Prostaglandin E_1 protects bone marrow-derived mesenchymal stem cells against serum deprivation-induced apoptosis

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Abstract. Mesenchymal stem cells (MSCs) have become a recent focus of experimental and clinical research regarding myocardial regeneration. However, the therapeutic potential of these cells is limited by poor survival. Prostaglandin E_1 (PGE_1) is known to have anti-inflammatory and anti-apoptotic effects on the myocardium. The aim of the present study was to determine whether PGE_1 could protect MSCs against serum deprivation (SD)-induced apoptosis. An SD model was used to induce apoptosis in MSCs *in vitro*. Apoptotic morphological changes were detected by Hoechst 33258 fluorescent nuclear staining; and Annexin V-fluorescein isothiocyanate/propidium iodide (PI) double staining and flow cytometry was used to quantify the rate of apoptosis. Western blot analysis was used to detect the expression levels of the apoptosis-associated proteins Bcl-2, Bax and caspase-3. The results of the present study demonstrated that SD induced apoptosis of MSCs, and that treatment with PGE_1 attenuated the morphological changes characteristic of apoptosis. Annexin V/PI staining showed that the rate of apoptosis gradually increased with the duration of ischemia. Furthermore, treatment with PGE_1 significantly reduced SD-induced apoptosis, decreased the protein expression levels of Bax and caspase-3, and increased the expression levels of Bcl-2. These data suggest that PGE_1 is able to influence the survival of MSCs under certain conditions. These results may aid in improving the therapeutic efficacy of MSC transplantation used to treat chronic ischemic heart disease.

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Introduction

Ischemic heart disease is one of the most common diseases worldwide. The traditional treatment of ischemic heart disease includes the prevention of atherosclerosis, and revascularization of the coronary arteries; however, these strategies cannot reverse or repair myocardial necrosis. Heart transplantation is an effective treatment for patients with late-stage heart failure, however due to the insufficient supply of organs there are limits to its clinical application (1-3). Recently, the rapid development of stem cell technology has led to novel treatment methods, including the transplantation of mesenchymal stem cells (MSCs) to repair or regenerate damaged myocardium (4-7). Previous studies have demonstrated that MSCs transplanted into areas of myocardial ischemia may differentiate into myocardial cells and repair necrotic myocardial tissue. However, the effects of MSCs are insufficient, since the majority of transplanted MSCs die shortly after transplantation in the ischemic microenvironment (7,8). Therefore, a key focus of research is to improve the survival of MSCs following transplantation into ischemic tissue.

Prostaglandin E_1 (PGE_1), also termed alprostadil, is an endogenous substance, which has numerous effects, including vasodilation, protection of endothelial cells, and inhibition of the activation and aggregation of neutrophils and thrombocytes (9). Furthermore, PGE_1 is widely used in the treatment of ischemic heart disease. Clinical research has previously demonstrated the potential of PGE_1 for improving myocardial microcirculation, and counteracting the effects of ischemia-reperfusion injury and apoptosis in the myocardium (10-13). These findings indicate that PGE1 may have a general cytoprotective action; however, there are currently no studies investigating whether PGE_1 may prevent apoptosis of MSCs.

Apoptosis is a type of physiological cell death, for which it is considered difficult to generate a comprehensive *in vitro* model. Serum deprivation (SD) injury *in vitro* is widely used to mimic the ischemic environment (14,15). The mitochondrial pathway is the major underlying mechanism of physiological cell death in apoptosis (16,17), and the Bcl-2 family proteins have an important role in the apoptotic response (18,19). To the best of our knowledge, the molecular

mechanism by which PGE_1 may inhibit apoptosis of MSCs is currently unknown.

The present study established an *in vitro* model of SD-induced apoptosis, in order to explore the potential mechanisms by which PGE_1 may improve the survival of MSCs in the myocardial microenvironment following transplantation.

Materials and methods

Animals. Sprague-Dawley rats (specific pathogen free; weight, 80-100 g) of either sex were purchased from the Animal Center of Sun Yat-sen University (Guangzhou, China). All procedures of the present study were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. The rats were maintained in 12 h light/dark cycles at a temperature of 26-26˚C and with a humidity of 40-70%, with free access to a standard laboratory diet and water.

