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Asprosin improves the survival of mesenchymal stromal cells in myocardial infarction by inhibiting apoptosis via the activated ERK1/2-SOD2 pathway

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ABSTRACT

Aims: Several adipokines have been proven to improve the therapeutic efficacy of mesenchymal stromal cells (MSCs) when used to treat ischemic heart disease. Asprosin (ASP) is a newly-discovered adipokine. ASP might also predict the severity of coronary pathology. We investigated the role of ASP on MSCs and the effects of ASP-pretreated MSCs on myocardial infarction (MI).

Main methods: MSCs were labelled with a lentivirus carrying green fluorescent protein (GFP). For in vivo study, after pretreatment with vehicle or ASP, MSCs were injected into infarcted hearts. Cardiac function and fibrosis were then evaluated 4 weeks after the induction of MI and survival of MSCs evaluated after 1 week. MSCs proliferation and migration were investigated after ASP treatment in vitro. MSCs apoptosis induced by hydrogen peroxide (H_2O_2) was assessed using flow cytometry.

Key findings: Compared to vehicle-pretreated MSCs, ASP-pretreated MSCs significantly improved the left ventricular ejection fraction (LVEF), and inhibited myocardial fibrosis 4 weeks after MI. ASP pretreatment may have promoted homing of transplanted MSCs. In vitro results showed that ASP had no significant effect on MSC proliferation and migration, but protected these cells from H_2O_2-induced apoptosis. Among 21 molecules associated with antioxidation and cell death, the antioxidant enzyme SOD2 was significantly upregulated by ASP. Furthermore, ASP treatment inhibited H_2O_2-induced ROS generation and apoptosis via the activated ERK1/2-SOD2 pathway.

Significance: This is the first evidence that ASP can regulate MSCs function and enhance MSCs therapy for ischemic heart disease. Furthermore, we demonstrate that ASP protects MSCs from oxidative stress-induced apoptosis via the ERK1/2-SOD2 pathway.

1. Introduction

Ischemic heart disease is the leading cause of death worldwide. Although the development of therapies such as drugs, interventions and surgery, have significantly reduced acute mortality caused by myocardial infarction (MI), heart failure caused by pathological myocardial remodeling, secondary to MI, has become the main burden in the treatment of cardiovascular diseases [1]. Cell transplantation therapy has proven to be a promising approach for heart diseases. Among the candidate cell types, mesenchymal stromal cells (MSCs) are most widely studied due to their excellent biological properties; they are easily accessible, they exhibit a low immune response, and there are less ethical issues concerning their use. Numerous preclinical studies have reported that transplanted MSCs treat MI mainly by the release of many bioactive molecules from the paracrine [2]. However, poor cell survival and retention in an ischemic microenvironment create a troublesome barrier, limiting the curative effect of MSCs [3]. In acute MI, ROS may contribute to myocyte loss via apoptosis or other cell

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death mechanisms [4,5]. Preconditioning strategies, such as hypoxia and drug administration, have been derived to improve cell survival and function by inhibiting oxidative stress-induced apoptosis and increasing the ability of injected cells to adapt to ischemia and hypoxia [6,7].

Recently, the level of asprosin (ASP), a previously unknown protein hormone generated and secreted by white adipose tissue, was discovered to decrease in plasma due to *FBN1* mutations in patients with Neonatal Progeroid Syndrome. Furthermore, it was identified as an adipokine involved in hepatic glucose release by a research team in 2016 [8]. Another report in 2017 demonstrated, for the first time, that the adipokine, ASP, crosses the blood-brain barrier during circulation to stimulate food intake by directly activating orexigenic neurons, and inhibits anorexigenic neurons via G proteins–cAMP–PKA axis [9]. These two pioneering studies suggest that ASP may provide new drug intervention targets for obesity, type 2 diabetes, and metabolic syndrome. Previous studies have demonstrated that multiple adipokines are involved in the regulation of the functions of MSCs under various physiological and pathological settings [10–12]. Pretreatment of MSCs with several adipokines has been reported to improve the survival of MSCs in the ischemic microenvironment, thereby increasing the efficacy of MSCs on ischemic heart disease [12–14]. Thus, we hypothesized that ASP may regulate the functions and therapeutic efficacy of MSC in cell transplantation. In the present study, we aimed to detect whether ASP treatment promotes the homing of MSCs in the ischemic microenvironment and enhances the curative efficacy of MSCs in MI. In addition, we explored the underlying molecular mechanisms involved in these processes.

2. Materials and methods

All experiments were approved by the Fourth Military Medical University Committee on Animal Care (number: 20171003). C57BL/6J male mice were provided by the Experimental Animal Center of the Fourth Military Medical University (Xi'an, China). U0126 (HY-12031) and AKT kinase inhibitor (HY-10249Y) were purchased from MedChemExpress (New Jersey, USA).

2.1. Preparation and characterization of MSCs

MSCs were isolated from bone marrow obtained from 8-week-old C57BL/6 mice based on a previous report [15]. The specimen was aspirated by flushing the tibia and femoral marrow compartments harvested, then cultured in C57BL/6 Mouse MSC Basal Medium (cat. no. MUBMX-90011; Cyagen Biosciences, Guangzhou, China) supplemented with 10% fetal bovine serum (FBS) at 37 °C in an incubator containing 5% CO_2. Adherent marrow cells were obtained via frequent medium changes and were sub-cultured to passage 4 (P4). To validate the purified population of MSCs, flow cytometry was used to characterize the surface markers. P4 MSCs were digested and resuspended in Flow Cytometry Stain (cat. no. 4311084; ThermoFisher). After the Nonspecific Fc receptor was blocked with anti-CD16/32 antibodies (cat. no. 14-0161; eBioscience), MSCs were incubated for 30 min with the following antibodies: anti-CD29-fluorescein isothiocyanate (FITC; cat. no. 11-0291; eBioscience), anti-CD34-FITC (cat. no. CBL555F; Millipore), anti-CD44-phycoerythrin (PE; cat. no. 103007; Biolegend), and anti-CD45-FITC (cat. no. 11-0461; eBioscience). Cells were then resuspended in flow cytometry staining buffer (cat. no. 00-4222-26; eBioscience) for flow cytometry. Adipogenic and osteogenic differentiation assays were conducted using Alizarin Red S and Oil Red O staining (cat. nos. MUBMX-90021 and MUBMX-90031, respectively; Cyagen Biosciences) according to the manufacturer's protocol.

2.2. MI model and MSCs transplantation

To monitor the homing of MSCs in vivo, MSCs were infected with null lentivirus carrying a green fluorescent protein (GFP) reporter gene (Shanghai Genechem Co., Ltd.); this was performed at a multiplicity of infection of 80 in the presence of 5 μg/mL polybrene. Robust expression of GFP was considered to indicate the successful labeling of MSCs in vivo. The mouse model of permanent MI utilized in this study has been described previously [16]. Briefly, mice were anesthetized with 2% isoflurane. The heart was exposed, and the left anterior descending branch of the coronary artery was ligated with 6–0 silk sutures. Blanching of the anterior wall observed within a few seconds after ligation indicated successful surgery, and then 2×10^5 MSCs suspended in 25 μL phosphate-buffered saline (PBS) was immediately injected into the infarct border zone. Mice were randomized into the following groups: (1) MI; (2) MI + MSCs (non-pretreated MSCs); (3) MI + MSCs-ASP (pretreatment with 5 μg/mL ASP for 24 h before transplantation); and (4) MI + MSCs-Veh (pretreatment with vehicle for 24 h before transplantation).

2.3. Echocardiographic analysis

Left ventricular ejection fraction (LVEF) of mice was evaluated by transthoracic echocardiography using a VisualSonics VeVo 2100 Imaging System on day 28 following MI induction.

2.4. Masson staining

For the analysis of fibrosis, mice were sacrificed and their hearts fixed with 4% paraformaldehyde overnight and embedded in paraffin. Subsequently, 7-μm-thick sections were prepared, and five sections per heart were stained with Masson's trichrome. The level of fibrosis was then quantified by Image-Pro® Plus version 6.0.

2.5. Monitoring of cell homing and survival

Eight μm-thick frozen sections near the ligation suture were prepared on day 7 following MI induction. The slides were observed under a laser scanning confocal microscope, and the digital images were analyzed using Image-Pro® Plus version 6.0.

