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N-Acetylcysteine enhances the therapeutic efficacy of bone marrow-derived mesenchymal stem cell transplantation in rats with severe acute pancreatitis

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ABSTRACT

Background: Severe acute pancreatitis (SAP) is a high mortality disease, for which there is a lack of effective therapies. Previous research has demonstrated that bone marrow-derived mesenchymal stem cells (BMSCs), which have immunomodulatory and antioxidant properties, have potential for the treatment of SAP. It remains unclear, however, whether the free radical scavenger *N*-acetylcysteine (NAC) can enhance the therapeutic efficacy of BMSC transplantation in SAP. In this study, we investigated the effect of combining treatment with NAC and BMSCs in a rat model of SAP.

Methods: SAP was induced by injection of sodium taurocholate into the pancreatic duct and, after successful induction of SAP, the rats were treated with BMSCs and NAC, either singly or in combination.

Results: After 3 days, serum levels of amylase, proinflammatory factors, malondialdehyde, and reactive oxygen species were significantly decreased in animals treated with BMSCs or NAC, compared with vehicle-treated animals. In contrast, total glutathione, superoxide dismutase and catalase were markedly increased after treatment with BMSCs or NAC. However, oxidative stress markers and inflammatory factors were significantly improved in the SAP + BMSCs + NAC group compared with those in the SAP + NAC group and the SAP + BMSCs group.

Conclusions: Combined NAC and BMSC therapy was found to alleviate oxidative stress damage to the pancreas and to inhibit the inflammatory response to a significantly greater extent than single therapy with either BMSCs or NAC. Because NAC enhances the therapeutic efficacy of BMSC transplantation in a rat model of SAP, combined therapy may provide a promising new approach for the treatment of SAP.

Introduction

Acute pancreatitis (AP) is a common acute abdominal disease, that is usually mild and self-limiting. Approximately 10–20% of AP patients, however, develop severe acute pancreatitis (SAP), which is associated with poor prognosis and mortality rates as high as 15–25% [1]. Despite significant improvements in the treatment of AP, the high mortality of patients with SAP remains a major problem worldwide [2]. Additionally, even after recovering from SAP, patients often have impaired pancreatic exocrine and endocrine function [3]. There is, therefore, an urgent need to identify a better treatment measures for SAP.

Oxidative stress is evident in the early phase of pancreatitis and acinar cells have been shown to produce a large number of reactive oxygen species (ROS) [4]. ROS act as a molecular trigger of pancreatitis because they directly attack biological membranes and also trigger the accumulation of neutrophils and their adherence to the capillary wall. ROS thus play a key role in perpetuating pancreatic inflammation and the development of extrapancreatic complications [5]. ROS can also lead to activation of the NF-κB signaling pathway and amplification of the inflammatory response during the course of pancreatitis [6]. Inhibition of oxidative stress

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should, therefore, be of significant benefit in the treatment of SAP.

N-acetylcysteine (NAC), a precursor of glutathione (GSH) synthesis and thus an ROS scavenger [7], has been shown to inhibit expression of cytokines and chemokines and to alleviate pancreatic injury in different experimental models of pancreatitis [8–10].

Mesenchymal stem cells (MSCs) are adult stem cells that have the potential for self-renewal and multilineage differentiation, together with low immunogenicity. These have been demonstrated that MSCs have significant therapeutic effects on a number of diseases, including pulmonary injury, acute liver failure and myocardial infarction [11–13]. Previous research have also revealed that MSCs can repair pancreatic injury and exert anti-inflammatory and antioxidant effects in the mild and severe AP [14,15]. When used alone, MSCs may, however, provided insufficient therapeutic effects because limited numbers of MSCs can be transplanted, ROS and inflammatory cytokines may affect the survival and differentiation of the MSCs after transplantation [16–18]. Meanwhile, preconditioning of MSCs with NAC has been found to be cytoprotective [19–21]. In current study, therefore, we investigated the effect of a combination of NAC and bone marrow-derived mesenchymal stem cells (BMSCs) for the treatment of rats with SAP.

Methods and materials

Animals

Male Sprague-Dawley (SD) rats, aged 4–6 weeks and weighing 100–150 g, were used as BMSC donors. Male SD rats, aged 8–10 weeks and weighing 200–250 g, were used as BMSC recipients. All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. The study was approved by the Animal Ethics Committee of Shanghai Tenth People's Hospital affiliated to Tongji University.

Establishment of SAP model and experimental design

The SAP model was established as previously described, by injection of sodium taurocholate into the pancreatic duct [22]. Briefly, all animals were underwent fasting for at least 12 h and water deprivation at least 4 h prior to induction of SAP. Anesthesia was induced by intraperitoneal injection of 3% pentobarbital, and then freshly-prepared 3% sodium taurocholate (Sigma-Aldrich, St. Louis, MO, USA) was injected into the common biliopancreatic duct (1 mL/kg) under steady pressure at a speed of 0.15 mL/min. All procedures were performed under sterile condition and process, and the severity of SAP was evaluated by pathology.