Reagents and instruments. PGE_1 was purchased from Zhuhai Schwarz Pharma Co., Ltd. (Zhuhai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco Life Technologies (Carlsbad, CA, USA). Penicillin-streptomycin was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). A Hoechst 33258 Staining kit and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies targeting Bcl-2 (cat. no. 2870), Bax (cat. no. 2772), and caspase-3 (cat. no. 9662), were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA) and were used at a dilution of 1:1,000. A Bicinchoninic Acid (BCA) Protein Assay kit was purchased from Kangchen Bio-tech (Shanghai, China). Enhanced Chemiluminescence (ECL) solution was purchased from KeyGen Biotech Co., Ltd. (Nanjing, China). Fluorescein isothiocyanate (FITC)-labeled Annexin V and anti-rat CD90 (cat. no. 11-0900-81), CD45 (cat. no. 17-0461-80), CD11b/c (cat. no. 12-0110-80), and CD29 (cat. no. 46-0291-80), antibodies were purchased from BD Biosciences (San Jose, CA, USA).

Cell preparation and culture. MSCs were isolated from the femora and tibiae of Sprague Dawley rats, which had were sacrificed by cervical dislocation. The cells were cultured in DMEM supplemented with 10% FBS and penicillin-streptomycin (50 U/ml), and were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. The culture medium was refreshed every 2-3 days. Each primary culture was passaged 1:2 once the MSCs had grown to 80% confluence. MSCs at passage 4, which were positive for CD90 and CD29, and negative for CD45 and CD11b/c, were collected and used for subsequent experiments.

SD-induced apoptosis. The MSCs were randomly divided into three groups and cultured for 24 h. The control group was cultured with complete medium supplemented with 10% FBS; the SD group was cultured with SD medium; and the PGE_1+SD group was cultured with SD medium plus 10 ng/ml PGE_1.

Hoechst 33342 staining. The culture medium was discarded, and the cells were washed three times with phosphate-buffered saline (PBS), and fixed in 4% paraformaldehyde for 15 min at room temperature. The fixing solution was then discarded and the cells were washed with PBS for 5 min and incubated in Hoechst 33342 in the dark for 15 min at room temperature. Following the 15 min incubation, the Hoechst 33342 solution was discarded, and the cells were washed for a further 5 min with PBS. The cells were then supplemented with PBS and observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan). The apoptotic cells exhibited morphological changes, including shrinkage and condensed nuclei.

Flow cytometric (FCM) analysis of apoptosis. The MSCs from passage 4 were digested with 0.05% Trypsin-EDTA (Gibco Life Technologies) for 5 min at 37˚C, resuspended at a concentration of $10x10^5$ cells/ml and centrifuged at 300 x g for 5 min. Following centrifugation, the cells were collected in PBS at 4˚C, washed twice, and resuspended in 100 ml binding buffer (BD Biosciences). In a 5 ml dry flow tube the cells were added to 5 μl Annexin V-FITC (BD Biosciences) and 5 μl PI (BD Biosciences), lightly vortexed, and incubated in the dark at room temperature for 15 min. Following incubation, 400 μl binding buffer was added, and the flow tube was placed on ice. PI and Annexin V-FITC fluorescence was measured using a flow cytometer (BD FACSVerse; BD Biosciences; excitation, 488 nm; emission, 615 nm). The research software (BD FACSuite softwared; BD Bioscience) matched with FCM was used to analyze the data. Positive Annexin V staining indicated apoptosis, and positive PI staining indicated necrosis. The experiment was repeated three times.

Western blot analysis of Bcl-2, Bax and caspase-3. The MSCs were seeded into a 60 mm petri dish, at a density of $10x10^5$ cells/dish. The total protein was extracted using radioimmunoprecipitation buffer (EMD Millipore), supplemented with PMSF. The cells were sonicated briefly and centrifuged at 10,000 x g at 4˚C. The protein concentration was measured using the BCA Protein Assay kit, according to the manufacturer's instructions. Equal samples of protein (20 μg) were separated by 12% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated with blocking solution (Beyotime Institute of Biotechnology, Jiangsu, China) at room temperature for 2 h, and then incubated with the following primary antibodies: Bcl-2, Bax, caspase-3 and GAPDH (cat. no. KC-5G5; KangChen Biotech, Shanghai, China; dilution, 1:10,000) at 4˚C overnight. The membranes were then incubated for 1 h at room temperature with the appropriate horseradish peroxidase conjugated-secondary antibodies. The blots were visualized using an enhanced chemiluminescence solution (EMD Millipore) and were exposed to X-ray film (Kodak, Tokyo, Japan). The density of the protein bands was analyzed using ImageJ 1.41 software (National Institutes of Health, Bethesda, MD, USA). The expression levels of the target proteins were normalized to those of GAPDH.

