

Protective effects of scoparone against ischemia-reperfusion-induced myocardial injury

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Abstract. The present study aimed to investigate the protective effects and molecular mechanisms of scoparone on ischemia-reperfusion (I/R) injury in primary cultured cardiac myocytes and rats. An *in vivo* rat model of I/R injury and an *in vitro* primary cultured cardiac myocyte model of oxygen-glucose deprivation/reoxygenation were used to investigate the protective effects of scoparone. Cell viability, lactate dehydrogenase (LDH) release, superoxide dismutase (SOD), creatine kinase (CK) and malondialdehyde (MDA) levels, and reactive oxygen species (ROS) production were subsequently measured. In addition, cell apoptosis was assessed by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling staining, and myocardial infarct area (IA) was determined by triphenyl tetrazolium chloride staining. Furthermore, the protein expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), cytochrome *c* (Cyt C) and caspase-3 were assessed by western blotting. The results demonstrated that treatment with scoparone markedly increased cell viability, SOD levels and Bcl-2 protein expression, and decreased LDH release, MDA production, CK levels, ROS concentration, cell apoptotic rate, myocardial IA, and Bax, caspase-3 and Cyt C protein expression. These findings indicated that scoparone may have a protective effect against I/R injury, thus suggesting that scoparone may be a considered a potential drug for the treatment of I/R injury via the inhibition of oxidative stress and cell apoptosis.

Introduction

Scoparone is obtained from *Artemisia Scopariae* Herba, which is a traditional Chinese medicine. *Artemisia Scopariae*

Herba refers to the aerial parts of *Artemisia capillaris* Thunb. or *Artemisia Scoparia* Waldst. et Kit., which are mainly distributed in China, Japan, Korea and Mongolia (1). *Artemisiae Scopariae* Herba has numerous effects, and has been reported to possess anti-inflammatory, antioxidant, antiviral and antitumor activities. In addition, it regulates immunity, blood sugar, blood lipid levels and blood pressure (1). Previous experimental and clinical studies have reported that *Artemisiae Scopariae* Herba exerts therapeutic effects against hepatobiliary disease, postoperative sequelae of gynecological diseases, maternal-fetal blood group incompatibility, severe acute pancreatitis, pneumonia, diabetes, oral ulcers, acute conjunctivitis and cancer (2-7).

Scoparone, which is also known as 6,7-dimethoxycoumarin, is a potent anti-inflammatory agent that has been reported to exert anti-inflammatory effects via inhibition of the transcriptional activity of nuclear factor- κ B (8). Scoparone has previously been reported to inhibit interleukin (IL)-8 and monocyte chemoattractant protein 1 production in U937 cells, and tumor necrosis factor- α , IL-6 and IL-1 β production in lipopolysaccharide-stimulated RAW264.7 cells (9). Scoparone also possesses antitumor activity in DU145 androgen-independent prostate cancer cells via the inhibition of signal transducer and activator of transcription 3 activity (10). Furthermore, scoparone may enhance bilirubin clearance in the liver by activating constitutive androstane receptor, which is a nuclear receptor that acts as a transcription factor to upregulate the expression of bilirubin glucuronyl transferase and other components of the bilirubin metabolism pathway (11). In addition, scoparone exerts protective effects against alterations in plasma lipoproteins, vascular morphology and vascular reactivity in hyperlipidaemic diabetic rabbits, which may be partly due to its free radical scavenging abilities (12). However, to the best of our knowledge, there are currently no studies regarding the protective effects of scoparone against ischemia-reperfusion (I/R)-induced cardiac myocyte injury, and the associated mechanisms have not been reported. The present study aimed to investigate the protective effects and molecular mechanisms of scoparone on I/R-induced myocardial injury in an *in vitro* primary cultured cardiac myocyte model of oxygen-glucose deprivation/reoxygenation (OGD/R) and an *in vivo* rat model of I/R.

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Materials and methods

Reagents. Scoparone was purchased from Dalian Meilun Biology Technology Co., Ltd., (Dalian, China).

Primary cultures of neonatal rat cardiac myocytes. Cardiac myocytes were prepared from 20 neonatal Sprague-Dawley rats (Male, 1-3 days-old, 10 g) as previously described (13). Rats were obtained from Hebei Medical University. Rats were housed under the same standard environmental conditions of light (a 12-h light/dark cycle), temperature ($22\pm 2^{\circ}\text{C}$), and ambient humidity of $50\pm 10\%$ with free access to food and water. Briefly, the obtained ventricles were cut into sections, which were digested with trypsin at 37°C for 8 min. Subsequently, the supernatant was added to Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The digestion step was repeated 6-8 times, until the tissue sections were digested. Cell suspensions were centrifuged at $800 \times g$ for 5 min at 37°C , and the cells were cultured with DMEM containing 10% FBS at 37°C in an atmosphere containing 5% CO_2 for 2 h. The non-adherent cell suspension was collected to separate fibroblast cells and cardiac myocytes based on the varying durations of adherence. The fibroblast cells and cardiac myocytes were separated by its adherent at different time. The cardiac myocytes (1×10^5 cells/ml) were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 g/ml vitamin B12. After 48 h, dead cells were removed, and the medium was replaced with fresh medium containing 0.1 mM bromodeoxyuridine. The cardiac myocytes were assessed by immunofluorescence staining with α -actin (cat. no. sc-58670; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) as previously described (13); myocardial cell purity was confirmed at $>95\%$.