2.6. Preparation of recombinant ASP protein

Based on the ASP mRNA sequence encoded by FBN1 (GenBank Accession Nr. 000138), the CDS 8194-8616 region was intercepted by *Escherichia coli* codon preference optimization for whole-gene synthesis. Following this synthesis by GENEWIZ (Jiangsu, China), the DNA fragment was cloned into the expression vector, pET30a (Novagen, Merck, USA), to form the expression plasmid, pET30a-Asp. The plasmid was then transferred into *E. coli* BL21 (DE3) bacterial protein expression host, which was then grown on LB medium; culturing continued at 37 °C and 200 rpm for 4–6 h. The inducer, isopropyl-B d-thiogalactoside, was added to the medium to achieve a final concentration of 0.2 mmol/L. The solution was shaken for 24 h at 16 °C and centrifuged at 5000 rpm. Protein purification was performed under native conditions by HisTrap HP, using the manufacturer's instructions (No. 17-5247-01; GE Healthcare, USA). Endotoxin was removed using an endotoxin-removing column (Pierce High Capacity Endotoxin Removal Spin Columns; Pierce, ThermoFisher, USA). Proteins were desalted and concentrated by centrifugation (cat. no. UFC900396; Millipore), and protein purity was measured using SDS-PAGE and staining with Coomassie Brilliant Blue G-250. The ASP protein was identified via immunoblot using Anti-His antibody, and the endotoxin content was determined by the LAL method. Finally, the obtained protein was divided and stored at −80 °C.

2.7. Measurement of cell proliferation

Cell proliferation was performed using a Cell Counting Kit-8 (CCK-8;

Beyotime Institute of Biotechnology, Nanjing, China) based on the manufacturer's protocol. After overnight serum starvation, MSCs were treated with ASP or vehicle for 24 h, followed by cell washing with PBS and incubation with serum-free medium containing 10% CCK-8 solution in 96-well plates for 2–4 h at 37 °C. The absorbance was detected at 450 nm using a microplate spectrophotometer.

2.8. Two-chamber migration assay

Two-chamber migration assay was performed as described previously [17]. MSCs were treated with different concentrations of ASP for 24 h, and were then seed in transwell inserts (8 μm pore size; cat. no. 3422; Corning Inc., NY, USA) with basal medium with 1% FBS. Complete culture medium with 10% FBS were added to receiver wells as to stimulate migration. After 24 h. cells on the upper surface of inserts were removed, and cells at the bottom of the insert membrane were fixed and stained with 0.1% Crystal Violet Solution (diluted with methanol). The number of migrated cells was quantified by Image-Pro® Plus version 6.0.

2.9. Wound healing assay

Wound healing assay was performed using Culture-Insert 2 Well in μ-Dish 35 mm (cat. no. 81176, ibidi, Martinsried, Germany) with a defined cell-free gap of 500 μm, following the manufacturer's instructions. MSCs were seeded into the Culture-Insert 2 Well. After cell attachment (24 h), the Culture-Insert 2 Well was gently removed using sterile tweezers. MSCs were incubated with vehicle or ASP. Cell migration was monitored using a phase contrast microscope at 0, 12, and 24 h after the creation of a gap.

2.10. Flow cytometry analysis for cell apoptosis assay

Cell apoptosis was assessed using flow cytometry with an Annexin V-FITC/PI staining kit (cat. no. A005-3; 7sea Biotech, China). Briefly, MSCs were exposed to 500 μM H_2O_2 in serum-free medium with or without ASP for 24 h. MSCs were harvested and washed with ice-cold PBS and incubated in FITC-conjugated annexin V binding buffer containing annexin V-FITC and propidium iodide for 20 min at room temperature, away from light. Apoptotic cells in each sample were then analyzed using the flow cytometer.

2.11. Lactate dehydrogenase (LDH) assay

Cell viability analysis was performed using an LDH cytotoxicity assay kit (cat. no. A020-2; Beyotime, China). The culture supernatants were collected and detected as described by the manufacturer. Absorbance was then recorded at 490 nm using a SpectraMax M5 microplate reader (Molecular Devices, Eugene, OR, USA).

2.12. SOD2 activities assay

SOD2 enzymatic activity was assessed using commercial kits (S0103, Beyotime Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions. The protein concentrations were determined using BCA assay. The results were expressed as unit per mg protein (U/mg).

2.13. Determination of ROS generation

MSCs were treated with and without ASP after exposure to H_2O_2. To confirm the signaling pathway, U0126 was added to the culture medium 2 h before ASP treatment. Changes in intracellular ROS levels were determined with the fluorescent probe, DCFH-DA (S0033, Beyotime Institute of Biotechnology, Nanjing, China), for 20 min and captured using a laser scanning confocal microscope at an excitation wavelength of 488 nm, and emission wavelength of 525 nm. Mitochondrial superoxide formation was detected by incubating cells with 5 μM MitoSOX™ (M36008, Invitrogen) in the dark at 37 °C for 10 min. A confocal microscope was used to take the fluorescence photograph (red, 510 nm at excitation and 580 nm at emission). Fluorescence intensity was quantified by Image-Pro® Plus version 6.0

2.14. Real-time PCR (RT-PCR)

24 h post-treatment, total RNA was extracted from cultured cells with an RNA simple Total RNA Kit (cat. no. DP419; TIANGEN), according to the manufacturer's protocol. cDNA synthesis was carried out using Prime Script RT Master Mix (cat. no. RR036A; Takara, Shiga, Japan). RT-PCR was performed with a CFX-96 Content Real-time System using TaKaRa SYBR Premix Ex Taq II (cat. no. RR820A; Takara); GAPDH served as the qPCR amplification endogenous control. PCR primers were purchased from GenScript Biotech Corp. (Nanjing, China); the primer sequences are listed in Table S1. Heml Heatmap Illustrator was used to display the relative mRNA expression as a heat map.

2.15. Western blot

Protein samples were harvested and lysed, after which, their concentration was determined using a BCA Protein Assay Kit (cat. no. 23227; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Proteins were subjected to electrophoresis and transferred to polyvinylidene fluoride membranes, which were incubated with 5% nonfat dry milk in TBST for 2 h at room temperature. Subsequently, an overnight incubation at 4 °C was performed with the following primary antibodies: anti-SOD2 (cat. no. 13141; Cell Signaling Technology), anti-phospho-AKT (cat. no. 4058; Cell Signaling Technology), anti-AKT (cat. no. 2967; Cell Signaling Technology), anti-phospho-extracellular signal-regulated kinase (ERK1/2; cat. no. 9106S; Cell Signaling Technology), anti-ERK1/2 (cat. no. 4696S; Cell Signaling Technology), anti-phospho-AMP-activated protein kinase (AMPK; cat. no. 2531; Cell Signaling Technology), anti-AMPK (cat. no. 2532; Cell Signaling Technology), anti- phospho-PKA (cat. no.5661, Cell Signaling Technology), anti-PKA (cat. no.4782, Cell Signaling Technology) and anti-GAPDH (cat. no. AT0002; CMCTAG). After washing with TBST, the blots were incubated for 2 h with anti-rabbit or anti-mouse secondary antibody (1:1000; cat. nos. ZB-2301 and ZB-2305; Zhongshan Company, China) at room temperature. The blots were then evaluated using a Bio-Rad Imaging System (Hercules, CA, USA).

2.16. Statistical analysis

All data in the text and figures are presented as mean ± SEM, unless otherwise specified. The data were analyzed using GraphPad Prism-6 statistic software (La Jolla, CA, USA). Differences between two groups were analyzed by unpaired, two-tailed *t*-tests, while one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was used to compare differences between three or more groups. Differences where the P value was < 0.05 were considered statistically significant.