Statistical analysis. The data are expressed as the mean ± standard deviation. Comparisons between the groups were analyzed by one-way analysis of variance or Student's t-test. Statistical analyses were performed using SPSS 16.0 (SPSS,

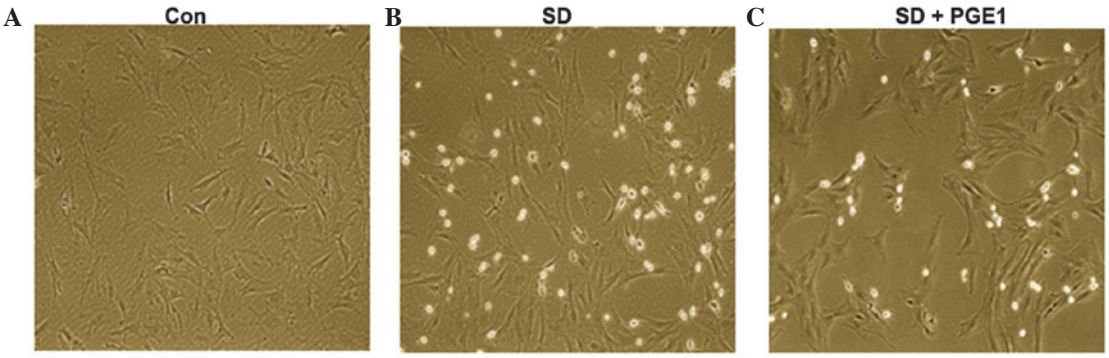

Figure 1. Morphology of MSCs at passage 4. (A) Control, untreated MSCs; (B) SD, serum-deprived MSCs; (C) SD+PGE_1, serum-deprived MSCs cultured with 10 ng/ml PGE_1. Image visualized under a phase-contrast microscope (magnification, x100). MSC, mesenchymal stem cells; Con, control; SD, serum deprived; PGE_1, prostaglandin E_1.

Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

MSC morphology and identification. MSCs were isolated and expanded from Sprague Dawley rats. The control MSCs were shown to sparsely attach to the culture flasks, and the majority of cells displayed a spindle-like shape (Fig. 1A). In the MSCs cultured with SD medium cells did not display the typical spindle-like shape, and a large number of floating dead cells were observed (Fig. 1B). Following treatment with PGE_1, the morphology of the MSCs cultured with SD changed, and the number of floating dead cells was reduced, as compared with the cells cultured with SD alone (Fig. 1C). The MSCs at passage 4 were stably positive for CD90 and CD29 markers, and negative for CD45 and CD11b/c markers.

PGE_1 inhibits SD-induced apoptosis. Hoechst 33342 staining showed that the majority of the MSCs in the control group were round or oval-shaped, and had light-blue regular nuclei (Fig. 2A). In the PGE_1+SD group, the apoptotic cells were round or oval-shaped, with bright-blue irregular nuclei, which indicated chromosome condensation. The number of apoptotic cells in the SD group was significantly increased, as compared with the control group. Treatment with PGE_1 significantly reduced the rate of MSC apoptosis, as compared with the SD group. Furthermore, flow cytometry of Annexin V/PI-stained cells was used to quantify the apoptotic rate of the MSCs. The percentage of apoptotic cells was significantly higher in the SD (P<0.01) and SD+PGE_1 (P<0.05) groups, as compared with the control group (Fig. 2B and C). In addition, the rate of apoptosis was lower in the PGE_1-treated MSCs, as compared with the untreated MSCs cultured with SD (P<0.05).

SD influences the expression levels of apoptosis-associated proteins Bax, Bcl-2 and caspase-3. The present study determined whether SD could affect the expression levels of the proapoptotic Bcl-2 family members, Bax and Bcl-2. Western blot analysis showed that the protein expression levels of Bax were significantly increased and the protein expression levels of Bcl-2 gradually decreased, following exposure to SD for 12-24 h (Fig. 3A and B). Cleaved caspase-3 expression was not detected in the control MSCs, however its expression was significantly increased in the MSCs cultured in SD medium (Fig. 3C).