OGD/R model. OGD/R was performed on primary cultured neonatal rat cardiac myocytes according to previously described methods (14). Briefly, primary cultured neonatal rat cardiac myocytes were randomly divided into five groups: Control group, OGD/R group, scoparone low-dose group (S-L), scoparone mid-dose group (S-M) and scoparone high-dose group (S-H). The control group was incubated without any treatment. As for the OGD/R group, the cell medium was replaced with DMEM (glucose-free), which was preincubated with a mixture of 5% CO_2 and 95% N_2 for 20 min to remove O_2 . Subsequently, cells were cultured in an incubator containing 5% CO_2 and 95% N_2 at 37°C . After 3 h, the medium was replaced with DMEM containing glucose, and cardiac myocytes were transferred into an incubator containing 5% CO_2 at 37°C for 1 h. The cells in the S-L, S-M and S-H groups were pretreated with scoparone at 100, 500 and 1,000 mg/ml, respectively, for 1 h prior to OGD/R, which was conducted as described for the OGD/R group.

I/R rat model. Male Wistar rats with body weight ranging from 240-260 g, 7-weeks old were used in the present study. Rats were obtained from Hebei Medical University and were housed under the same standard environmental conditions of light (12-h light/dark cycle), temperature ($22\pm 2^{\circ}\text{C}$), and

ambient humidity of $50\pm 10\%$ with free access to food and water. A total of 120 Wistar rats were randomly divided into five groups: Sham-operated group (sham), I/R group, scoparone low-dose group (S-L), scoparone mid-dose group (S-M) and scoparone high-dose group (S-H) ($n=24$ rats/group). Briefly, the rats were anesthetized with ether and an incision was made in the chest to expose the heart. The left anterior descending branch of the coronary artery was then isolated. With the exception of the sham group, in the other groups, the coronary artery was immediately ligated with line 0 (15); the groups underwent ischemia for 30 min, followed by 120 min of reperfusion. Scoparone was administered 1 h prior to ligation. The rats in the sham and I/R groups were intravenously injected with 2 ml/kg normal saline, whereas the rats in the S-L, S-M and S-H groups were intravenously injected with 25, 50, 100 mg/kg scoparone, respectively. The rats were sacrificed after reperfusion and myocardial tissues were collected. The present study was approved by the Ethics Committee of Hebei Medical University (Shijiazhuang, China).

Cell viability assay. Following OGD/R *in vitro*, cell viability was determined using MTT reagent. Cells (1×10^5 /ml) were cultured with MTT reagent (final concentration, 0.5 mg/ml) for 4 h at 37°C . The medium was then removed and dimethyl sulfoxide (150 μl) was added to each well for 15 min at 37°C , in order to solubilize formazan. The absorbance of formazan was measured at 492 nm, which is directly proportional to cell viability.

Cell apoptosis assay. The apoptotic rates of cardiac myocytes subjected to OGD/R injury *in vitro* and I/R injury *in vivo* were measured by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay using a TUNEL kit (*In situ* Cell Death Detection kit, fluorescein; Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's protocols. The cell nucleus was stained with DAPI (Roche Diagnostics) according to manufacturer's protocols. Positive TUNEL staining was observed under a fluorescence microscope (TE2000U; Nikon Corporation, Tokyo, Japan) using a B-2A filter (450-490 nm excitation filter, 505 nm dichroic mirror, 520 nm bandpass filter). The ratio was determined by calculating the number of TUNEL-positive cells to the total number of cells in each of the 10 fields of view.

ELISA assay. Following OGD/R *in vitro*, cell culture medium was collected. The levels of lactate dehydrogenase (LDH) and creatine kinase (CK) were measured using commercial ELISA kits (cat. nos. JL13677 and 0-025486; Shanghai Jiang Lai Biological Technology Co., Ltd., Shanghai, China), according to the manufacturer's protocols. In addition, the cells were collected by centrifugation ($1,000 \times g$, 5 min, 37°C), and the levels of malondialdehyde (MDA) and superoxide dismutase (SOD) were measured using commercial ELISA kits (cat. no. JL13297 and JL11065; Shanghai Jiang Lai Biological Technology Co., Ltd.) according to the protocols recommended by the manufacturer.

