Astragalus Polysaccharide Attenuated Iron Overload-Induced Dysfunction of Mesenchymal Stem Cells via Suppressing Mitochondrial ROS

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Key Words
Astragalus polysaccharide • Iron overload • Senescence • ROS • Proliferation

Abstract
Background/Aims: Bone marrow-derived mesenchymal stem cells (BMSCs) have the ability to differentiate into multilineage cells such as osteoblasts, chondrocytes, and cardiomyocytes. Dysfunction of BMSCs in response to pathological stimuli participates in the development of diseases such as osteoporosis. Astragalus polysaccharide (APS) is a major active ingredient of Astragalus membranaceus, a commonly used anti-aging herb in traditional Chinese medicine. The aim of this study was to investigate whether APS protects against iron overload-induced dysfunction of BMSCs and its underlying mechanisms.

Methods: BMSCs were exposed to ferric ammonium citrate (FAC) with or without different concentrations of APS. The viability and proliferation of BMSCs were assessed by CCK-8 assay and EdU staining. Cell apoptosis, senescence and pluripotency were examined utilizing TUNEL staining, β-galactosidase staining and qRT-PCR respectively. The reactive oxygen species (ROS) level was assessed in BMSCs with a DCFH-DA probe and MitoSOX Red staining.

Results: Firstly, we found that iron overload induced by FAC markedly reduced the viability and proliferation of BMSCs, but treatment with APS at 10, 30 and 100 μg/mL was able to counter the reduction of cell proliferation. Furthermore, exposure to FAC led to apoptosis and senescence in BMSCs, which were partially attenuated by APS. The pluripotent genes Nanog, Sox2 and Oct4 were shown to be downregulated in BMSCs after FAC treatment, however APS inhibited the reduction of Nanog, Sox2 and Oct4 expression. Further study uncovered that APS treatment abrogated the increase of intracellular and mitochondrial ROS level in FAC-treated BMSCs.

Conclusion: Treatment of BMSCs with APS to impede mitochondrial ROS accumulation can remarkably

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inhibit apoptosis, senescence, and the reduction of proliferation and pluripotency of BMSCs caused by FAC-induced iron overload.

Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) have the ability of self-renewal and the potential to differentiate into multilineage cells such as osteoblasts, chondrocytes, adipocytes, neurons and cardiomyocytes [1-3]. Therefore, BMSCs have emerged as one of the greatest therapeutic resources for gene therapy, cell therapy and tissue engineering [4, 5]. Recently, it has been reported that BMSCs support bone microenvironment homeostasis and maintain bone remodeling balance [6-8]. A series of studies have reported that under pathological conditions, dysfunction of BMSCs characterized by deficient proliferation and increased apoptosis and senescence contributed to the development of reduced bone mass and even osteoporosis [9]. For example, excessive caffeine consumption, an important risk factor for osteoporosis markedly inhibited the commitment of BMSCs to osteoblasts via reducing calcium deposition and increasing cAMP level [7].

Iron is an essential micronutrient, and it is required as a cofactor for fundamental cellular processes such as DNA synthesis, oxygen transport, and cellular respiration. Continued iron deficiency causes cell death, on the other extreme, free iron excess is also toxic. Iron overload (IO) is a disease characterized by excessive iron deposition in tissues, damaging vital organs including the heart, liver and kidney. IO is associated with hereditary hemochromatosis or repeated blood transfusions for diseases such as beta thalassemia, bone marrow failure, or myelodysplastic syndrome [10-14]. Recently, iron accumulation has been shown to impair the bone marrow microenvironment and suppress the proliferation and differentiation of BMSCs, thus leading to lower bone mineral density and postmenopausal osteoporosis [15, 16]. Oxidant stress has also been seen to be involved in iron overload-induced bone loss in mice [17]. Information as to how to inhibit iron overload-induced dysfunction of BMSCs has been limited until now.