Effects of PGE_1 on the protein expression levels of Bax, Bcl-2 and caspase-3. Treatment with PGE_1 significantly reduced SD-induced Bax protein expression levels and increased the protein expression levels of Bcl-2 (Fig. 4A). PGE_1 also reduced the protein expression levels of cleaved caspase-3 in the MSCs (Figure 4B). These results indicate that PGE_1 was able to attenuate SD-induced apoptosis though activation of Bax and deactivation of Bcl-2, thus reducing the expression of cleaved caspase-3.

Discussion

The present study reported the protective effects of PGE_1 on SD-induced apoptosis in MSCs, and this effect was shown to be mediated through the mitochondrial caspase-3 pathway. The results of the present study demonstrated that treatment with PGE_1 (10 ng/ml) decreased SD-induced apoptosis in MSCs, as shown by Hoechst 33342 staining, flow cytometry and measurement of caspase-3 protein expression levels. Furthermore, PGE_1 protected MSCs against SD-induced apoptosis by downregulating Bax expression and upregulating Bcl-2 expression.

MSCs are non-hematogenic stem cells, which are present in the bone marrow. Due to their availability, potential for differentiation (20) and amplification, and association with fewer ethical issues (21), MSCs have become a focus of attention in the field of experimental and clinical research regarding myocardial regeneration. However, the ischemic cardiac microenvironment reduces the survival rate of transplanted cells, and limits their therapeutic effects (22). More than 90% of MSCs have previously been shown to die within 24 h of transplantation (23). Another study demonstrated that only ~21% of MSCs survive after 4 h of transplantation, and only 3.6% survive seven days (24). To address the problem of poor survival, research has focused on strategies that inhibit apoptosis of MSCs and improve their therapeutic effects in the ischemic myocardium. Such strategies include the use of genetically modified stem cells, preconditioning of stem cells, and combination drug therapy prior to transplantation of the