Following I/R *in vivo*, serum samples were collected by centrifugation ($3,000 \times g$, 10 min, 4°C) from myocardial tissues. The concentrations of LDH and CK in the serum were detected using ELISA kits (cat. nos. JL13677 and 0-025486; Shanghai Jiang Lai Biological Technology Co., Ltd.) according

to manufacturer's protocols. In addition, myocardial tissues were collected from all rats to detect MDA and SOD levels. Ice physiological saline is added to the myocardial tissues. A total of 10% myocardial tissue homogenate was made by using a high-speed homogenizer and centrifuged $3,000 \times g$ at 4°C . The supernatant was collected. The levels of MDA and SOD in the supernatant were determined using commercial kits (cat. nos. JL13297 and JL11065; Shanghai Jiang Lai Biological Technology Co., Ltd.) according to the manufacturer's protocols.

Reactive oxygen species (ROS) assay. Intracellular ROS levels were measured using a fluorescent carboxy- H_2DCFDA probe, as previously described (16). Carboxy- H_2DCFDA is hydrolyzed to H_2DCF in cells; H_2DCF emits no fluorescence and cannot leave the cell through the cell membrane. However, ROS can oxidize H_2DCF to DCF, which emits a green fluorescence; therefore, detection of the fluorescence intensity of DCF can reflect intracellular ROS levels; the fluorescence intensity is proportional to the concentration of ROS. Following I/R injury, the myocardium was homogenized in Hank's buffered salt solution and the supernatant was collected. The samples were cultured with $10 \mu\text{M}$ carboxy- H_2DCFDA for 20 min at 37°C in the dark. The fluorescence signal intensity of DCF was detected using a flow cytometer with 488 nm excitation wavelength and 600 nm detection wavelength. Expo 32 ADC (Beckman Coulter, Inc., Brea, CA, USA) was used to analyze the fluorescence data.

Measurement of infarct area (IA). Myocardial IA was determined using the triphenyl tetrazolium chloride (TTC) staining method (17). Briefly, the heart samples were frozen and sliced into 1 mm sections along the vertical axis. The sections were incubated for 15 min at 37°C in 1% TTC, and were then immersed in 4% formaldehyde for 12 h at 37°C . IA were determined using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Western blotting. Following OGD/R *in vitro*, cardiac myocytes were collected and lysed at 4°C with lysis buffer (Tiangen Biotech, Co., Ltd., Beijing, China) containing $1 \mu\text{M}$ phenylmethylsulfonyl fluoride. Protein concentration was determined according to the bicinchoninic acid method. Equal amounts of protein ($100 \mu\text{g}$) were separated by 10% SDS-PAGE and were electrotransferred onto a polyvinylidene fluoride membrane. The membrane was blocked with Tris-buffered saline (TBS) containing 5% skimmed milk for 1 h at 37°C , and was then incubated with primary antibodies against B-cell lymphoma 2 (Bcl-2; cat. no. ab692), Bcl-2-associated X protein (Bax; cat. no. ab77566), caspase-3 (cat. no. ab13585) and cytochrome c (Cyt C; cat. no. ab110325; Abcam, Cambridge, MA USA), all 1:100 dilution, at 4°C overnight. Subsequently, the membrane was washed with TBST [TBS, (pH 7.5) containing 0.1% Tween-20] and was incubated with an anti-Mouse immunoglobulin G (IgG) H&L secondary antibody (1:10,000; cat. no. ab6785; Abcam) for 1 h at 37°C . Signals were then developed using the Enhanced Chemiluminescence Plus Western Blotting Detection system (Amersham; GE Healthcare Life Sciences, Little Chalfont, UK). Signal intensities were semi-quantified by densitometry using ImageJ v1.45 software (National Institutes of Health, Bethesda, MD,

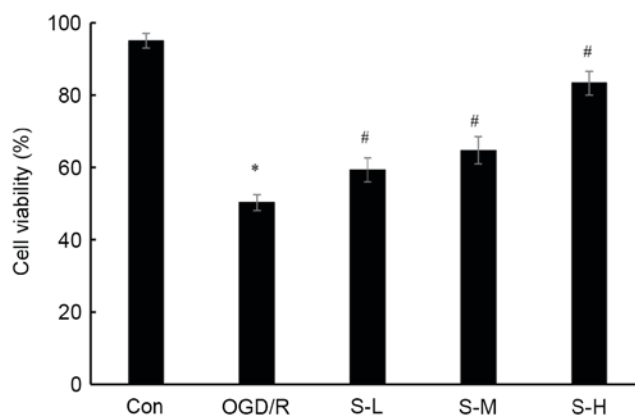


Figure 1. Effects of scoparone on viability of cardiac myocytes following OGD/R injury. Con group, cells were cultured under normal conditions; OGD/R group, cells were subjected to 3 h OGD and 1 h recovery; S-L, S-M and S-H groups, cells were pretreated with scoparone at concentrations of 100, 500 and 1,000 mg/ml, respectively, for 1 h prior to OGD/R. Data are presented as the means \pm standard deviation of three independent experiments. * $P < 0.05$ vs. the Con group; # $P < 0.05$ vs. the OGD/R group. Con, control; OGD/R, oxygen-glucose deprivation/reoxygenation; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose.