3. Results

3.1. Bone marrow-derived cells exhibit characteristics of MSCs

Bone marrow MSCs from C57/6J mice were isolated, cultured and allowed to proliferate. We used bone marrow-derived mononuclear cells from P4 to identify MSCs and detect their purity. Flow cytometry demonstrated that > 90% of P4 MSCs were positive for the surface markers, CD29 and CD44, but negative for the hematopoietic cell markers, CD34 and CD45 (Fig. 1A). To investigate whether MSCs retain the CD29 and CD44 positive phenotype after ASP treatment. We treated MSCs with or without 5 μg/mL ASP for 7 days, and then detected

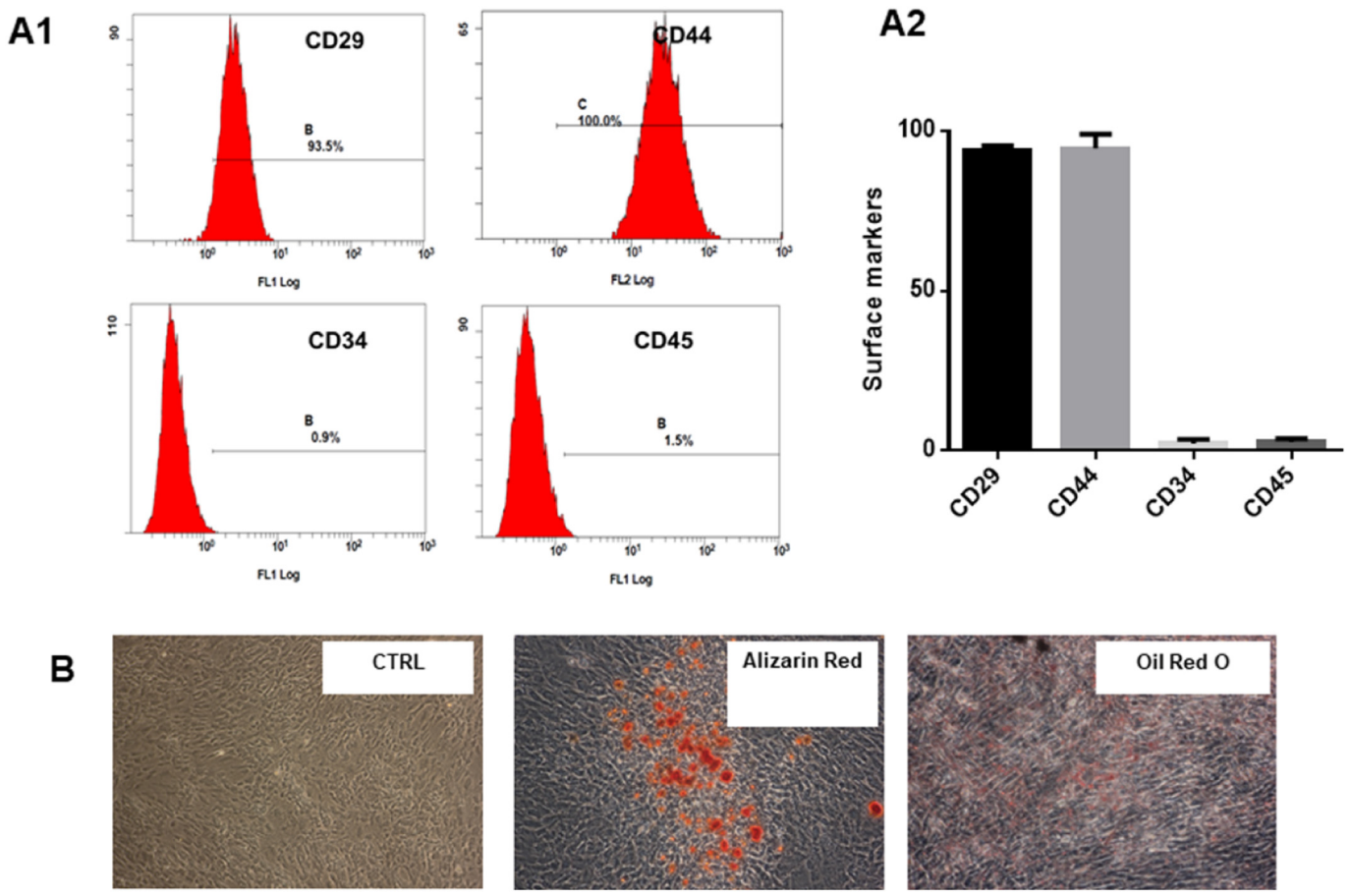

Fig. 1. Characterization and labeling of mesenchymal stromal cells (MSCs).
(A1–2) Surface marker molecules expressed in passage 4 MSCs were analyzed and quantified by flow cytometry. Data are expressed as mean ± SEM (n = 4). (B) Representative image of MSC morphology and their induced differentiation into osteoblasts and adipocytes visualized by alizarin red and oil red O staining, respectively. Images were at original magnification, X100. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surface marker CD29 and CD44. Treatment with ASP did not significantly alter the levels of CD29 and CD44 in MSCs (Fig. S1). MSC pluripotency was confirmed by adipogenic and osteogenic differentiation induced in vitro. As illustrated in Fig. 1B, the observed calcium nodule deposition and lipid droplet respectively stained with Alizarin Red and Oil-Red O indicated the differentiation of MSCs into osteocytes and adipocytes.

3.2. ASP preconditioning enhances MSCs homing after injection

To investigate whether ASP pretreatment could regulate the homing of MSCs in mice with permanent MI, we labelled MSCs with GFP by lentivirus and treated MSCs with vehicle or ASP (Fig. 2A–B). After 24 h treatment, MSCs, vehicle-pretreated MSCs (MSCs-Veh) or ASP-pretreated MSCs (MSCs-ASP) were injected into the infarct border zone. Infarcted hearts from all groups were acquired on day 7 to track the number of MSCs (Fig. 2B). We did not detect any GFP positive cells in samples from the MI group, while we observed cluster distribution of transplanted MSCs (GFP positive cells) in the other three groups (Fig. 2C). There were no significant differences in the number of implanted MSCs between MI + MSCs and MI + MSCs-Veh groups. However, compared to vehicle-treated MSCs, ~2-fold increase in the number of MSCs was detected in the ASP-pretreated MSCs in vivo (Fig. 2C). These results indicate that ASP pretreatment improved homing of MSCs on MI.

3.3. ASP-pretreated MSCs improve the function of cardiac ejection and relieve myocardial remodeling after MI

To furtherly investigate the effect of ASP on MSC-mediated cardioprotective effects, we evaluated LVEF in the heart of mice using transthoracic M model echocardiography on day 28 following MI induction. As illustrated in Fig. 3A, when the MSCs pretreated with ASP were injected into infarcted hearts, cardiac function of mice was significantly improved compare to MSCs pre-treated with vehicle. Next, we proceeded to analyze cardiac fibrosis and remodeling on day 28. As shown in Fig. 3B, the percentage of cardiac fibrosis in the ASP-treated MSC group was significantly reduced compared to the vehicle-treated MSCs. Collectively, the above results suggested that pretreatment with ASP significantly augmented MSC-mediated curative efficacy on MI.

3.4. ASP does not affect MSC proliferation and migration

MSC proliferation and migration are important mechanisms in cell transplantation therapy [18]. To investigate the effect of ASP on MSC proliferation, we added different doses of ASP (0 μg/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL) into the culture medium for 48 h after overnight serum starvation. A CCK-8 assay was then performed, which showed that ASP did not alter MSC proliferation both at low and high concentrations (Fig. 4A). Subsequently, we proceeded to explore the role of ASP in MSC migration. As illustrated in Fig. 4B, the two-chamber migration assay showed that ASP treatment for 24 h did not alter migratory capacity of MSCs. Moreover, treatment of MSCs with various