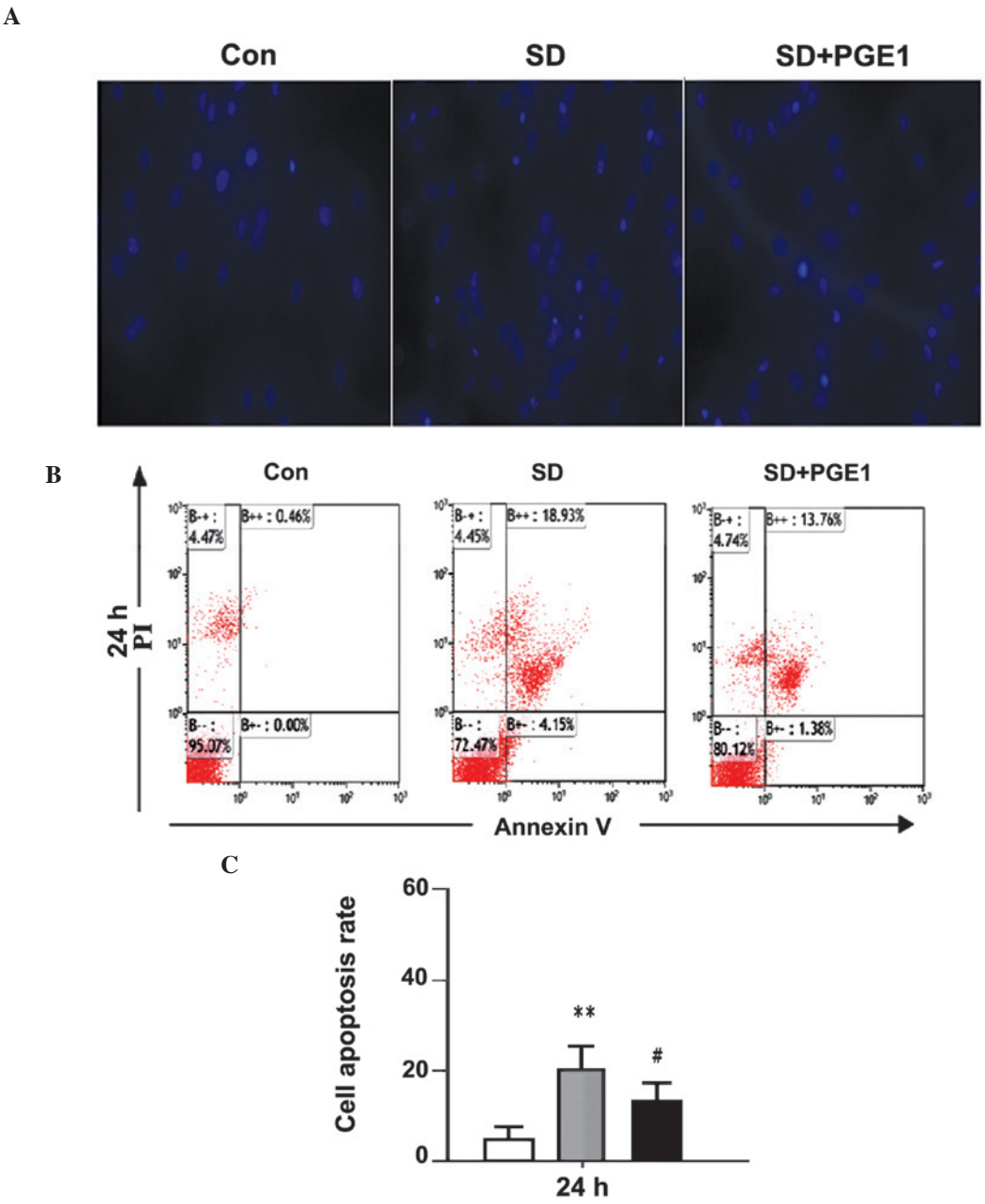

Figure 2. Apoptosis of MSCs exposed to serum deprivation with or without PGE_1 preconditioning. (A) Morphological changes to the apoptotic cells were assessed by Hoechst 33258 staining (magnification, x100). Control, untreated MSCs; SD, serum-deprived MSCs; SD+PGE_1, serum-deprived MSCs cultured with 10 ng/ml PGE_1. MSCs were incubated for 24 h. (B) Flow cytometric analysis of the influence of 10 ng/ml PGE_1 on SD-induced apoptosis. Apoptotic cells were identified by Annexin V and PI staining; viable cells are Annexin V^-/PI^-, early apoptotic cells are Annexin V^+/PI^-, late apoptotic cells are Annexin V^+/PI^+, and necrotic cells are Annexin V^-/PI^+. (C) The results show that 10 ng/ml PGE_1 reduced apoptosis of MSCs after 24 h of serum deprivation. The data are presented as the mean ± standard error (n=5). $^{**}P<0.01$, as compared with the control group; $^{\#}P<0.05$, as compared with the SD group. MSC, mesenchymal stem cells; Con, control; SD, serum deprived; PGE_1, prostaglandin E_1; PI, propidium iodide.

cells into the damaged myocardium (25). Genetic modification (26,27) and preconditioning (28) of MSCs may improve the survival rate of stem cells; however, these are difficult to perform clinically. Whereas, combination drug therapy improves the viability of MSCs and is convenient for clinical application. Zhang *et al* (29) previously used rosuvastatin as a combination therapy to improve the therapeutic efficacy of MSCs for treating myocardial infarction. Furthermore, Dong *et al* (30) used combination therapy with atorvastatin, which was shown to activate AMP-activated protein kinase (AMPK); phosphorylation of AMPK resulted in activation of endothelial nitric oxide synthase. This mechanism may also be associated with the protection of MSCs against SD-induced apoptosis, through the mitochondrial apoptosis signaling pathway.

PGE_1 is widely used in the treatment of ischemic heart disease. Previous research has identified the ability of PGE_1 to improve myocardial microcirculation, reduce ischemic-reperfusion injury, and exert anti-inflammatory and antiapoptotic effects on the myocardium (10-13,31). PGE_1 also exhibits general cytoprotective effects and anti-apoptotic activity (32). It has previously been reported that PGE_1 is able to significantly