USA) and were normalized against the corresponding β -actin (cat. no. ab8226; Abcam) signals. The antibody was diluted by 1:1,000 and incubated for 1 h at 37°C .

Immunohistochemical staining. Harvested myocardial tissues were fixed in formalin overnight at 37°C , paraffin embedded; serial sections ($3\text{--}5 \mu\text{m}$) obtained. The myocardial sections were blocked with 3% hydrogen peroxide for 15 min at room temperature. Subsequently, the sections were microwaved in 10 mmol/l (pH 8.0) EDTA (Sangon Biotech Co., Ltd., Shanghai, China) for 2 min, incubated with 5% goat serum (Shanghai Haoran Bio, Shanghai, China) for 1 h at 37°C , and incubated overnight at 4°C with various antibodies (Cyt c, caspase-3, Bcl-2 and Bax) as aforementioned. Anti-Mouse IgG H&L was used as aforementioned. To analyze staining, the following systems were used: PicTure PV6000 and Elivision Plus (Fuzhou Maixin Biotech Development Co., Ltd., Fuzhou, China). Finally, the sections were counterstained with hematoxylin (3 min, 37°C). An Olympus BX41 brightfield microscope (Olympus Corporation, Tokyo, Japan) was used to observe sections.

Statistical analysis. Data are presented as the means \pm standard deviation. Experiments were repeated in triplicate. All data were analyzed using one-way analysis of variance followed by Bonferroni post hoc test (SPSS software package version 19.0; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Protective effects of scoparone on OGD/R injury in cardiac myocytes. Following OGD/R injury in cardiac myocytes, cell viability and SOD activity were significantly reduced, whereas LDH release, MDA levels, CK activity and cell apoptosis were markedly increased. Scoparone, at concentrations of 100, 500 and 1,000 mg/ml, attenuated these alterations in a dose-dependent manner (Figs. 1-3). In cardiac myocytes treated with 100,

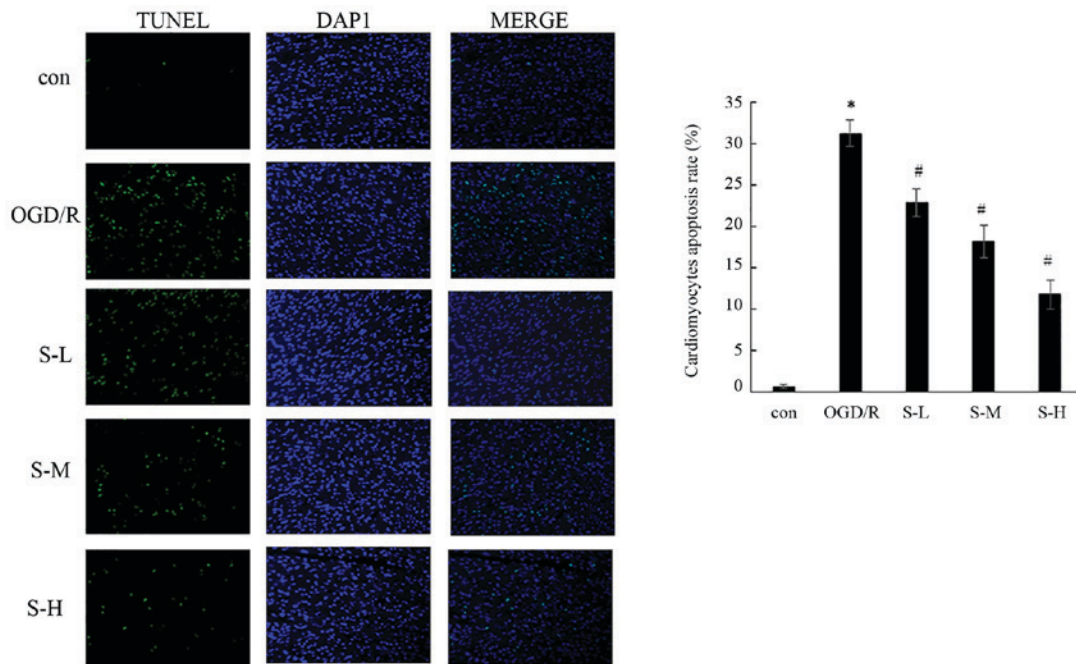


Figure 2. Effects of scoparone on cell apoptosis in cardiac myocytes following OGD/R injury. Con group, cells were cultured under normal conditions; OGD/R group, cells were subjected to 3 h OGD and 1 h recovery; S-L, S-M and S-H groups, cells were pretreated with scoparone at concentrations of 100, 500 and 1,000 mg/ml, respectively, for 1 h prior to OGD/R. Data are presented as the means \pm standard deviation of three independent experiments. * $P < 0.05$ vs. the Con group; # $P < 0.05$ vs. the OGD/R group. Con, control; OGD/R, oxygen-glucose deprivation/reoxygenation; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling.