After induction of SAP, the rats were randomly divided into four groups, with 8–10 rats in each group: SAP + Vehicle group (SAP model treated with vehicle), SAP + NAC group (SAP model treated with NAC), SAP + BMSCs group (SAP model treated with BMSCs), SAP + BMSCs + NAC group (SAP model treated with BMSCs plus NAC). An NC group (normal control group) was also included in the study. Six hours after induction of SAP, either phosphate-buffered saline (PBS) or BMSCs (1×10^6) in PBS (200 μL) was transfused into the caudal vein over a period of 2 min. NAC (100 mg/kg; Selleck Chemicals, Houston, TX, USA) was administered intraperitoneally 6 h after induction of SAP [23]. Rats in the NC group were injected with an equal volume of normal saline. Rats were euthanized 72 h after SAP induction and pancreatic tissues and blood samples were collected.

Isolation, culture and identification of BMSCs

BMSCs were isolated, identified and cultured as we described previously [22,24]. Briefly, BMSCs were isolated aseptically from the femurs and tibias of Sprague–Dawley rats after the animals were killed by cervical dislocation. The femurs and tibias were excised and the soft connective tissue was removed, then the bone marrow cells were harvested by flushing the marrow cavity with low glucose Dulbecco's modified Eagle's medium (DMEM; Gibco/Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco), and when fully suspended were filtered through a 200 μm screen mesh. Cells were collected by gradient centrifugation, the supernatant was removed, the pelleted cells were resuspended and seeded in growth medium containing DMEM supplemented with 10% fetal bovine serum, streptomycin (100 mg/mL) and penicillin (100 U/mL), and cultured at 37 °C with 5% CO_2. Non-adherent cells were removed after the first 48 h, and thereafter, the medium was changed every 72 h. When the cells reached 80% confluence, at around 10 days, they were passaged at a ratio of 1:2. The third passage cells were identified by BMSC markers and differentiation assay. Surface markers (CD44, CD90 and CD34) were detected using flow cytometry. Adipogenic and osteogenic differentiation *in vitro* were identified by maintaining third generation BMSCs in adipogenic and osteogenic differentiation medium, followed by Oil Red O staining and Alizarin Red S staining (Cyagen Biosciences, Sunnyvale, CA, USA), respectively. BMSCs obtained between the third and fifth passages were used.

Determination of wet-to-dry ratio of pancreatic tissue

Water content in pancreatic tissue was measured to estimate the severity of pancreatic edema. As soon as the rats were sacrificed, the fresh pancreatic samples were weighed. The pancreatic tissue was then dried at 60 °C for 48 h and weighed to obtain the dry weight. The wet/dry weight ratio of the pancreas was calculated to indicate the degree of pancreatic edema.

Pathological analysis

Pancreatic samples were fixed in 4% phosphate-buffered formaldehyde and processed in paraffin-embedded sections. The 5 μm-thick sections were stained with hematoxylin and eosin (H&E) for routine histology. For each animal, three independent sections were evaluated by three pathologists in a blinded manner, using the pathological criteria for pancreatitis. The severity of AP was graded as previously described and the results are shown in Table 1: pathological score = edema score + necrosis score + inflammatory cellular infiltration score + vacuolization score [25].

Table 1
Histological scoring for severe acute pancreatitis.

Edema	Inflammatory cellular infiltration	Vacuolization	Necrosis
0 = absent	0 = absent	0 = absent	0 = absent
1 = diffuse expansion of interlobar septa	1 = around ductal margin	1 = periductal, < 5%	1 = 1-4necrotic cells/HPF*;
2 = diffuse expansion of interlobular septa	2 = in parenchyma, < 50% of lobules;	2 = focal, 5%–20%	2 = 5-10necrotic cells/HPF
3 = diffuse expansion of interacinar septa	3 = in parenchyma,50%–75% of lobules	3 = diffuse, 21%–50%	3 = 11–15 necrotic cells/HPF
4 = diffuse expansion of intercellular septa	4 = in parenchyma, > 75% of lobules	4 = severe, >50%	4 = ≥16 necrotic cells/HPF

Western blot analysis

Pancreatic tissues were lysed with RIPA lysis buffer and phenylmethanesulfonyl fluoride (Beyotime, Nantong, China), and protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Total proteins from the samples were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and incubated with primary antibodies against Bax, Bcl2, NF-κBp65, IKKβ, HO-1 and β-actin at 4 °C overnight. Bax and Bcl2 antibodies were purchased from Cell Signaling Technology Company (Danvers, MA, USA), others antibodies were purchased from Abcam Company (Cambridge, MA, USA). After incubation with secondary antibodies, the nitrocellulose membranes were analyzed using Odyssey 3.0 software (LI-COR Biosciences, Lincoln, NE, USA).