Astragalus membranaceus, known as Huang Qi, is one of the most commonly used anti-aging herbs in traditional Chinese medicine and it has been widely used to treat a variety of diseases such as diabetes and myocardial infarctions with positive effects in clinics [18]. Astragalus polysaccharide (APS) is a major active ingredient of Astragalus membranaceus [19]. The pro-angiogenic and anti-inflammatory properties of APS as well as its protective effects on various organs have been investigated extensively [20-22]. Recent studies further indicated the diversity of the potential effects of APS on improving microcirculatory disturbances, including anti-oxidation, inhibition of apoptosis and amelioration of injury to target organs such as the kidney [23, 24]. However, its protective effects against damage to BMSCs induced by excess iron have not been reported. Therefore, we hypothesize that APS plays a protective role in FAC-induced iron overload and dysfunction of BMSCs.

Materials and Methods

Animal care

The Male C57BL/6J mice (18-20 g) were purchased from the experimental animal center of the Affiliated Second Hospital of Harbin Medical University. All animal protocols were approved by the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. Additionally, all experimental procedures were carried out in strict accordance with the ethic committee of Harbin Medical University.

Reagents

Astragalus polysaccharides (APS) were prepared as described previously [19]. APS was dissolved in dimethyl sulfoxide (DMSO) for assay with cultured cells, and the final culture concentration of DMSO was...
≤0.5%. Other chemicals were purchased from Sigma (St. Louis, Mo. USA). TUNEL was purchased from Roche Company (Roche, Germany). qRT-PCR primers were purchased from Invitrogen (Invitrogen, Carlsbad, USA). Stem cell medium was purchased from Stem Cell (Canada). In Cell-Light EdU Apollo567 in Vitro Kits were purchased from Ribobio (Ribobio, Guangzhou, China). MitoSOX™ Red mitochondrial superoxide indicator was purchased from Invitrogen (Invitrogen Detection Technologies, USA). The SA-β-gal staining kit (C0602), Reactive Oxygen Species Assay Kit (S0033), Hoechst 33342 staining (C1022) and Cell-Counting Kit-8 (CCK-8) proliferation assay kits (CK04) were purchased from Beyotime (Beyotime Biotechnology, China).

**Isolation and culture of BMSCs**

BMSCs were primarily isolated and cultured from C57BL/6J mice based on previous reports [25]. BMSCs were acutely isolated from mouse bone marrow of the femur and tibia. Separation of muscles and tendons from the femurs and tibias was done before BMSCs isolation. BMSCs stem cell medium supplemented with 20% special serum was used to flush the marrow cavity of femurs and tibias. The culture medium used to flush the bone marrow containing BMSCs was collected and seeded into 25 cm² flasks, then cultured in an incubator at 37 °C with 95% air and 5% CO₂. BMSCs were adherent to the bottom of the flask by their adherent characteristic. The culture medium was replaced every 3 days. After 7-10 days of culturing, when the cells reached 70% confluency, they were trypsinized and passaged.

**Cell proliferation assay**

BMSCs were seeded at 2000 cells/well in 96-well plates for 24 h, and the cell number was measured using a Cell-Counting Kit-8 (CCK-8) proliferation assay kit. BMSCs were incubated in 100 μL of stem cell culture medium mixed with 10 μL of CCK-8 solution/well for an additional 2 h at 37°C. The amount of formazan dye generated by cellular dehydrogenase activity was measured for absorbance at 450 nm with a microplate spectrophotometer (BioTek, Winooski, VT, USA). The optical density (OD) values of each well represented the proliferation of BMSCs.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Staining**

TUNEL staining was used to detect the anti-apoptotic effects of APS on FAC-induced injury of BMSCs. The apoptotic BMSCs were determined as multinucleated cells containing multiple nuclei under fluorescence microscopy. TUNEL staining was carried out according to the manufacturer’s instructions. Briefly, cells were fixed with 4% paraformaldehyde solution for 15 min at room temperature, and then permeabilized in 0.1% Triton X-100. Cells were then introduced to TUNEL reaction mixture for 1 h at 37°C under a humidified atmosphere in the dark. Finally, the cells were incubated in DAPI for 20 min. The TUNEL index (%) is the average ratio of the number of TUNEL-positive cells divided by the total number of cells under optical microscopy. For each sample, ten randomly selected areas of TUNEL-stained slices were counted, and the average value were calculated.