500 and 1,000 mg/ml scoparone prior to OGD/R injury, cell viability was increased by 17.9, 28.7 and 65.7%, respectively; cell apoptosis was decreased by 27.0, 41.7 and 62.2%, respectively; LDH levels were decreased by 12.8, 32.1 and 52.8%, respectively; MDA production was decreased by 13.7, 28.6 and 47.4%, respectively; SOD activity was increased by 24.4, 56 and 82.4%, respectively; and CK activity was decreased by 24.2, 47.2 and 62.7%, respectively.

Protective effects of scoparone on I/R injury in rats. Following I/R injury in rats, the levels of LDH and MDA, and SOD and CK activities were detected. I/R significantly increased the LDH levels ($4,152.51 \pm 487.31$ U/l), MDA production (10.547 ± 0.92 mmol/ml), and CK activity ($1,137.18 \pm 106.35$ U/ml), whereas SOD activity was significantly decreased ($1,684.68 \pm 143.56$ U/l) compared with in the sham group (Fig. 4; $P < 0.05$). Conversely, compared with the I/R group, scoparone was revealed to significantly decrease the LDH levels ($4,034.15 \pm 428.23$, $3,502.53 \pm 412.04$ U/l and $3,425.14 \pm 482.47$ U/l, respectively; $P < 0.05$), MDA production (8.677 ± 0.495 , 7.621 ± 0.587 and 6.805 ± 0.647 mmol/ml, respectively; $P < 0.05$), and CK activity (924.478 ± 38.841 , 766.758 ± 43.812 and 708.158 ± 83.958 U/l, respectively; $P < 0.05$), whereas SOD activity was increased ($1,977.341 \pm 176.902$, $2,173.923 \pm 185.483$ and $2,365.837 \pm 166.384$ U/l, respectively; $P < 0.05$) in a dose-dependent manner, compared with the I/R group (Fig. 4).

Effects of scoparone on I/R injury-induced cardiac myocyte apoptosis in rats. The apoptotic rate of cardiac myocytes was detected by TUNEL staining. The results indicated that the percentage of apoptotic cardiac myocytes was significantly

increased in the I/R group ($34.95 \pm 1.53\%$), compared with in the sham group (Fig. 5; $P < 0.05$). Treatment with scoparone, at doses of 25, 50 and 100 mg/kg, resulted in a dose-dependent reduction in the apoptotic rate of cardiac myocytes following I/R injury, to 26.14 ± 1.71 , 22.11 ± 1.98 and $12.13 \pm 1.54\%$, respectively.

Effects of scoparone on I/R injury-induced myocardial IA in rats. TTC staining was conducted to determine the effects of scoparone on myocardial infarct size in I/R rats (Fig. 6). In the I/R group, myocardial IA was $85.09 \pm 2.53\%$, which was a significantly increased compared with the sham group ($P < 0.05$). Treatment of scoparone, at a dose of 25, 50 and 100 mg/kg, significantly reduced myocardial IA (43.98 ± 1.96 , 25.64 ± 2.36 and $9.05 \pm 1.87\%$, respectively) compared with in the I/R group ($P < 0.05$).

Effects of scoparone on ROS concentration in rats following I/R injury. ROS concentration was determined following I/R using a carboxy- H_2DCFDA probe. The fluorescence intensity of DCF represents the concentration of ROS. As shown in Fig. 7, I/R injury significantly increased fluorescence intensity compared with in the sham group ($677.93 \pm 37.44\%$ of control). However, scoparone pretreatment, at a dose of 25, 50 and 100 mg/kg, significantly decreased fluorescence intensity (454.29 ± 32.32 , 346.64 ± 30.90 , $224.45 \pm 27.44\%$ of control, respectively) induced by I/R in a dose-dependent manner.