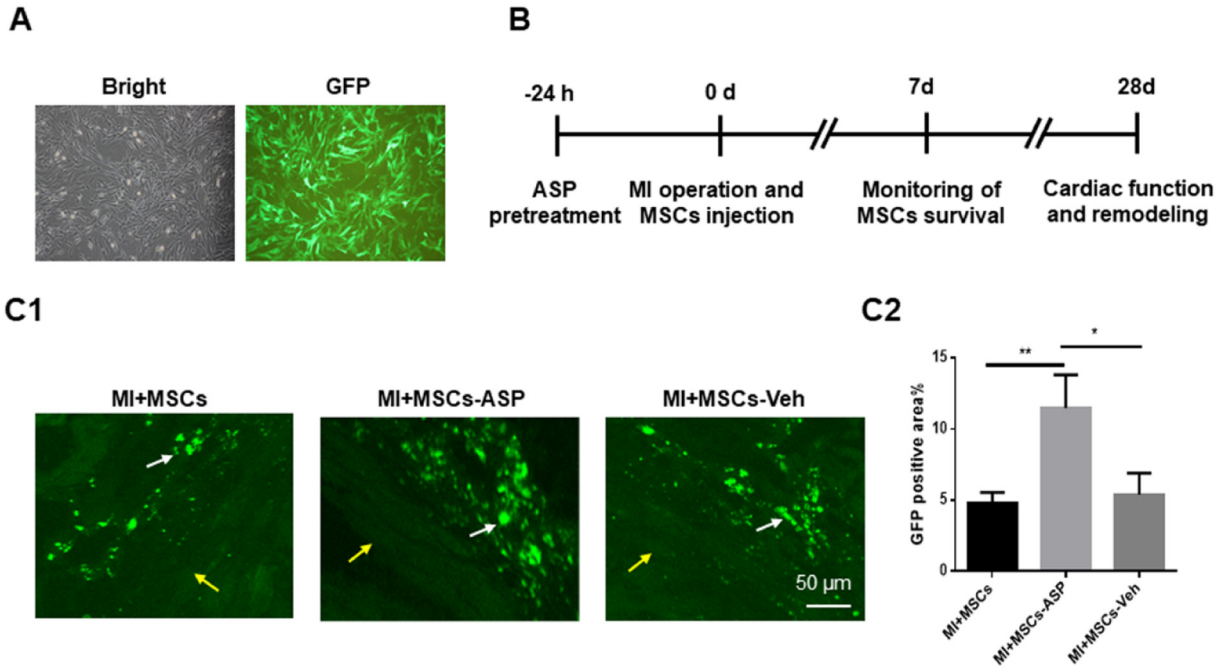

Fig. 2. ASP pretreatment improve survival of MSCs after MI.
(A) MSCs were labelled with GFP by lentivirus infection in vitro. Images were at original magnification, X100. (B) The experimental design. (C1–2) Representative images of MSCs and their quantification in the heart at 7 days after MI induction (n = 6). White arrows showed MSCs (GFP). Yellow arrows showed cardiomyocytes (autofluorescence). All data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

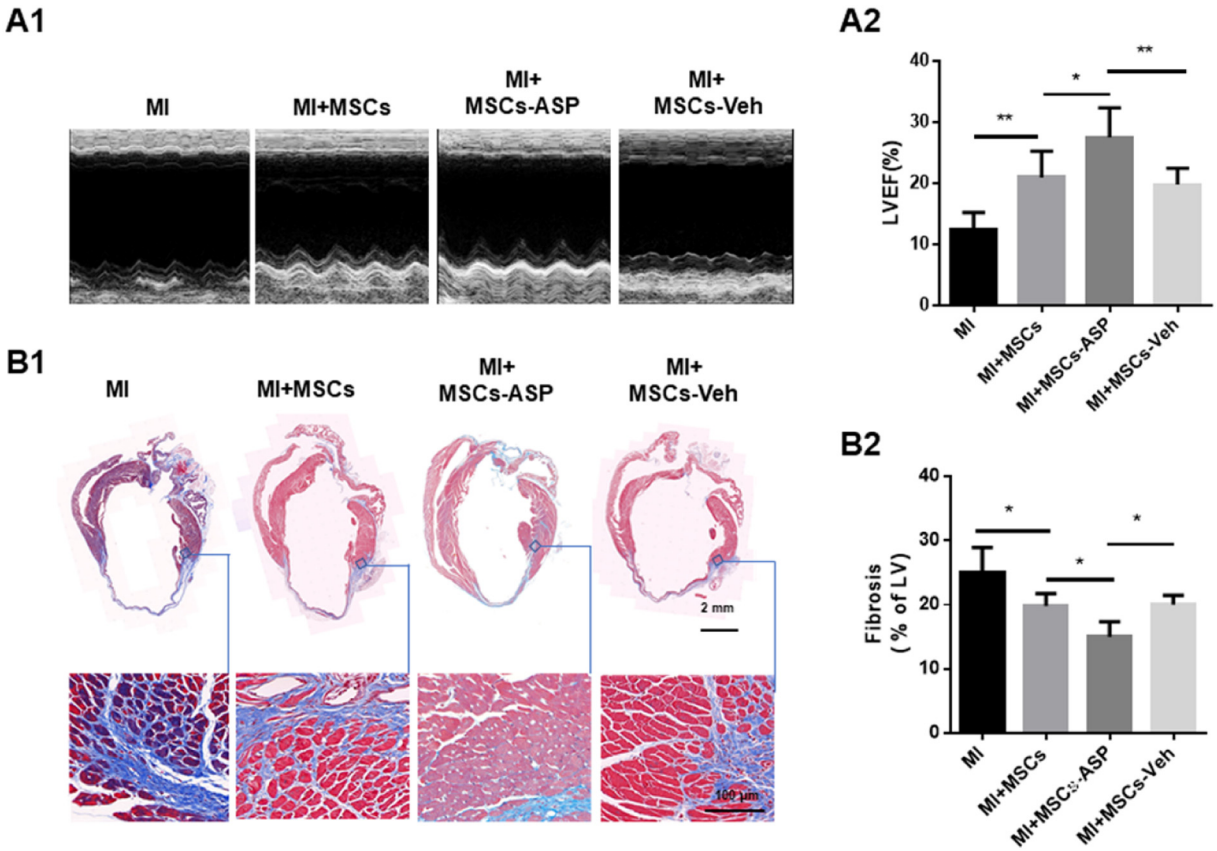

Fig. 3. ASP-pretreated MSCs enhance the therapeutic effects of cell transplantation in MI due to improved survival of MSCs in vivo.
(A1–2) Echocardiographic analysis of the left ventricle ejection fraction at 4 weeks after MI induction (n = 7). (B1–2) Representative confocal microscopic images and quantification of cardiac fibrosis 28 days after MI induction (n = 5). All data are expressed as mean ± SEM. $*P < 0.05$, $**P < 0.01$.