**Hoechst 33342 Staining**

Hoechst staining was used to detect the effects of APS on FAC-induced apoptosis of BMSCs. The protocol was carried out according to the manufacturer’s instructions. BMSCs were fixed with 4% paraformaldehyde for 30 min at room temperature. Then, the cells were stained with Hoechst 33342 for 20 min. After washing three times with serum-free DMEM, the cells were washed in serum-free DMEM for morphological observation using a fluorescence microscope.

**SA-β-gal assay**

SA-β-gal activity was determined using an SA-β-gal staining kit from Beyotime according to the manufacturer’s instructions. BMSCs cultured in 6-well plates were washed three times with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. After rinsing with PBS, cells were incubated with SA-β-gal staining solution at 37°C for 16 hours. The senescent cells were identified as blue-staining by standard light microscopy, and a total of 1000 cells were counted in 20 randomly chosen fields to calculate the percentage of SA-β-gal-positive cells.
Western blot

Total proteins were obtained using protein isolation kits (Beyotime) according to the manufacturer’s protocol. Equal amounts of protein were resolved on SDS-polyacrylamide gels. The electrophoresed proteins were transblotted onto a PVDF membrane, blocked with 5% milk, and incubated with primary antibodies specific to: Bax (Cell signaling Technology 1:1000), Bcl-2 (Cell signaling Technology 1:1000) overnight at 4°C. The membrane was then incubated using a secondary antibody for 45 min at room temperature. After washing, membranes were scanned with the Odyssey Imaging System (Li-Cor Bioscience).

Measurement of Reactive Oxygen Species (ROS) and DAPI staining

To quantify intracellular ROS level, we used DCFH-DA probe. BMSCs were plated in 6-well plates and incubated for 24 h. After being washed three times with PBS, the BMSCs were loaded with DCFH-DA 10 μM to incubate for 30 min at 37°C. Cells were then washed with PBS three times in order to fully remove the superfluous DCFH-DA probe which didn’t penetrate into BMSCs. Next, BMSCs were fixed in 4% paraformaldehyde for 30 min at room temperature. After being washed again with PBS by three times, the fixed cells were stained with DAPI 20 μg/mL for 15 min at room temperature. Then the cells were imaged with a fluorescence microscope.