Effects of scoparone on caspase-3 and Cyt C expression. As shown in Fig. 8, the results of western blot analysis indicated that caspase-3 and Cyt C expression were increased following OGD/R injury. Conversely, scoparone, at concentrations of

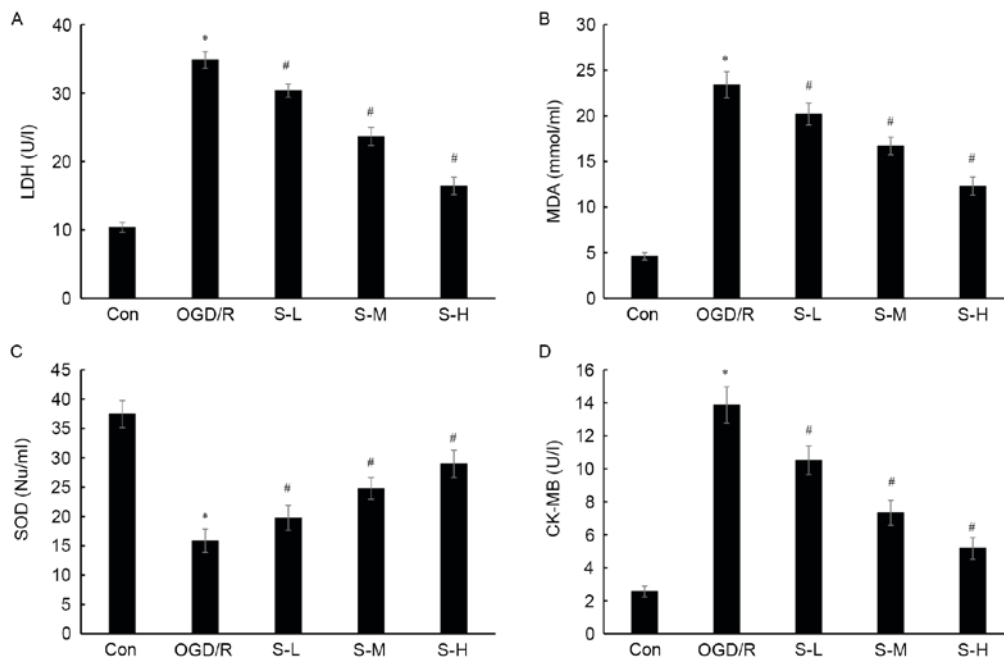


Figure 3. Effects of scoparone on (A) LDH, (B) MDA, (C) SOD and (D) CK levels in cardiac myocytes following OGD/R injury. Con group, cells were cultured under normal conditions; OGD/R group, cells were subjected to 3 h OGD and 1 h recovery; S-L, S-M and S-H groups, cells were pretreated with scoparone at concentrations of 100, 500 and 1,000 mg/ml, respectively, for 1 h prior to OGD/R. Data are presented as the means \pm standard deviation of three independent experiments. * $P < 0.05$ vs. the Con group; # $P < 0.05$ vs. the OGD/R group. CK, creatine kinase; Con, control; LDH, lactate dehydrogenase; MDA, malondialdehyde; OGD/R, oxygen-glucose deprivation/reoxygenation; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose; SOD, superoxide dismutase.