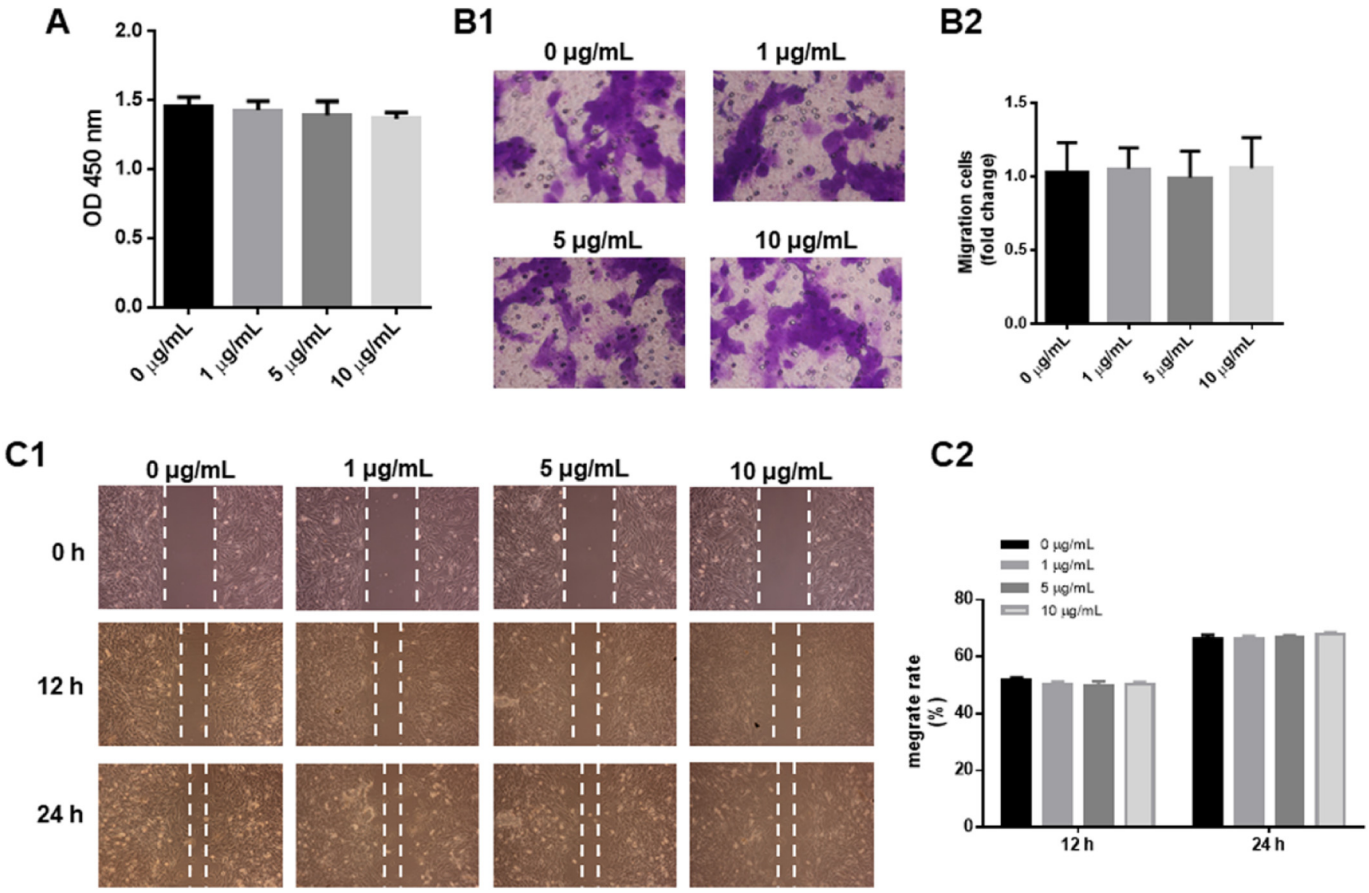

Fig. 4. ASP does not regulate proliferation and migration of MSCs.
(A) The direct effects of CTPR3 on MSC proliferation were measured and analyzed by a CCK-8 assay (n = 6). (B) Cell migration was detected by two-chamber migration assay after 24 h treatment with or without ASP. (n = 5). Images were at original magnification, X400. (C1–2) Wound healing assay illustrated MSC migration after ASP treatment at different time intervals (n = 5). Images were at original magnification, X100. All data are presented as mean ± SEM.

concentrations of ASP did not promote MSC migration at 12 h and 24 h as evidenced by wound healing assay (Fig. 4C). These data suggest that ASP may not regulate MSC proliferation and migration.

3.5. ASP reduces H_2O_2-induced damage and apoptosis of MSCs

To explore the effect of oxidative stress on damages to MSC, time gradients were established to elucidate the optimal experimental conditions. As shown in Figs. 5A, 6 h treatment with 500 μM H_2O_2 significantly altered the cell morphology and reduced cell density. The CCK-8 assay indicated that H_2O_2 treatment inhibited cell growth, with the 24 h treatment (500 μM H_2O_2) resulting in cell damages of approximately 50% compared to the control group (Fig. 5A). To investigate the effect of ASP on H_2O_2-induced MSC injury, MSCs were incubated with various concentrations of ASP, and cell viability was measured by a CCK-8 assay. Administering 5 μg/mL ASP significantly recovered cell viability, but higher doses (10 μg/mL) did not yield a better performance (Fig. 5B). Further, we chose 5 μg/mL as an optimal ASP concentration in vitro experiment. To further confirm H_2O_2-induced apoptosis/death could be inhibited by ASP, Annexin V/propidium iodide (PI) staining and LDH assay were performed. As shown in Fig. 5C, H_2O_2 exposure increased the number of MSCs in Quadrants 2 and 3 (Q2 and Q3) compared to the control group, suggesting that H_2O_2 induced early and late apoptosis. When MSCs were co-incubated with 5 μg/mL Asp for 24 h, the percentage of apoptotic cells significantly decreased (Fig. 5C3)). In addition, ASP induced a significant reduction in H_2O_2-induced LDH release (Fig. 5D). Altogether, the above data demonstrate that ASP has a protective effect on H_2O_2-induced MSC injury.

3.6. ASP upregulates SOD2 expression and activates the ERK1/2 and PI3K/AKT pathways

Since we have demonstrated that ASP can protect MSCs from oxidative injury, we further investigated the molecular mechanisms by which this occurs. After MSCs were treated with ASP for 24 h, RT-PCR was performed to detect the gene expression levels of 21 molecules associated with antioxidation (i.e., MT1, MT2, MT3, SOD1, SOD2, SOD3, Nrf1, Nfe2l2, Txn2, Hmox1, Nqo1, Gpx1, Gpx4, Prdx1), and cell death (i.e., Bcl2, Bcl2l1, Dapk1, Stk17b, Becn1, Map1lc3a [LC3], Sqstm1 [p62]); the obtained results are presented as a heat map (Fig. 6A). SOD2 and MT2 mRNA levels were significantly upregulated in the ASP treatment group compared to the vehicle group (Fig. 6B). However, when exposed to H_2O_2, mRNA expression of the anti-oxidase SOD2, and not MT2, was downregulated (Fig. 6C). These data indicate that SOD2 may play a critical role in regulating oxidative injury. Importantly, we also demonstrated that ASP treatment upregulated SOD2 protein expression and activity in MSCs (Fig. 6D and E), suggesting that SOD2 may be the target protein mediating ASP cytoprotection on MSCs.

We furtherly examined the upstream signal enzymes involved in cell survival. As shown in Fig. 7A, ASP can activate the ERK1/2 and AKT pathways, but not the AMPK or PKA pathways, after a 15 min incubation. In the presence of the ERK1/2 inhibitor, U0126, the ASP-induced upregulation of SOD2 in MSCs was strongly inhibited (Fig. 7B and C). However, the AKT activation inhibitor did not inhibit SOD2 (Fig. 7D and E), suggesting that ERK1/2, and not AKT, may be responsible for