Biochemical analysis of rat serum and pancreatic tissue

Amylase activity was assayed using an amylase assay kit (BioVision, Milpitas, CA, USA). Malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) levels and SOD and catalase (CAT) activity in the pancreas were also evaluated using specific kits (Beyotime). TNF-α, IL-6, IL-1β and IL-10 levels in serum were assessed using ELISA kits (R&D Systems, Minneapolis, MN, USA). Levels of ROS in homogenates of pancreas were determined using an ROS assay kit (Cell Biolabs, San Diego, CA, USA).

Immunohistochemistry

Immunostaining was performed on 6–8 μm thick sections after deparaffinization. Antigen retrieval was performed in citric acid (pH 6.0). Endogenous peroxidase was inactivated with 3% hydrogen peroxide. After incubation with the primary anti-NF-κBp65 antibody at room temperature for 1 h, the sections were treated with biotin-conjugated rabbit anti-rat secondary antibody, followed by visualization with 3,3′-diaminobenzidine (DAB). Finally, the sections were developed with diaminobenzidine tetrahydrochloride substrate for 10 min and counterstained with hematoxylin. The sections were observed at a magnification of 200× or 400× and analyzed using a computer image analysis system (Media Cybernetics, Silver Spring, MD, USA). At least 5 random fields of each section were examined.

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using ANOVA and an unpaired Student's *t*-test. A P value < 0.05 was considered to be statistically significant. Statistical calculations were performed using SPSS software for Windows operating system (version 10.0; SPSS, Chicago, IL, USA).

Results

Characterization of BMSCs

When cultured on plastic culture plates, the cells were of uniform size, had fibroblast-like shapes, and formed spiral-like colonies (Fig. 1A). Positive rates for BMSC surface markers CD44 and CD90 were 95.30% and 91.16%, respectively, and positive rates for CD34 were 0.95% (Fig. 1B). Cell type-specific staining showed that the BMSCs had successfully differentiated into adipocytes and osteoblasts when they were induced *in vitro* for 28 and 21 days in adipogenic and osteogenic medium, respectively (Fig. 1C). These results demonstrated that the clonal cells isolated from rat bone mesenchyme possessed an MSC phenotype.

Enhancement of protective effect of BMSCs by NAC in the treatment of SAP

Serum amylase activity was significantly increased 72 h after successful induction of SAP (Fig. 2A). The wet/dry weight ratios of pancreatic tissue from the SAP group were significantly increased compared with the NC group (Fig. 2B), indicating greater severity of pancreatic edema. Pancreatic tissue damage was evaluated by determination of edema, necrosis, inflammatory cell infiltration and vacuolization. Based on these factors, pathological scores were significantly increased in the pancreases of the SAP group compared with those of the NC group (Fig. 2C). Pancreatic tissue from the SAP group showed massive edema and inflammation with necrosis (Fig. 2C and D). After transplantation of BMSCs or administration of NAC, necrosis, inflammation and edema decreased and serum amylase activity was also significantly reduced. Notably, pathological scores, wet/dry ratios of pancreatic tissue and serum amylase activity were all significantly decreased in the SAP + BMSCs + NAC group compared with those in the SAP + BMSCs and SAP + NAC groups (Fig. 2A–D). We concluded that treatment with both NAC and BMSCs attenuated SAP, and that NAC enhanced the therapeutic efficacy of BMSC transplantation in rats with SAP.

NAC enhances effectiveness of BMSCs against oxidative damage in SAP

Damage to the pancreas caused by oxidative stress was assessed by measuring the activity of CAT and SOD, together with levels of MDA, GSH and ROS in pancreatic tissue. After successful induction of SAP in rats, levels of MOD and ROS were significantly increased. CAT, SOD and GSH activity were reduced in the SAP + Vehicle group compared with the NC group (Fig. 3A–E). These parameters were significantly improved after transplantation of BMSCs or NAC therapy. Heme oxygenase-1 (HO-1) is a cytoprotective enzyme, which is involved in the response to oxidative stress and the inflammatory response. Expression levels of HO-1 were upregulated in the SAP + NAC, SAP + BMSCs and SAP + BMSCs + NAC groups compared with the SAP + Vehicle and NC groups (Fig. 3F and G). Levels of ROS and MDA were significantly decreased and levels of GSH and HO-1, together with CAT and SOD activity, were significantly increased in the SAP + BMSCs + NAC group compared with the SAP + NAC and SAP + BMSCs groups. Thus, we concluded that NAC enhanced the effectiveness of BMSCs against oxidative damage in the injured pancreas tissue with SAP.

NAC enhances the ability of BMSCs to inhibit inflammatory response in rats with SAP

Expression levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) and anti-inflammatory cytokine (IL-10) in serum were measured to further elucidate the effects of NAC therapy and transplantation of BMSCs in SAP. Levels of IL-6, IL-1β, and TNF-α were significantly increased, and the level of IL-10 was significantly decreased, in the SAP + Vehicle group (Fig. 4A–D). Treatment with BMSCs or NAC significantly decreased proinflammatory cytokine levels and increased anti-inflammatory cytokine levels in the SAP + NAC, SAP + BMSCs and SAP + BMSCs + NAC groups. The effects in the SAP + BMSCs + NAC group were significantly greater than those in the SAP + NAC group or the SAP + BMSCs group.