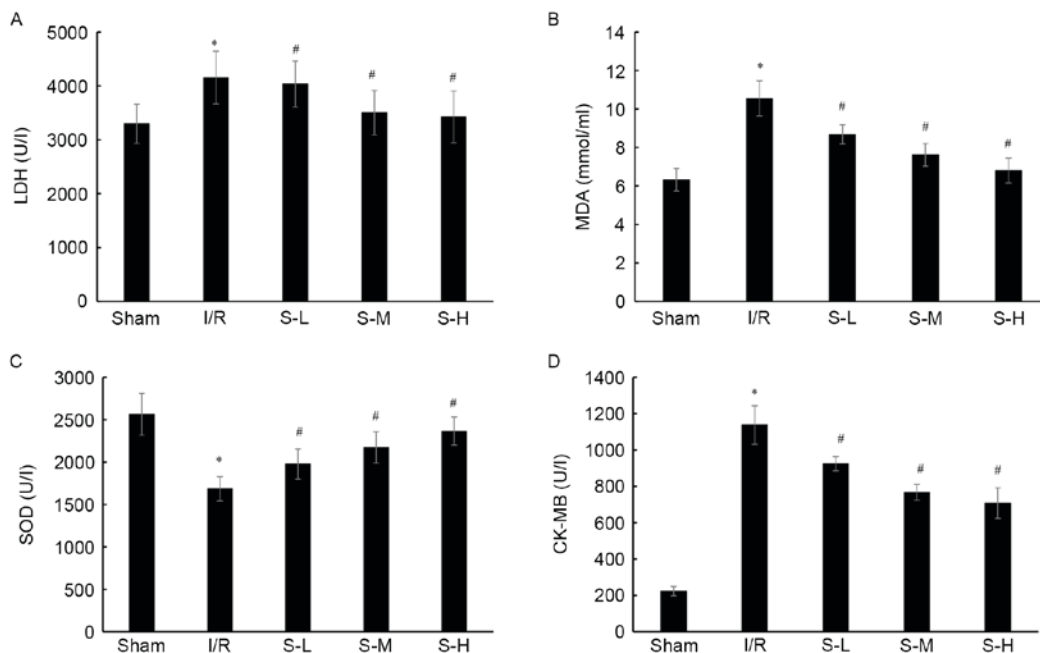


Figure 4. Effects of scoparone on (A) LDH, (B) MDA, (C) SOD and (D) CK levels in rats following I/R injury. With the exception of the sham group, the other groups underwent ischemia for 30 min, followed by 120 min of reperfusion. Rats in the sham and I/R groups were intravenously injected with 2 ml/kg normal saline. Rats in the S-L, S-M and S-H groups were intravenously injected with 25, 50 and 100 mg/kg scoparone respectively. Data are presented as the means \pm standard deviation ($n = 24$ /group). * $P < 0.05$ vs. the sham group; # $P < 0.05$ vs. the I/R group. CK, creatine kinase; I/R, ischemia-reperfusion; LDH, lactate dehydrogenase; MDA, malondialdehyde; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose; SOD, superoxide dismutase.

100, 500, 1,000 mg/ml, significantly and dose-dependently decreased caspase-3 and Cyt C expression following OGD/R injury of cardiac myocytes.

The effects of scoparone on caspase-3 and Cyt C expression in I/R rats were detected by immunohistochemical

staining. As shown in Fig. 9, I/R administration markedly increased caspase-3 and Cyt C expression compared with in the sham group; however, scoparone inhibited I/R-induced caspase-3 and Cyt C expression in a dose-dependent manner.

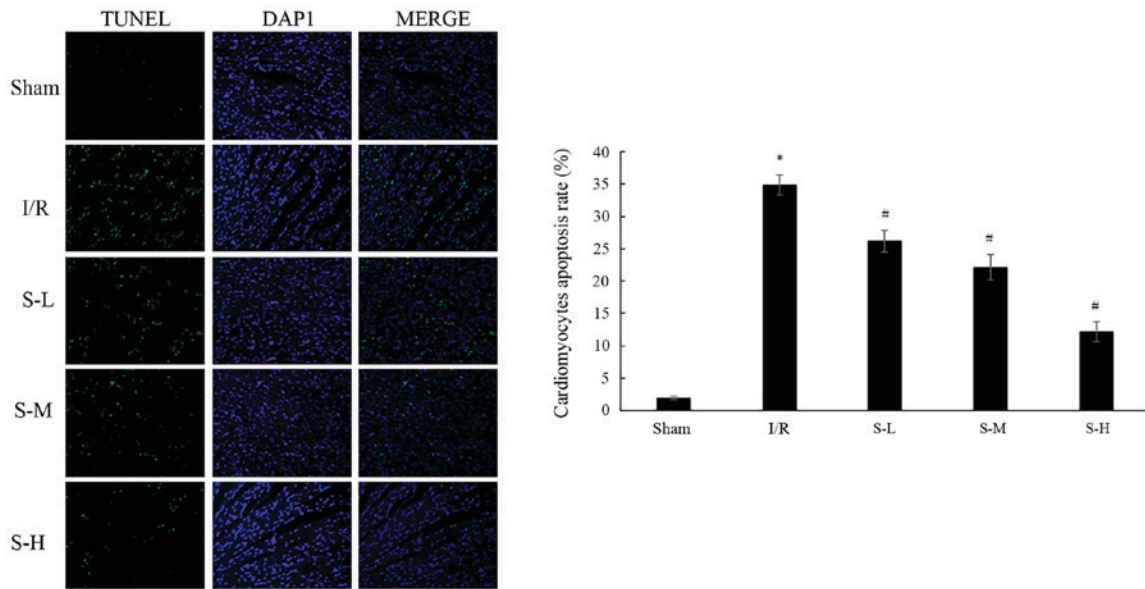


Figure 5. Effects of scoparone on cell apoptosis in rats following I/R injury. With the exception of the sham group, the other groups underwent ischemia for 30 min, followed by 120 min of reperfusion. Rats in the sham and I/R groups were intravenously injected with 2 ml/kg normal saline. Rats in the S-L, S-M and S-H groups were intravenously injected with 25, 50 and 100 mg/kg scoparone respectively. Data are presented as the means \pm standard deviation (n=24/group). *P<0.05 vs. the sham group; #P<0.05 vs. the I/R group. I/R, ischemia-reperfusion; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling.