MitoSOX™ Red mitochondrial superoxide indicator (MitoSOX™ Red) assay

MitoSOX™ Red mitochondrial superoxide indicator is a novel fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells. MitoSOX™ Red reagent is live-cell permeant and it can rapidly and selectively target to the mitochondria. Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide and exhibits red fluorescence. In brief, BMSCs were plated in 6-well plates and incubated for 24 h. The contents (50 μg) of one vial of MitoSOX™ mitochondrial superoxide indicator (Component A) were dissolved in 13 μL of DMSO to make a 5 mM MitoSOX™ reagent stock solution. The 5 mM MitoSOX™ reagent stock solution (prepared above) was dissolved in HBSS/Ca/Mg buffer to make a 5 μM MitoSOX™ reagent working solution. 1 mL of 5 μM MitoSOX™ reagent working solution was applied to cover cells adhering on 12-well plates. Protected from light, cells were incubated for 10 min at 37°C. After washed gently three times with warm HBSS/Ca/Mg buffer, BMSCs were counterstained and mounted for imaging with a fluorescence microscope at 530/590 nm.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from BMSCs using TRIzol reagent (Sigma-Aldrich) according to the manufacturer’s instructions. cDNA was prepared using the Revert-AidTM First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada) following the manufacturer’s protocol. To perform real-time PCR, each 20 μL RT-PCR mix contained 10 μL of FastStart SYBR Green Master Mix (Roche Diagnostics), 1 μL of each primer, 1 μL cDNA, and distilled water; qRT-PCR was performed on an ABI 7500 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Conditions were as follows: hold stage was 95°C for 30 s, cycling was 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. The primer sequences of Nanog, Sox2, Oct4 and GAPDH genes are listed as following: Nanog-F (5’-TCT CTC AGG CCC AGC TGT GT-3’), Nanog-R (5’-GCT TGC ACT TCA TCC TTT GGT T-3’), Sox2-F (5’-ACC AGC TCG CAG ACC TAC AT-3’), Sox2-R (5’-CCT CGG ACT TGA CCA CAG AG-3’), Oct4-F (5’-CCC GGA AGA GAA AGC GAA CT-3’), Oct4-R (5’-AGA ACC ATA CTC GAA CCA CAT CCT-3’), GAPDH-F (5’-CAT CAC TGC CAC CCA GAA GAC-3’), GAPDH-R (5’-CCA GTG AGC TTC CGG TGC TTC AG-3’).

The threshold cycle (Ct) value was subsequently determined, and the relative quantification of mRNA expression was calculated using the comparative Ct method.

Statistical Analysis

All data was expressed as mean ± S.E.M. Statistical analysis was performed to determine the significance of differences between groups using ANOVA. All statistical analysis was performed using the SPSS 13.0 software for Windows. Statistical significance was set at P < 0.05.

Results

Effect of APS on FAC-induced viability and proliferation reduction of BMSCs

Firstly, we investigated whether FAC treatment impacts the viability and proliferation of BMSCs. As shown in Figure 1A, exposure to FAC 200 μM for 24 h resulted in the decrease
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Fig. 1. The pro-proliferative effect was enhanced in BMSCs when treated by different concentrations of APS for 24 h. (A), CCK-8 assay showed that APS played a significant protective role in the cellular viability of BMSCs in a dose-dependent manner for 24 h. The quantities of BMSCs incubated with FAC 200 μM were significantly reduced compared to the control group, while treatment with APS 10 μg/mL, 30 μg/mL, 100 μg/mL and 300 μg/mL for 24 h significantly increased quantities compared to the FAC 200 μM group. (B and C), Moreover, EdU staining results showed the percentage of proliferative BMSCs induced by different concentrations of APS from 10 μg/mL to 300 μg/mL was significantly increased. Values are the mean ± S.E.M. of six independent experiments (n = 6). Scale bar = 50 μm. * P < 0.05, ** P < 0.01, *** P < 0.001.

Effect of APS on FAC-induced apoptosis of BMSCs

We then further investigated if APS protects against FAC-induced apoptosis of BMSCs. As displayed in Figure 2A, TUNEL staining showed that FAC 200 μM could induce significant increase of TUNEL-positive BMSCs. Nevertheless treatment with APS 30 μg/mL and 100 μg/mL significantly decreased the percentage of TUNEL-positive BMSCs. Hoechst 33342 staining also showed BMSCs exposed to APS 30 and 100 μg/mL for 24 h displayed the improvement in apoptotic morphological changes compared to the FAC 200 μM group (Fig. 2B). In agreement, FAC 200 μM led to the increase of apoptotic protein Bax, and the reduction of anti-apoptotic protein Bcl-2, which was partly prevented by APS 30 μg/mL and 100 μg/mL (Fig. 2C). These results suggest that APS plays an anti-apoptotic role in BMSCs.