The NF-κB signaling pathway plays a key role in the inflammatory response in SAP. Expression levels of two key proteins in the NF-κ B signaling pathway, IKKβ and NF-κBp65 were measured. Total protein levels of NF-κBp65 were not increased in the SAP + Vehicle group, but immunohistochemical analysis indicated

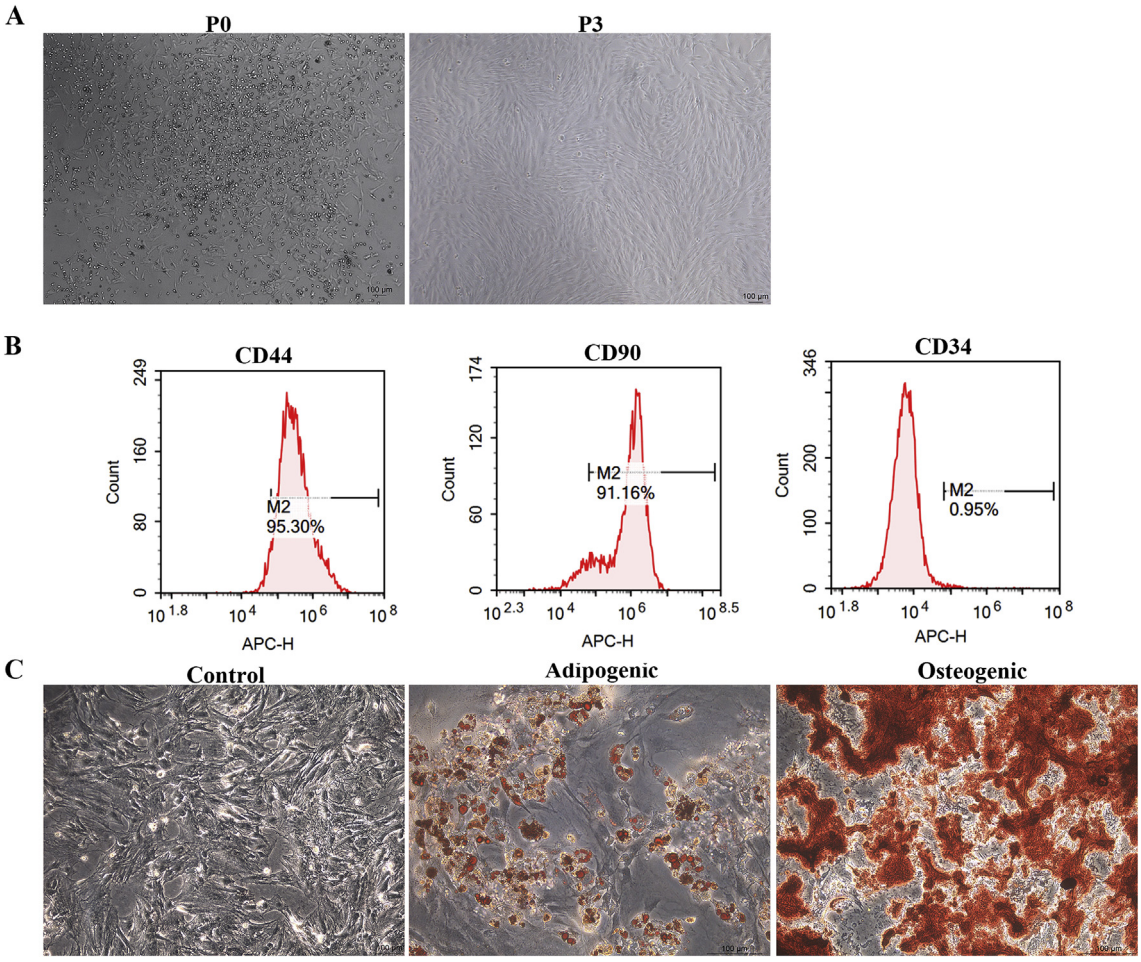

Fig. 1. Characterization of BMSCs. Characterization of BMSCs. (A) BMSCs were spindle-shaped, forming spiral-like colonies on plastic culture dishes (magnification, 50×). (B) Expression of several stem cell markers detected by flow cytometry. (C) Multilineage potential of BMSCs for adipogenic and osteogenic differentiation (magnification, 200×).

that NF-κBp65 levels in the nucleus were significantly increased in pancreatic tissue in the SAP + Vehicle group (Fig. 4E, G and H). Expression of IKKβ, an inhibitory protein of NF-κBp65, was significantly reduced in pancreatic tissues of rats in the SAP + Vehicle group (Fig. 4E and F). After treatment with NAC or BMSCs, expression of IKKβ was upregulated, and levels of NF-κBp65 in the nucleus were reduced in the SAP + NAC, SAP + BMSCs and SAP + BMSCs + NAC groups compared with the SAP + Vehicle group. The improvements in the SAP + NAC + BMSCs group were significantly greater than those in the SAP + NAC and SAP + BMSCs groups. NAC therapy combined with transplantation of BMSCs thus significantly inhibited the NF-κB signaling pathway and NAC enhanced the anti-inflammatory effect of BMSCs in rats with SAP.