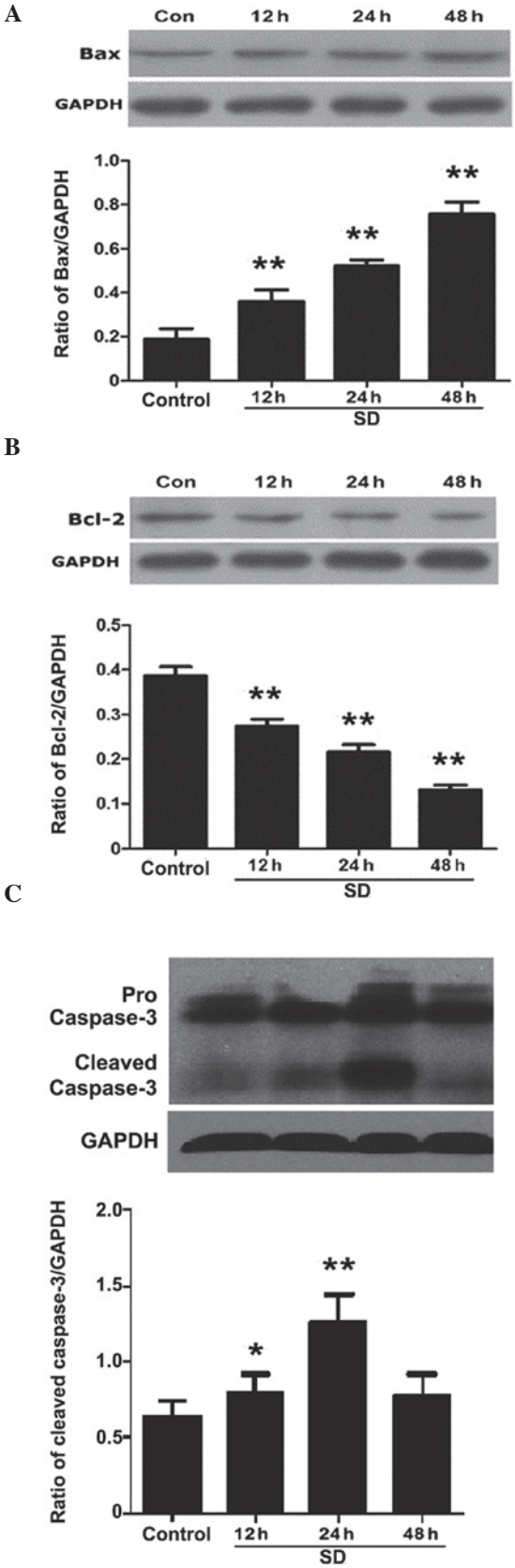

Figure 3. Protein expression levels of Bcl-2, Bax and caspase-3 in MSCs, as determined by western blotting following 12, 24 and 48 h of serum deprivation. Protein expression levels of (A) Bcl-2, (B) Bax and (C) cleaved caspase-3. GAPDH was used as a loading control, and the expression levels of the target proteins were determined relative to the levels of GAPDH. Blots shown are from ≥three independent experiments. The data are presented as the mean ± standard error (n=5). *P<0.05 and **P<0.01, as compared with the control group. MSCs, mesenchymal stem cells; Con, control; SD, serum deprived.

