.

Electron microscopy observation of the ASB treated HK-2 cells showed morphological changes of the cells including shrink of the cell, chromatin aggregation and emergence of autophagic bodies. Autophagy is a phenomenon of self-eating in cells, and it is a non-injured response for constant physiological stimuli. The injured proteins or organelles are packaged by autophagic vacuoles with a double-layered membrane structure, and are further degraded in lysosomes. Finally, cell function and homeostasis are maintained by autophagy (Mizushima et al., 2008). Beclin-1 is a homologous gene of the yeast Atg6/Vps30 gene, and it is an important signal for cell autophagy with other upstream and downstream Beclin-1-associated proteins (Cao and Klionsky, 2007). Our study found that ASB upregulated Beclin-1 expression in HK-2 cells, suggesting that ASB-induced autophagy was associated with augmented Beclin-1 expression.

It was reported previously that ROS mediated JNK signaling played an important role in inflammation, cell apoptosis and autophagy. Moreover, activated JNK can phosphorylate and further inhibit Bcl-2 and Bcl-Xt, whereupon Bax was translocated to the mitochondria. Next, cellular apoptosis was further induced by initiating Caspase-dependent mitochondrial apoptosis (Lei and Davis, 2003; Papa et al., 2004). We researched the mitochondrial pathway and the results showed that MMP was decreased and cytochrome C was released from mitochondria, subsequently activated both Caspase-9 and Caspase-3. Hence, ASB-induced HK-2 cellular apoptosis might be a Caspase-dependent mitochondrial pathways.

Our findings indicated that ASB increased ROS levels in HK-2 cells, and that Bcl-2 expression was decreased. Moreover, Bax and JNK expression were upregulated, suggesting that JNK signaling played an important role in regulating ASB-induced cellular apoptosis and autophagy.
In summary, we found that ASB induced high levels of ROS generation in HK-2 cells and activated JNK signaling. ASB induced cell apoptosis via the Caspase-dependent mitochondrial pathway, and induced cellular autophagy, in part by enhancing Beclin-1 protein expression. ASB-induced ROS generation was a common inductive factor for HK-2 cellular apoptosis and autophagy. Bcl-2 and Beclin-1 played an important role in the interaction between cellular apoptosis and autophagy. However, the precise sequence of events responsible for HK-2 cellular apoptosis and autophagy need to be further clarified.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Authors’ contribution

Hong Lu and Xin-Yue Zhang designed the experiments; Hong Lu, Yi-Qi Wang, Xiao-Liang Zheng, Yin Zhao and Qin Zhang performed the experiments; Wen-Min Xing and Hong Lu performed data analysis and prepared the manuscript.

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