Discussion

In this study, we demonstrated that combined treatment with NAC and BMSCs significantly reduced oxidative stress damage to the pancreas and inhibited the inflammatory response in a rat model of SAP. NAC thus enhanced the therapeutic efficacy of BMSC transplantation in rats with SAP.

NAC is an antioxidant that is known to increase intracellular stores of glutathione and to enhance endogenous antioxidant defense mechanisms [26]. NAC can also directly scavenge ROS produced by inflamed cells [27]. Previous studies have shown that NAC has antioxidant and cytoprotective effects, inhibits proinflammatory cytokines, and improves the microcirculation in pancreatitis [9,10,23,28]. These protective effects of NAC not only block the development of AP and reduce the severity of SAP, but also limit systemic complications and decrease mortality. Interestingly, several studies have found that addition of NAC to other treatment regimens improves outcomes in SAP. As examples, NAC combined with ascorbic acid therapy limited pancreatic damage and protected the fine structure of acinar cells [8], and NAC combined with hyperbaric oxygen (HBO) therapy significantly decreased oxidative stress damage [29]. In the current study, we found that a combination of NAC therapy and BMSC transplantation reduced oxidative stress damage to the pancreas and inhibited the inflammatory response to a significantly greater extent than NAC therapy alone.

In recent decades, MSC therapy has been suggested as a treatment for many intractable diseases. Previous studies have revealed that MSC treatment promotes tissue regeneration and improves functional recovery of multiple organs, after both acute and chronic injury. In recent years, MSC transplantation has been considered as a treatment for pancreatitis [14]. The mechanism of action of MSCs in alleviating the acute inflammation and tissue damage following SAP has been shown to involve immunomodulation, angiogenesis and anti-apoptosis, as well as the antioxidant effect and the homing of infused cells [15,30–33]. In the present study, we not only found that BMSCs alleviate SAP in a rat model, but also that the therapeutic efficacy of BMSC transplantation can be enhanced by