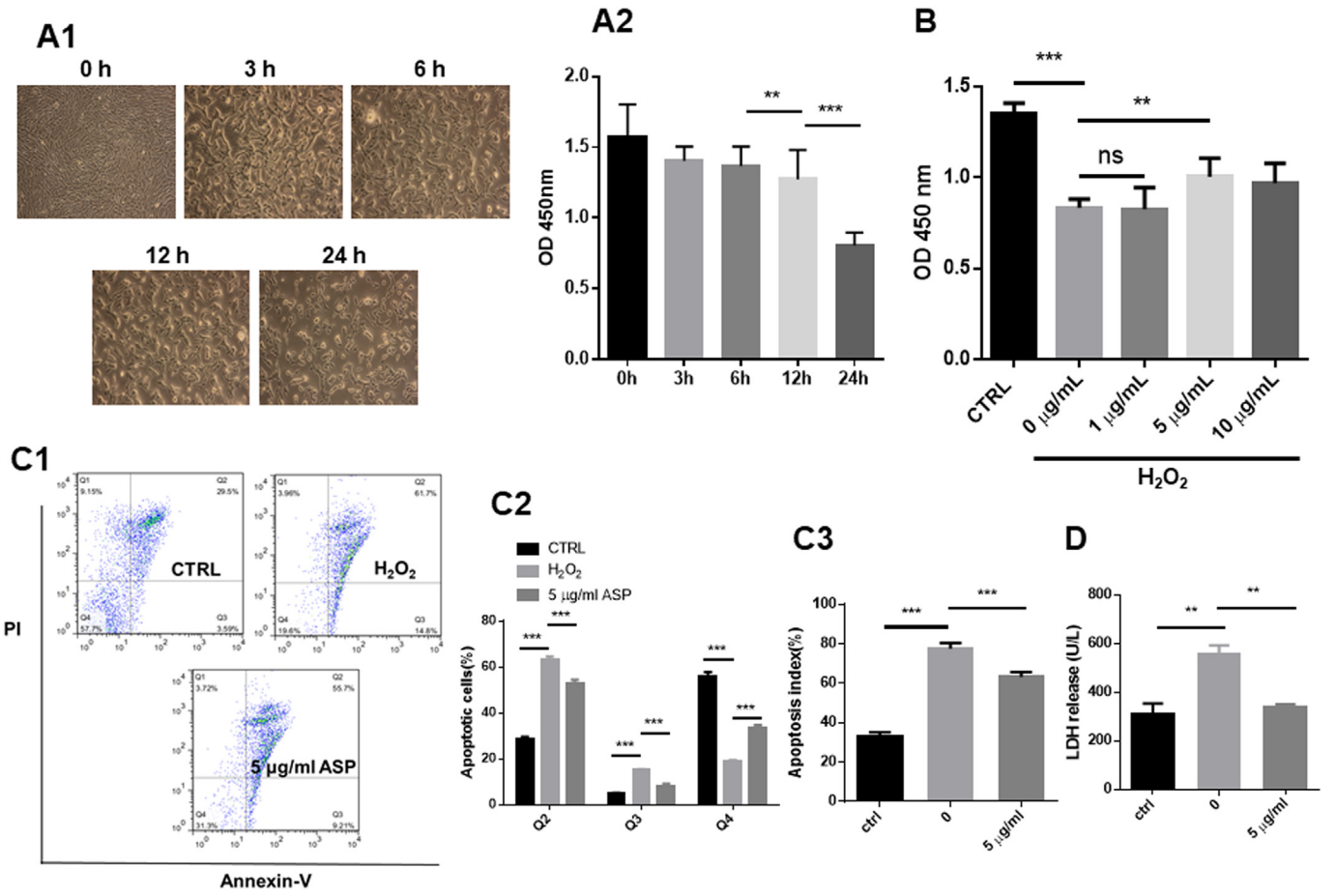

Fig. 5. ASP treatment protects MSCs against H_2O_2-induced cell apoptosis and death.
(A1–2) Cell morphology and CCK-8 assay results of MSC treated with 500 μM H_2O_2 at different time intervals (3, 6, 12, 24 h) (n = 10). Images of phase contrast were at original magnification, X100. (B) CCK-8 assay of cells treated with ASP (0, 1, 5 or 10 μg/mL) and co-incubated with 500 μM H_2O_2 for 24 h (n = 10). (C1–3) Apoptosis was quantified by flow cytometry (n = 4). (D) MSC viability was determined by LDH release (n = 4). All data are presented as presented mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant.

the anti-apoptosis process exhibited by ASP.

3.7. ASP inhibits H_2O_2-induced ROS production and MSC apoptosis via the activated ERK1/2 pathway

To further elucidate whether the ERK1/2-SOD2 pathway is involved in the anti-apoptotic action exhibited by ASP on MSCs, we examined the level of total ROS and mitochondrial ROS under oxidative stress. As shown in Fig. 8A, the H_2O_2-induced increase in ROS level was significantly lowered when MSCs were pretreated with ASP. Moreover, an increase in ROS level was observed in the presence of the ERK1/2 specific inhibitor, U0126, when compared to the group pretreated with ASP. Similar experiment was performed to measure the production of the superoxide radical, the result showed that H_2O_2 treatment dramatically increased mitochondrial ROS level relative to control group. ASP treatment reduced H_2O_2-induced mitochondrial ROS generation, which was inhibited by U1026 (Fig. 8B). The above data suggest that ASP can modulate ROS generation by activating the ERK1/2 signaling pathway. Meanwhile, we quantified the apoptotic cells in the absence or presence of U0126. U0126 significantly inhibited the protective effect of ASP pretreatment on MSC apoptosis caused by H_2O_2 (Fig. 8C). Together, these data demonstrate ASP pretreatment activates ERK1/2 pathway in MSCs to protect against oxidative injury (Fig. 9).

4. Discussion

The present study aimed to elucidate whether ASP, a newly-discovered glucogenic protein hormone, can regulate the functions of MSCs and influence the efficacy of MSC therapy in MI. Our main finding is that ASP-pretreated MSCs are more resistant to apoptosis and death induced by both oxidative stress in vitro and myocardial ischemia in vivo, indicating that ASP may be a cytoprotective factor of MSC transplantation following MI. Furthermore, the improved survival of the implanted MSCs by ASP preconditioning was positively associated with MSC-mediated cardiac protection. Mechanistically, our data show that ASP pretreatment activates the ERK1/2 signaling pathway to up-regulate the antioxidant protein, SOD2, and reduce ROS production, consequently inhibiting cell apoptosis/death.

MSCs are considered the major candidate cell type for cardiac protection because of their superior biological properties; these include ease of accessibility, low immunogenicity, lack of ethical concerns regarding their use, paracrine action and potent differentiation [19]. Many animal and clinical studies have confirmed that MSC transplantation is effective when used to treat cardiovascular disease. However, the curative efficacy seems to depend on the number of surviving MSCs in the detrimental microenvironment after MI induction; this includes, but is not limited to, hypoxia and oxidative stress [3,20]. To address this challenge, investigators have been focused on improving the survival and retention of injected MSCs using several strategies, such as genetic manipulation, biomaterials, and preconditioning. Given that

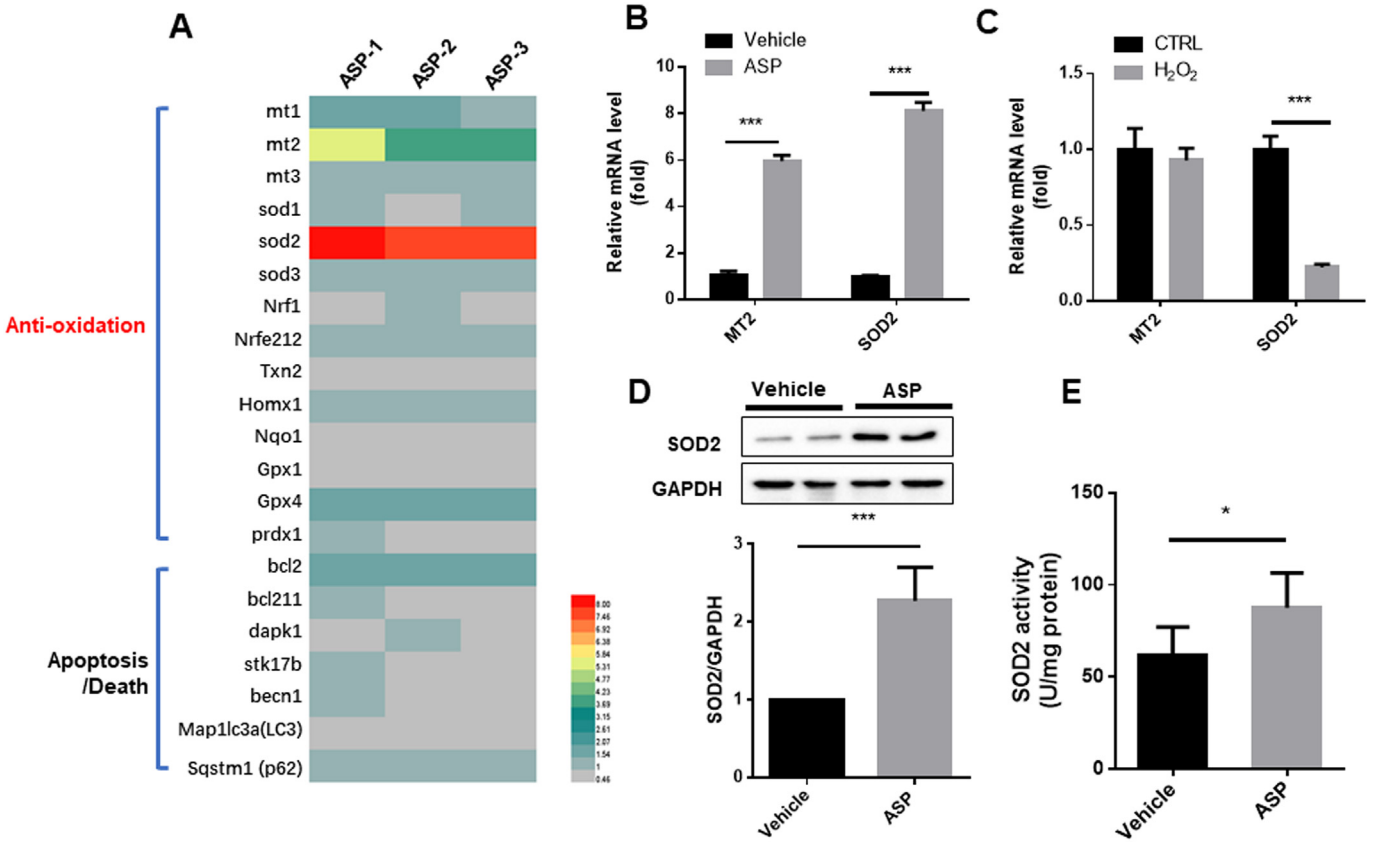

Fig. 6. ASP treatment upregulates SOD2 expression in MSCs.
(A) RT-PCR was used to analyze the mRNA expression levels of 21 genes related to cell apoptosis, and anti-oxidative capacity 24 h after ASP treatment. The gene expression levels were normalized to that of GAPDH (n = 3). (B) mRNA expression levels of genes screened from (A) were shown using a histogram. (C) Relative mRNA expression of SOD2 and MT2 in MSCs exposed to H_2O_2 (n = 4). (D) Western blot analysis in MSCs treated with ASP or vehicle alone (n = 6). (E) SOD2 activity in MSCs treated with ASP or vehicle alone (n = 6). All data are presented as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant.

gene manipulation has a tumorigenic risk, cell preconditioning is considered safe, and is accompanied by a low-cost [19,21,22]. A great need therefore exists to identify new pretreatment targets to promote MSC engraftment and survival.