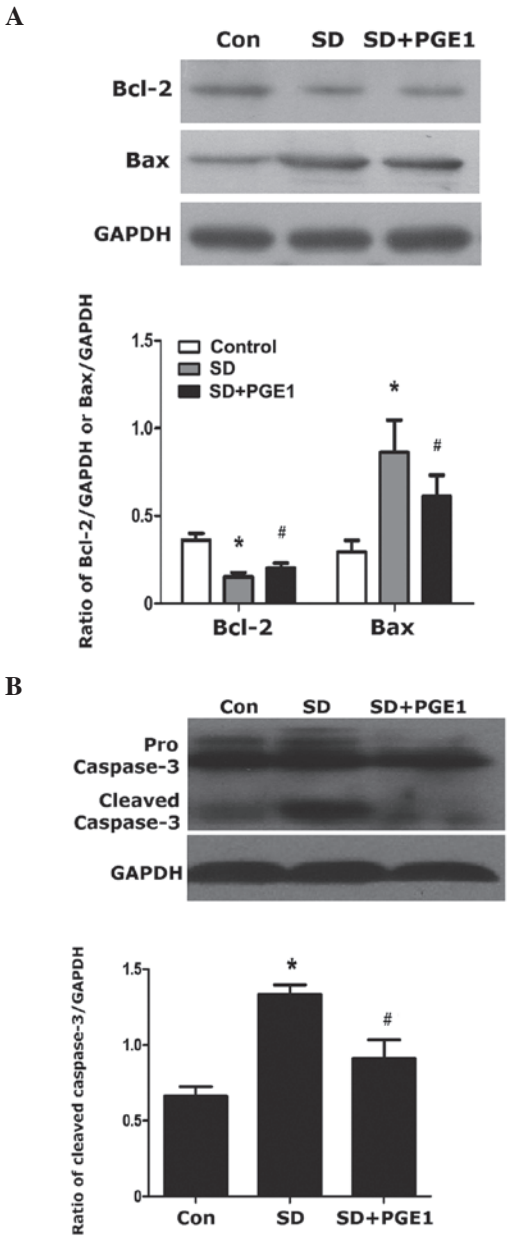

Figure 4. Western blot analysis of Bax, Bcl-2 and caspase-3 following 24 h of serum deprivation with or without PGE_1 preconditioning. Control, untreated MSCs; SD, serum-deprived MSCs; SD+PGE_1, serum-deprived MSCs cultured with 10 ng/ml PGE_1. MSCs were cultured at 37˚C in a humidified atmosphere containing 5% CO_2. Protein expression levels of (A) Bax and Bcl-2, and (B) caspase-3. GAPDH was used as a loading control, and the expression levels of the target proteins were determined relative to the levels of GAPDH. Blots are shown from at least three independent experiments. The data are presented as the mean ± standard error (n=5). *P<0.01, as compared with the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, as compared with the SD group. MSC, mesenchymal stem cells; Con, control; SD, serum deprived; PGE_1, prostaglandin E.

upregulate antiapoptotic proteins, such as Bcl-2 (33); and downregulate Bax and caspase-3 (13). The present study established an *in vitro* SD-induced apoptosis model, in order to explore the potential mechanisms for the protective effects of PGE_1 on MSCs. Apoptosis was detected by Hoechst 33258 and Annexin V-FITC/PI double staining. PGE_1 was shown to protect the MSCs against SD-induced apoptosis. However, apoptosis involves a series of gene activation, expression

and regulation; therefore, further investigation is required to explore the underlying molecular mechanisms by which PGE_1 inhibits apoptosis of MSCs.

The Bcl-2 family is an important apoptosis-regulating family, which includes the antiapoptotic molecule Bcl-2 and proapoptotic molecule Bax (34). Numerous studies have demonstrated that Bcl-2 and Bax are associated with the mitochondrial membrane (35-39). Bcl-2 is predominantly localized to endoplasmic reticulum and mitochondrial membranes, where it prevents the release of cytochrome *c* from the mitochondria and inhibits glutathione leakage, thus blocking programmed cell death (40,41). Bcl-2 can inhibit the activation of caspases, including caspase-9 and caspase-3, and thereby acts as an antiapoptotic agent (42). Bax is predominantly localized to the cytosol, or may be loosely attached to the mitochondrial membrane in an inactive form in healthy cells. Apoptotic stimuli result in structural changes to Bax, which may facilitate the translocation of Bax from the cytosol to the mitochondria, leading to apoptosis (43). Bax exhibits extensive amino acid homology with Bcl-2, and can form homodimers and heterodimers with Bcl-2 *in vivo* (44). The overexpression of Bax counteracts the death repressor activity of Bcl-2, and the activation of caspase-3 is dependent on the ratio of Bcl-2 to Bax, which controls cell survival and death following an apoptotic stimulus (44,45). Caspase-3 is a critical mediator of mitochondrial apoptosis (46), which can be activated by SD in MSCs (47).

The effects of PGE_1 on the inhibition of caspase-3 and regulation of Bcl-2 and Bax have previously been reported (13,33,48); however, the present study is the first, to the best of our knowledge, to report such effects in MSCs. To confirm these findings, western blot analysis was used to detect the protein expression levels of Bax, Bcl-2 and caspase-3. SD downregulated the protein expression levels of Bcl-2, and upregulated the expression levels of Bax in MSCs, resulting in overexpression of caspase-3, which caused an increased rate of MSC apoptosis. Furthermore, treatment with PGE_1 significantly increased the expression levels of Bcl-2 and inhibited the expression levels of Bax and caspase-3, thereby attenuating apoptosis in MSCs. Apoptosis is a complex process, and the SD model used in the present study attempted to simulate the myocardial microenvironment *in vitro*. However, further investigation is required to confirm these findings *in vivo*.

In conclusion, the results of the present study demonstrated that PGE_1 exerts protective effects against SD-induced MSC apoptosis. PGE_1 downregulated the protein expression levels of Bax and caspase-3, and upregulated the protein expression levels of Bcl-2 in the SD *in vitro* model. These findings may be useful in the clinical application of PGE_1 alongside MSC transplantation into ischemic tissue, and may enhance the efficacy of cell therapy.

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