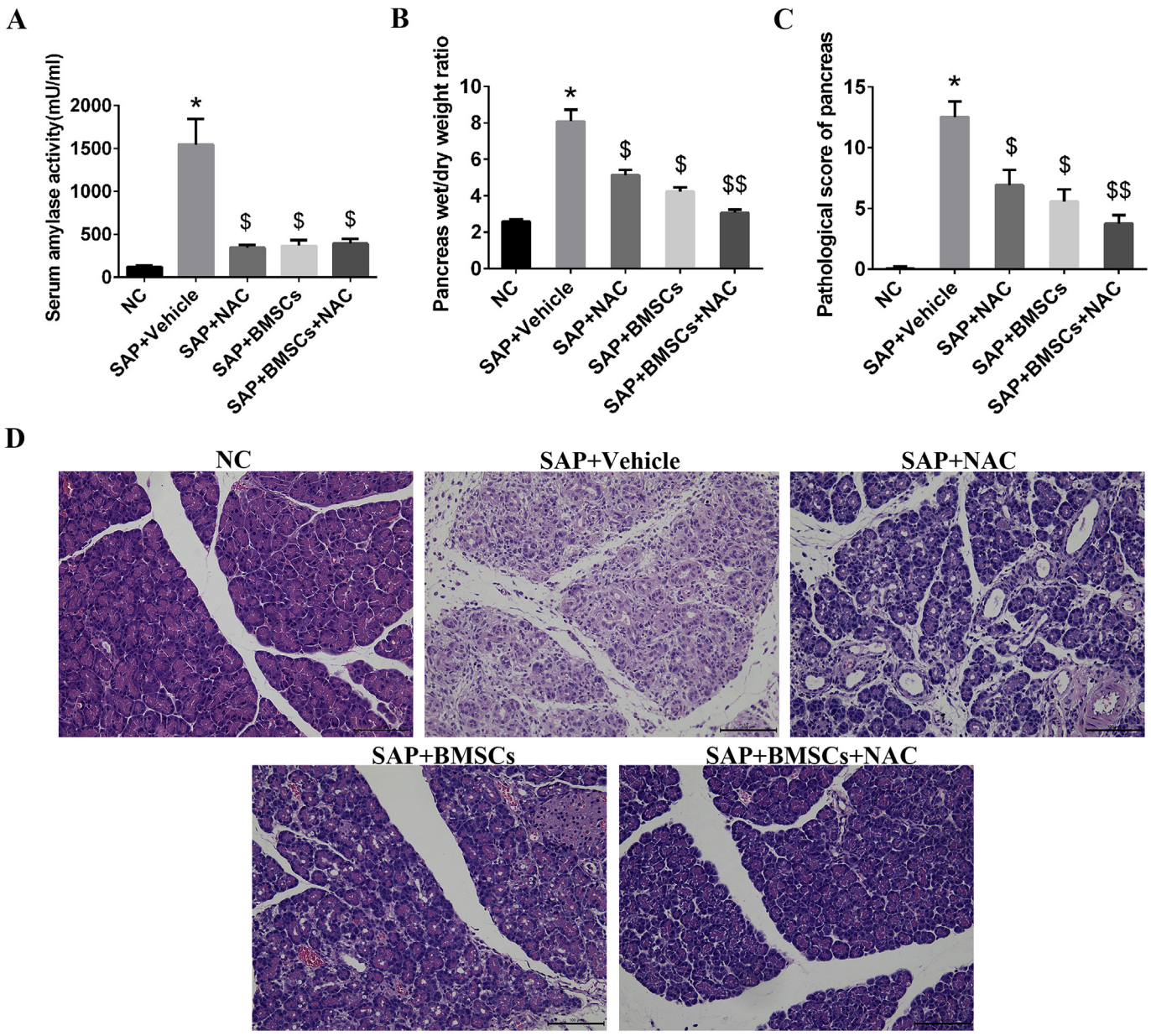

Fig. 2. Administration of NAC enhances therapeutic efficacy of BMSC transplantation in rats with SAP. (A) Serum amylase activity, (B) Wet/dry weight ratio of pancreatic tissue, (C) Pathological score of pancreas, (D) Histological analysis of SAP (images enlarged 200×). Data are presented as mean ± SEM. $^{*}P < 0.01$ vs. NC group; $^{\$}P$ and $^{\$\$}P < 0.05$ vs. NC and SAP + Vehicle group; $^{\$\$}P < 0.05$ vs. SAP + NAC and SAP + BMSCs group.

administration of NAC.

In the previous studies, we have showed that most MSCs were distributed in the lung, while a small number of MSCs were distributed in injured pancreatic tissue in the early stage after MSCs were intravenously administered [34]. Moreover, ROS and inflammatory mediators may affect the survival, proliferation, differentiation, migration and paracrine action of the MSCs after transplantation [18,35,36]. Therefore, these adverse factors after MSCs transplantation hinder the progress of MSCs-based therapy for SAP.

Interestingly, a previous study have found that NAC pretreatment of MSCs significantly increased their therapeutic effects on lung injury. The potential molecular mechanism could be that antioxidant pretreatment of MSCs reduced the release of cellular ROS, increased cellular glutathione levels, the ability of cellular adhesion and migration, and enhanced the MSCs antioxidant capacity [21]. In addition, Song et al. have found that ROS inhibit the adhesion of MSCs implanted into ischemic myocardium via interference with the focal adhesion complex. Moreover, the combined treatment with NAC and MSCs significantly reduced heart fibrosis and infarct size compared to MSCs therapy alone [19]. Although this could provide a promising approach to improve the therapeutic use of MSCs, the specific molecular mechanism remains unclear.

A recent study revealed that treatment with NAC *in vitro* not only improved the quantity and function of BMSCs derived from prolonged isolated thrombocytopenia (PT) patients by down-regulation of the p38 and p53 pathways, but also partially rescued the impaired ability of BMSCs to support megakaryocytopoiesis [37]. Ali et al. have demonstrated that NAC preconditioning protected MSCs against hydrogen peroxide-induced injury by promoting survival and attenuating apoptosis [38]. Sun et al. found that NAC treatment could promote entry of adipose-derived