Effect of APS on FAC-induced senescence of BMSCs

We also studied whether FAC induces the senescence of BMSCs, and if APS protects BMSCs from BMSCs aging. As shown in Figure 3, a significant increase of SA-β-gal-positive...
cells was observed in the FAC-treated BMSCs. In the control group, only 6.25% cells were positive for SA-β-gal staining, but after treatment with FAC 200 μM, the percentage of SA-β-gal-positive cells increased to 15.89%. While in the presence of APS 30 μg/mL and 100 μg/mL, the percentage of SA-β-gal positive cells individually decreased to 7.34% and 7.26%. This result indicates that APS is able to effectively inhibit the senescence in FAC-induced BMSCs (Fig. 3).

Fig. 3. Anti-senescent effect of APS on FAC-induced iron-overload injury in BMSCs. (A), The percentage of SA-β-gal-positive cells was retained at a high level after treatment with FAC, while after treating with APS 30 μg/mL and 100 μg/mL could significantly decrease the positive apoptotic cell percentage (n=10, p<0.05). Scale bar = 50 μm. (B), Hoechst 33342 staining also showed BMSCs after being exposed to different concentrations of APS for 24 h displayed anti-apoptotic morphological changes compared to FAC 200 μM group (n=10, p<0.05). Scale bar = 50 μm. (C), Western blot showed that FAC 200 μM increased the expression of apoptotic protein Bax, and inhibited the expression of antiapoptotic protein Bcl-2, and this alteration was attenuated by APS 30 μg/mL and 100 μg/mL (n=5, p<0.05). Scale bar = 50 μm. * P < 0.05, ** P < 0.01, *** P < 0.001.
Effects of APS on FAC-induced changes of pluripotent genes in BMSCs

The effects of iron overload along with APS on the expression of pluripotent genes such as Nanog, Sox2 and Oct4 in BMSCs was then investigated. As shown in Figure 4, FAC-treated BMSCs showed the reduction of Nanog, Sox2 and Oct4 mRNA expression in BMSCs. It suggested that FAC treatment reduced the pluripotency of BMSCs. Nevertheless, in the presence of APS 30 and 100 μg/mL, the reduction of these pluripotent genes at mRNA level was partially reversed. It suggests that APS protects against iron overload-induced downregulation of pluripotent genes in BMSCs (Fig. 4).

APS reversed FAC-induced increase of ROS in BMSCs

It is well documented that reactive oxygen species (ROS) are involved in iron overload-induced cell apoptosis and organ aging. Oxidative stresses caused by ROS are shown to initiate or promote apoptosis and senescence via oxidizing mitochondrial membrane phospholipids and depolarizing mitochondrial membrane potential which produces more ROS. We therefore investigated the influences of APS on FAC-induced the production of ROS by DCFH-DA staining. As shown in Figure 5A, DCFH-DA staining showed the intensity of...
green fluorescence increased, representing the percentage of ROS-positive cells significantly increasing in the presence of FAC 200 μM for 24 h, which could be reversed by treating with APS 30 and 100 μg/mL. Superoxide production in the mitochondria was also measured using MitoSOX Red. Treatment of BMSCs with FAC 200 μM for 24 h was able to lead to an increase of mitochondrial superoxide productions, which could be inhibited by treatment with APS 30 and 100 μg/mL (Fig. 5B). These results indicate that ROS-mediated mitochondrial dysfunction is involved in FAC-induced BMSCs apoptosis, which can be improved by APS treatment.

Discussion

The present study firstly revealed that iron overload could inhibit the proliferation and pluripotency of BMSCs as well as trigger the apoptosis and senescence through ROS accumulation, while APS treatment could significantly reverse these processes. These findings provide evidence for APS as a potential treatment for BMSCs injury and bone diseases.