Adipokines (specific cytokines secreted by adipocytes) have been reported to regulate MSC proliferation, migration, differentiation, autophagy and apoptosis [10,12,23]. Treating MSC with several adipokines improves cardiac function and structure and increases cell engraftment and the survival of MSCs [12,24]. Encoded by the FBN1 gene, ASP is a ~30 kDa protein composed of 140-amino-acid and the C-terminal cleavage product, profibrillin. In addition, ASP has been identified as a novel adipokine secreted by white adipose tissue [8], with well-established effects on the regulation of glucose release from the liver, and appetite stimulation [8,9]. Moreover, plasma ASP levels are significantly increased in obese subjects and in those with type 2 diabetes, suggesting that ASP might serve as a risk factor associated with obesity and type 2 diabetes [8,25]. ASP could also be a new biochemical marker for predicting the severity of acute coronary syndrome in unstable angina pectoris [26], but it is unclear whether it affects the efficacy of cell therapy for ischemic heart disease. Furthermore, its role in injected MSCs under ischemic conditions has not been explored. In this study, we treated MSCs with ASP and transplanted them into the heart of mice after MI induction. The results indicated that preconditioning with ASP significantly improves the survival rate of MSCs in the ischemic microenvironment, thereby increasing the therapeutic effect of MSCs in MI. These data suggest that the newly-discovered adipokine, ASP, may serve as a promising target for cell therapy in ischemic heart disease.

The imbalance between antioxidants and oxidants in an infarcted heart induces oxidative stress and increases intracellular ROS levels under ischemic conditions [27]. Under physiological conditions, small quantities of ROS are involved in intracellular signal transduction. However, the over-production of ROS triggers various pathological processes such as premature senescence, anoikis, decreased differentiation and migratory potential, apoptosis and death of MSC [28,29]. Moreover, recent evidence suggests that exogenous ROS significantly alters cytokine secretion and impairs the immunosuppressive ability of MSCs in patients with atherosclerosis [30]. Elimination of ROS has been proven to promote MSC engraftment and enhance tissue repair in the ischemia model [7,30,31]. Hence, enhancing the antioxidative ability and survival of transplanted MSC under ischemic or oxidative stress conditions may be crucial in improving the efficiency of cell therapy [12,32]. Consistent with previous researches, our study indicated that ASP treatment can reduce ROS production, protect MSCs against oxidative stress-induced cell apoptosis/death and as a result, improve MSC survival/retention in vivo. We examined MSC proliferation and migration in our experiments, but did not find a significant difference between ASP-treated MSC and vehicle-treated MSC. Hence, ASP-mediated cell protection, rather than cell proliferation and migration, contributes to the effects of ASP on MSCs.

To investigate the molecular mechanisms responsible for the protective effects of ASP on MSCs, we screened the levels of 21 mRNAs relative to MSC apoptosis/death and examined their anti-oxidative effects; two antioxidant enzymes, MT2 and SOD2, were removed. When exposed to oxidative conditions, only the expression level of SOD2 was reduced, suggesting that a low expression of SOD2, but not MT2, is involved in oxidative-induced MSC injury. SOD2 is an important antioxidant enzyme for the removal of mitochondrial ROS, conferring

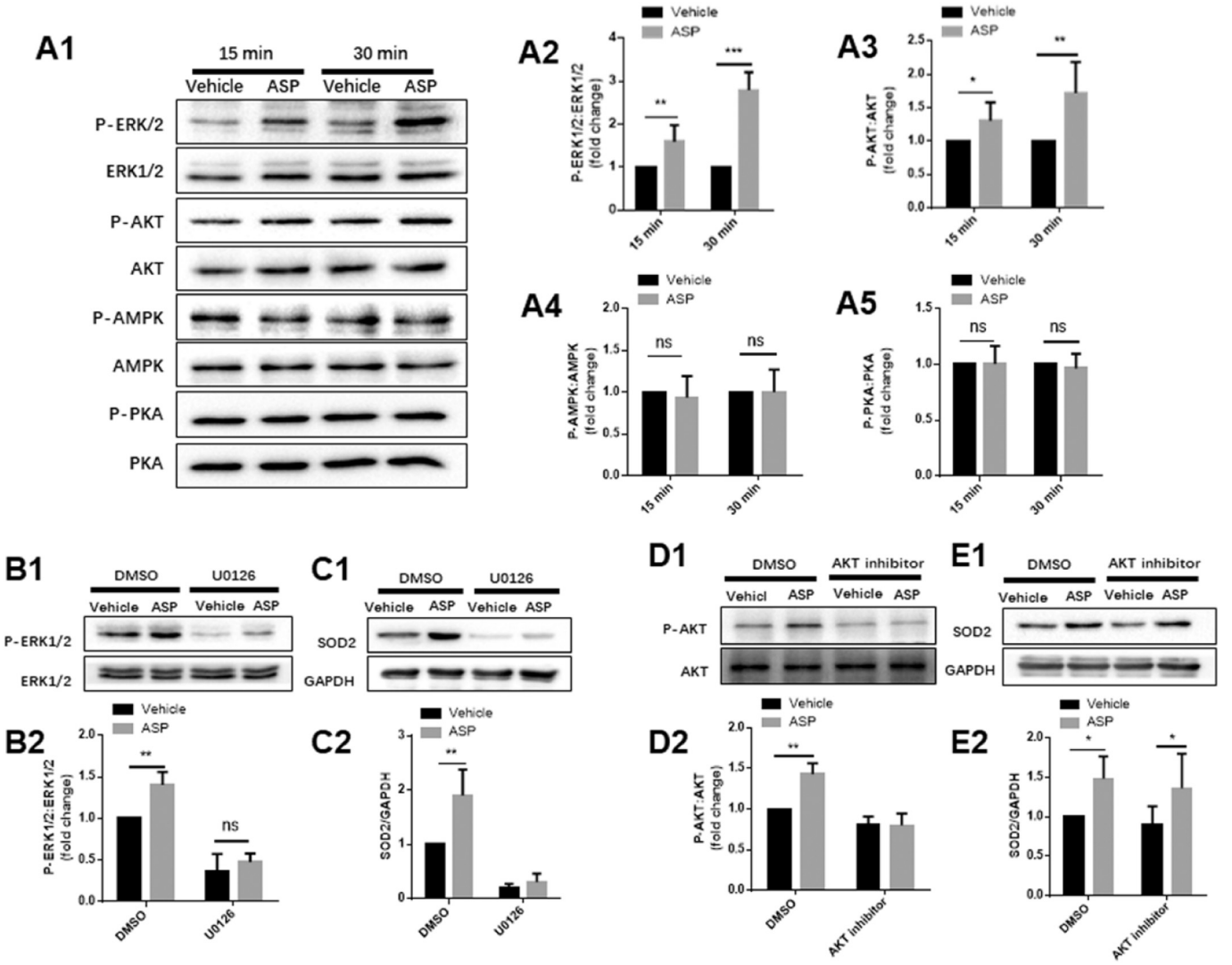

Fig. 7. ERK1/2, and not AKT, is the upstream kinase for SOD2 regulation by ASP in MSCs.
(A1–5) Western blot analysis of p-Akt/Akt, p-ERK1/2/ERK1/2, p-AMPK/AMPK and p-PKA/PKA following ASP (n = 5). (B1–2) Western blot analysis and quantitative analysis showed that U0126 effectively blocked ERK activation. (C1–2) Effects of U0126 (an ERK1/2 inhibitor) on ASP-mediated upregulation of SOD2. GAPDH served as a control (n = 4). (D1–2) Western blot analysis showed that an AKT inhibitor completely blocked AKT phosphorylation (n = 4). (E1–2) Effects of AKT inhibitor on ASP-induced upregulation of SOD2. GAPDH served as the control (n = 6). All data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

protection against cell apoptosis/death. Previous studies have shown that SOD2 expression induced by gene modification or preconditioning improves the efficacy of MSCs [33,34]. Our data showed that ASP treatment can recover the downregulated intracellular SOD2 level induced by H_2O_2. Furthermore, we observed several key pro-survival kinases that were previously reported [35–37] and found that the ERK1/2 and AKT pathways are activated by ASP. To proceed, we then employed U0126 and an AKT inhibitor to inhibit the corresponding kinases. The results demonstrated that ASP treatment activated the ERK1/2-SOD2 signaling pathway to resist H_2O_2-mediated ROS generation and cell apoptosis. Consistent with the in vitro study, the enhanced protection of MSCs-ASP against MI was lost by the ERK1/2 inhibitor.