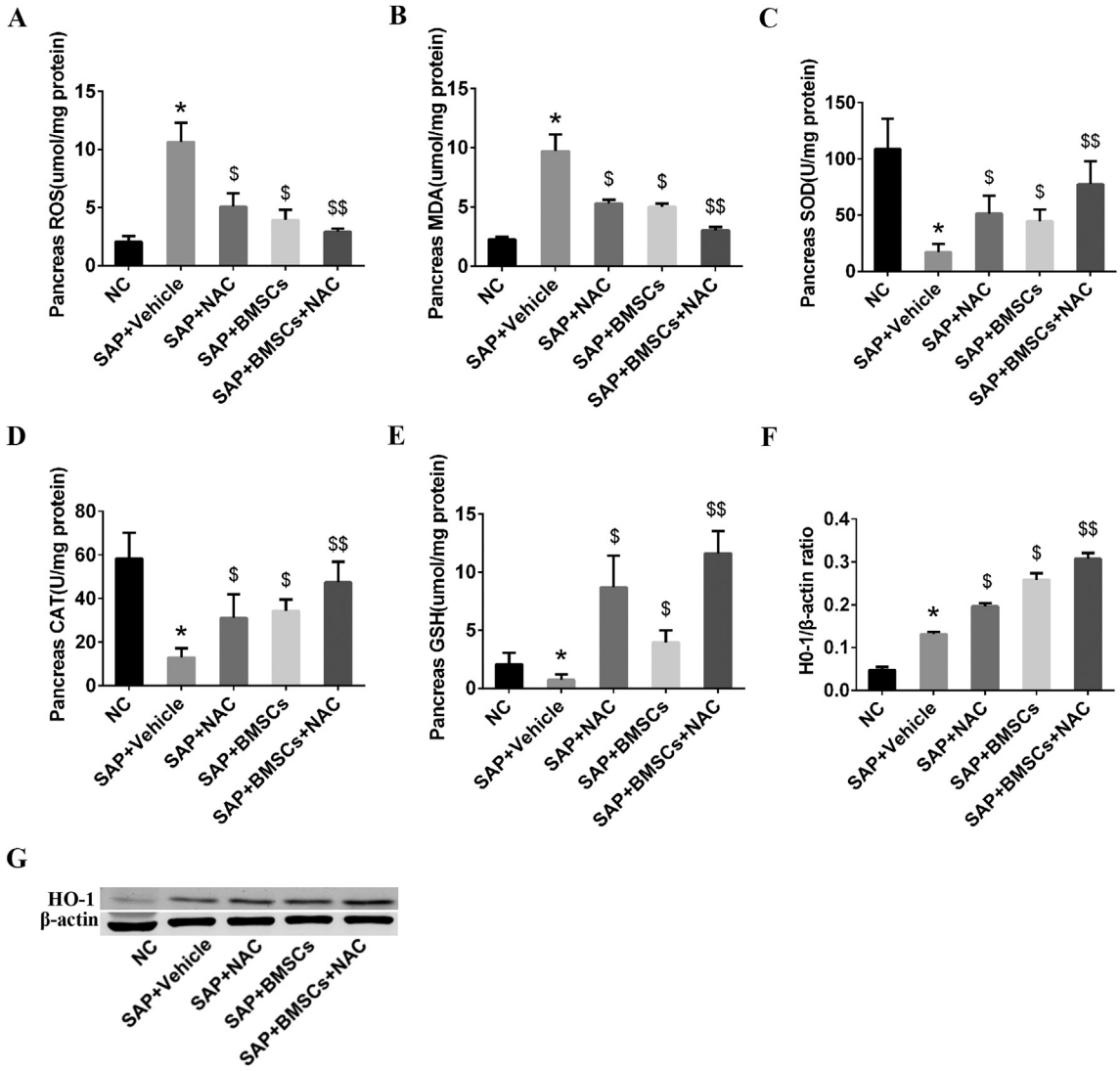

Fig. 3. NAC enhanced effectiveness of BMSCs against oxidative damage in pancreatic tissue of rats with SAP. (A) ROS levels in pancreas, (B) MDA levels in pancreas, (C) SOD activity in pancreas, (D) Activity of antioxidant enzyme CAT in pancreas, (E) GSH levels in pancreas, (G) HO-1 protein levels in pancreas, (F) Protein levels normalized by densitometry (β-actin standard), three independent experiments were conducted. Data are presented as mean ± SEM. $^{*}P < 0.01$ vs. NC group; $^{\$}P$ and $^{\$\$}P < 0.05$ vs. NC and SAP + Vehicle group; $^{\$\$}P < 0.05$ vs. SAP + NAC and SAP + BMSCs group.

mesenchymal stem cells into S phase by inhibiting cyclin-dependent kinase inhibitors and results in rapid cell proliferation [20]. Shi et al. also found that a high level of ROS impaired MSC migration capacity via over polymerization of the F-actin cytoskeleton, and NAC treatment reversed the in *vivo* homing capacity of MSCs [35]. Moreover, a recent study showed that combined *ex vivo* treatment of autologous stem cells with NAC and AAP could potentially be an effective strategy to restore the paracrine function of impaired diabetic MSCs before transplantation [36]. Although we have demonstrated that NAC could enhance the therapeutic efficacy of BMSC transplantation in rats with SAP, we have not, so far, elucidated the specific molecular mechanisms by which NAC affected BMSC therapy in our rat model of SAP. This also will be the subject of our future studies.

Multiple organ failure is frequently associated with SAP, with respiratory, hepatic and renal failure contributing most significantly to the morbidity and mortality of patients with SAP. Previous studies have found that NAC therapy can protect against pancreatitis-induced liver injury and lung damage [39,40]. In our studies, however, we did not determine whether NAC enhanced the ability of BMSCs to reduce these complications of SAP. Because we simply observed the therapeutic effect of NAC and BMSCs 3 days after treatment, we also did not monitor possible adverse effects of treatment with BMSCs and NAC. Next, we will carry out a systematic evaluation of the effect of NAC combined with BMSCs for the treatment of SAP and will investigate both short-term and long-term systemic complications.