Increasing evidence uncovered BMSCs as a novel therapeutic strategy for a variety of diseases [25, 26]. Iron overload is characterized by excessive iron deposition in organs or tissues, which causes damage to vital organs such as heart, liver and kidney. In clinics, iron overload is usually associated with hereditary hemochromatosis or repeated blood transfusions for diseases such as beta thalassemia, bone marrow failure, or myelodysplastic syndrome [27, 28]. More and more evidence shows that iron overload is correlated with bone mass loss and osteoporosis. Accumulation of iron has been shown to induce the apoptosis of osteoblasts [29, 30]. Excess iron also can lead to toxic effects on the proliferation and differentiation of mesenchymal stem cells. BMSCs have been proved that they played an important role in maintaining bone marrow microenvironment and bone mass. Iron overload-induced damage of BMSCs has been considered as a major cause of osteoporosis. However, how to prevent FAC-induced iron overload injury in BMSCs has not been fully affirmed yet.

APS is a major active component of traditional Chinese medicine and has multiple biological activities including antioxidant properties, cardioprotection, antiviral properties, immunoregulatory involvement, hypoglycemic counter-action and antitumor properties [19, 31]. In this study, we hypothesized that APS plays a protective role in FAC-induced BMSCs dysfunctions. Firstly, CCK-8 assay and EdU staining showed that FAC could significantly inhibit the viability and proliferation of BMSCs. In agreement, it was recently reported that BMSCs from iron overloaded mice exhibited proliferation deficiencies [16]. But this phenomenon could be reversed by APS treatment in different concentrations. It suggests that APS has a protective effect on FAC-induced injury of BMSCs. Consistently, it was also reported that Astragalus was able to promote the proliferation of BMSCs via VEGF secretion [32]. In order to demonstrate whether FAC could produce apoptotic toxicity on BMSCs, we further used TUNEL and Hoechst staining to assess BMSCs apoptosis, and the results showed that BMSCs underwent apoptosis in the presence of FAC 200 μM, which could also be reversed by APS 30 and 100 μg/mL. Similarly, iron overload has been shown to have toxic effects such as pro-apoptotic effect on osteoblasts and neurons [33, 34]. Ferric ion accumulation also promoted osteoclast differentiation and causes bone mass loss [35]. These data suggests that iron overload accelerated bone loss and osteoporosis via inhibiting osteogenesis and promoting osteoclasts. We found that APS at the concentration from 30 to 100 μg/mL effectively inhibited the apoptosis of BMSCs.

Although previous studies have uncovered the effects of iron overload on proliferation and apoptosis of BMSCs, whether iron overload affects senescence and pluripotency of BMSCs remains unknown. We found that treatment with APS is able to attenuate the reduction of pluripotency and senescence of BMSCs. This is a novel biological action of APS on BMSCs.
Increased ROS level plays a key role in the development of cellular apoptosis [36, 37]. It has been shown that iron overload induced the apoptosis of BMSCs via increasing intracellular ROS level [17]. Thus, we explored if APS protects BMSCs against iron overload-induced BMSCs injury via affecting ROS level. The ROS fluorescent probe was further used to detect the level of intracellular ROS in BMSCs. The results showed that ROS in BMSCs treated by FAC 200 μM was markedly increased, and treatment with APS can reverse the apoptosis in BMSCs induced by FAC, indicating FAC-induced ROS increasing is involved in BMSCs apoptosis. Furthermore, the increase of mitochondrial ROS was observed in FAC-treated BMSCs by using MitoSOX Red probe, which can be attenuated by APS treatment. This data suggests that APS protects against FAC-induced BMSCs dysfunction via inhibiting mitochondrial ROS.

Conclusions

In summary, the present study firstly showed the pro-proliferative, anti-apoptotic, anti-senescent effects of APS on FAC-treated BMSCs. Our data confirmed that APS can promote the proliferation and inhibit the apoptosis and senescence in BMSCs suffering from iron overload-induced injury, which is associated with the decreased production of mitochondrial ROS. Furthermore, our study also extends our understanding about the action and mechanism of APS in the treatment of BMSCs dysfunction and related bone diseases.

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Disclosure Statement

The authors had no conflicts of interest to declare in relation to this article.

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