The use of a pretreatment method to understand the direct effect of ASP on MSCs and the injection of pretreated MSCs into ischemic heart served as a limitation in the study. Therefore, a more effective treatment strategy might entail combining MSC transplantation with the systemic administration of ASP (the focus of our next research) to elucidate the response to oxidative stress. In addition, the more detailed mechanism in signaling cascade should be elucidated in further studies.

5. Conclusions

In conclusion, through our findings, we have identified a novel function of ASP; it is a positive modulator of MSC survival. Importantly, preconditioning with ASP enhanced the survival of MSC in vivo as well as the curative efficacy of MSC for the treatment of ischemic heart disease.

Abbreviations

MSCs	mesenchymal stromal cells
FBS	fetal bovine serum
GFP	green fluorescent protein
MI	myocardial infarction
ASP	asprosin
PBS	phosphate-buffered saline
LVEF	left ventricular ejection fraction

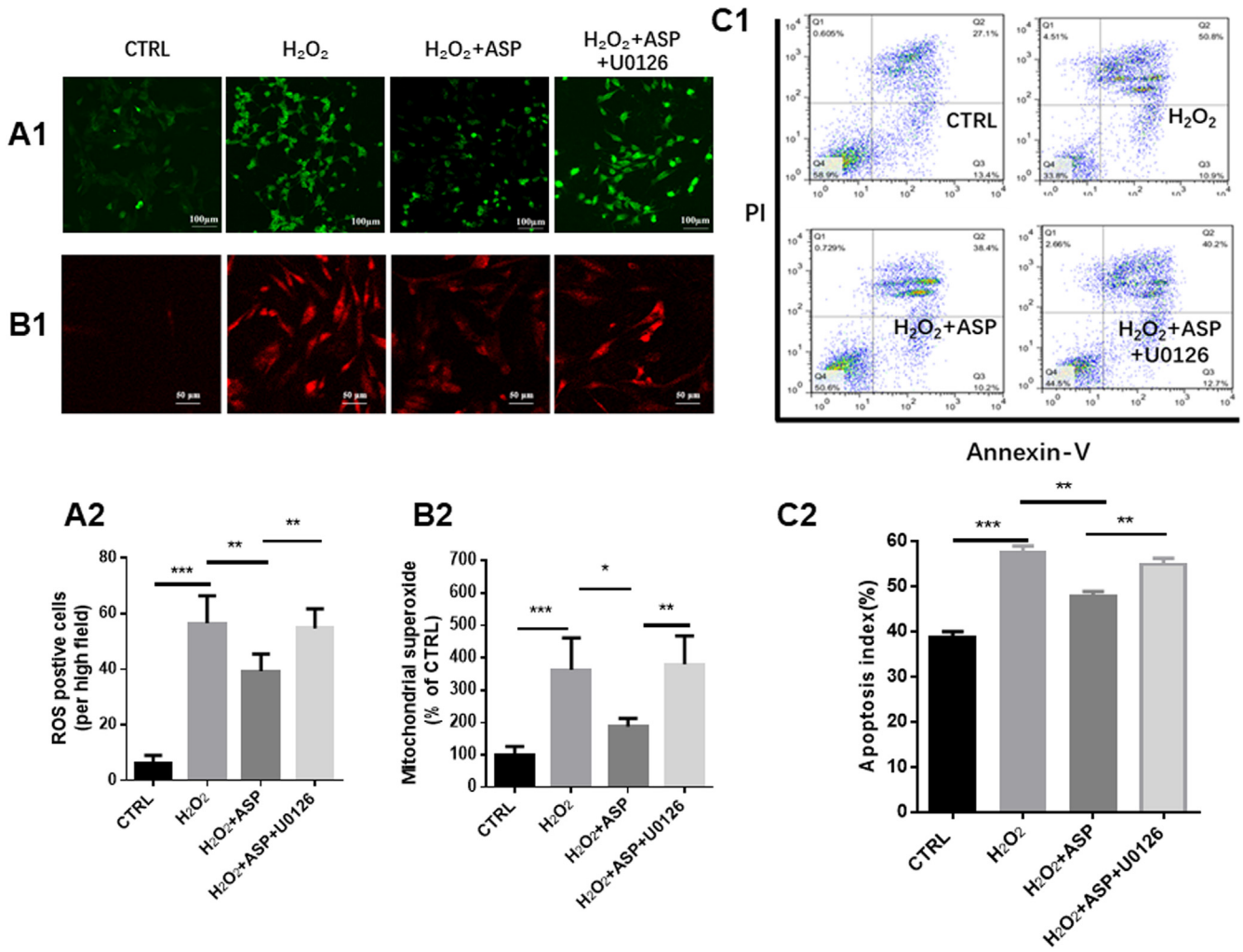

Fig. 8. ASP reduces ROS production and apoptosis in an ERK1/2-dependent manner.
(A) ASP suppressed H_2O_2-induced ROS production, but this was blocked by U0126 (n = 6). (B) Mitochondrial superoxide was measured by MitoSOX™ Red staining (n = 4). (C) Apoptosis was measured by flow cytometry (n = 4). All data are presented as mean ± SEM. All data are presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

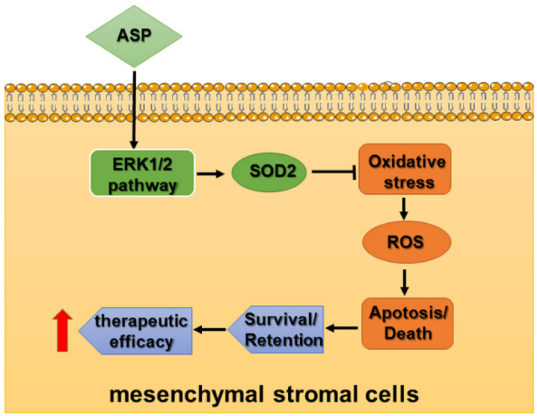

Fig. 9. Diagram of the enhanced effects exhibited by MSCs on myocardial repair induced by ASP pretreatment.
ASP activated the ERK1/2/SOD2 pathway to decrease ROS-induced cell apoptosis, improve the survival of transplanted MSCs and enhance their therapeutic efficacy. ASP preconditioning may be a novel therapeutic strategy for MSC transplantation in MI.

CCK-8 Cell Counting Kit-8
LDH lactate dehydrogenase
RT-PCR Real-time PCR
c-caspase3 cleaved caspase 3
Q2 and Q4 Quadrants 2 and 4

Authors' contributions

Conceptualization, Zhengbin Zhang, Wei Yi and Yang Sun; Data curation, Liwen Zhu and Bing Zhang; Formal analysis, Zhengbin Zhang and Liwen Zhu; Funding acquisition, Wei Yi and Yang Sun; Investigation, Zhengbin Zhang, Yanzhen Tan, Liwen Zhu and Bing Zhang; Methodology, Yanzhen Tan, Bing Zhang, Pan Feng and Erhe Gao; Supervision, Xiaoming Wang, Wei Yi and Yang Sun; Validation, Xiaoming Wang; Visualization, Zhengbin Zhang, Yanzhen Tan, Pan Feng and Chennian Xu; Writing original draft, Zhengbin Zhang.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116554.

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