Conclusion

NAC enhances the therapeutic efficacy of BMSC transplantation in rats with SAP by alleviating pancreatic oxidative stress damage and inhibiting inflammation. Our data suggested that this combination therapy may provide a promising new approach for the treatment of SAP.

Conflicts of interest

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

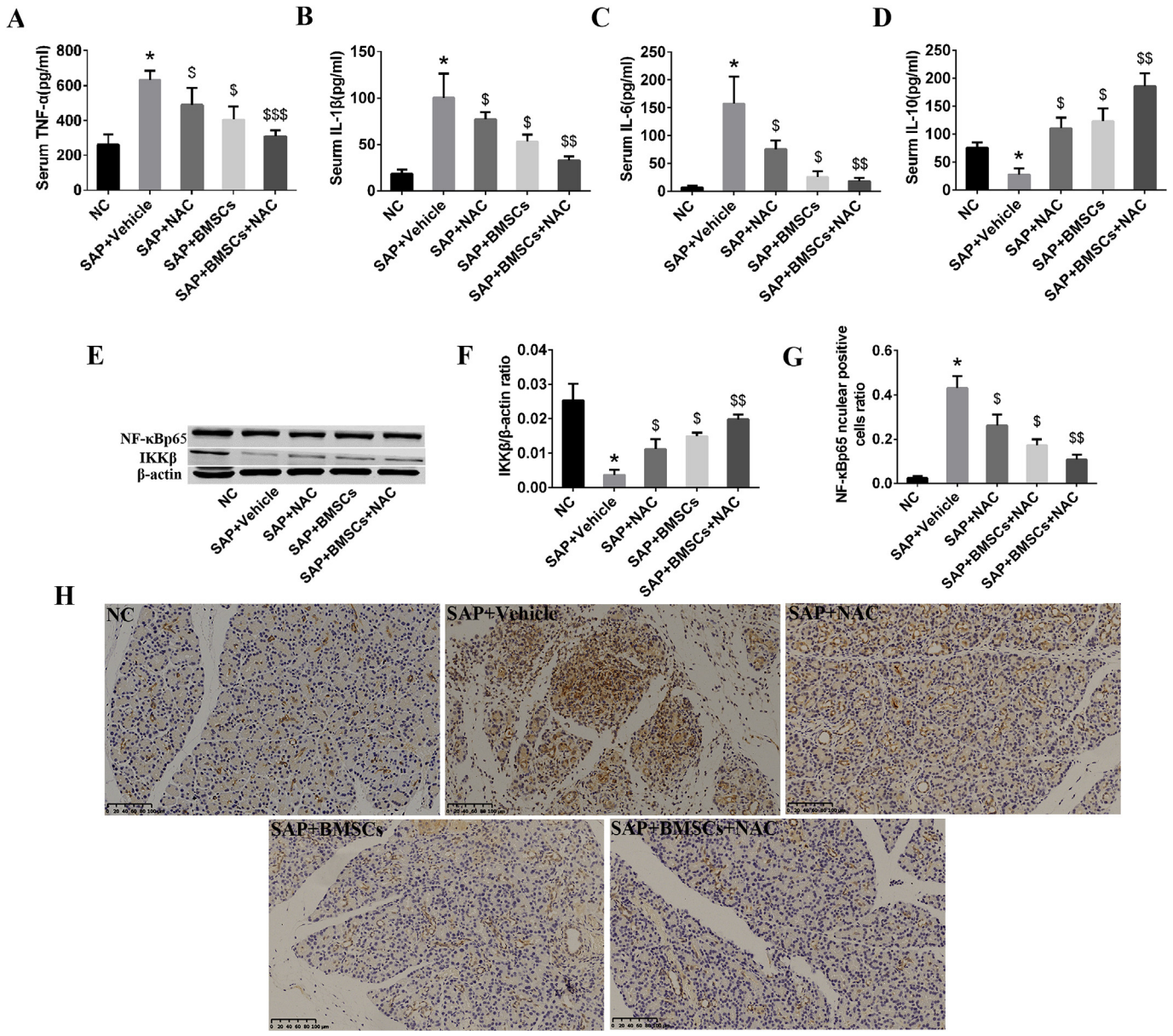

Fig. 4. NAC enhanced ability of BMSCs to inhibit inflammatory response in rats with SAP. (A, B, C, D) Serum levels of inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-10, (E) Expression of NF-κ B p65 and inhibitory protein IKKβ in pancreas, (F) Protein levels normalized by densitometry (β-actin standard), three independent experiments were conducted, (G) Quantification of NF-κB nuclear-positive cells in six independent immunohistochemical images (magnification 200×), (H) Immunohistochemical analysis of NF-κB in the pancreas. Data are presented as mean ± SEM. $^{*}P < 0.01$ vs. NC group; $^{\$}P$ and $^{\$\$}P < 0.05$ vs. NC and SAP + Vehicle group; $^{\$\$}P < 0.05$ vs. SAP + NAC and SAP + BMSCs group.

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