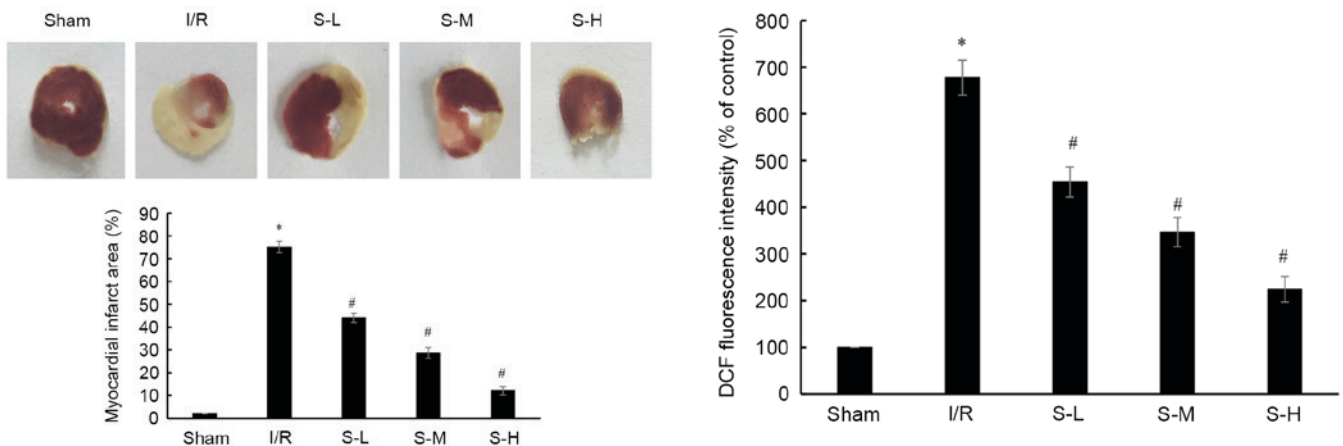


Figure 6. Effects of scoparone on myocardial infarct area in rats following I/R injury. With the exception of the sham group, the other groups underwent ischemia for 30 min, followed by 120 min of reperfusion. Rats in the sham and I/R groups were intravenously injected with 2 ml/kg normal saline. Rats in the S-L, S-M and S-H groups were intravenously injected with 25, 50 and 100 mg/kg scoparone respectively. Data are presented as the means \pm standard deviation (n=24/group). *P<0.05 vs. the sham group; #P<0.05 vs. the I/R group. I/R, ischemia-reperfusion; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose.

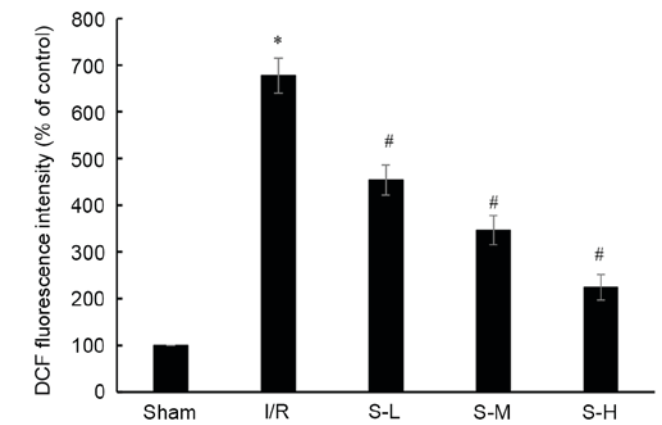


Figure 7. Effects of scoparone on ROS concentration in rats following I/R injury. DCF fluorescence intensity of the sham group cells was set at 100% for comparison with the fluorescence intensity of the other groups. With the exception of the sham group, the other groups underwent ischemia for 30 min, followed by 120 min of reperfusion. Rats in the sham and I/R groups were intravenously injected with 2 ml/kg normal saline. Rats in the S-L, S-M and S-H groups were intravenously injected with 25, 50 and 100 mg/kg scoparone respectively. Data are presented as the means \pm standard deviation (n=24/group). *P<0.05 vs. the sham group; #P<0.05 vs. the I/R group. I/R, ischemia-reperfusion; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose.

Effects of scoparone on Bcl-2 and Bax expression. As shown in Figs. 8 and 9, I/R injury induced an increase in Bax expression and a decrease in Bcl-2 expression in a cell model of OGD/R model and a rat model of I/R. Conversely, treatment with scoparone attenuated these alterations in a dose-dependent manner.

Discussion

Myocardial ischemia can cause tissue damage and cell death over a certain period of time; therefore, rapid restoration of blood

perfusion is required for cardiac myocyte survival. I/R injury in myocardial tissue is mediated by calcium overload, ROS generation, cell apoptosis and disordered energy metabolism, which may eventually lead to organ damage and myocardial metabolic disorder. LDH is present in the cytoplasm of all tissues. When cell apoptosis or necrosis occurs, the membrane structure is destroyed, thus leading to the release of LDH from the cytoplasm to outside of the cell. Therefore, detecting LDH levels may indirectly reflect the extent of the damage to the cell membrane (18). CK is a cardiac-specific marker of acute

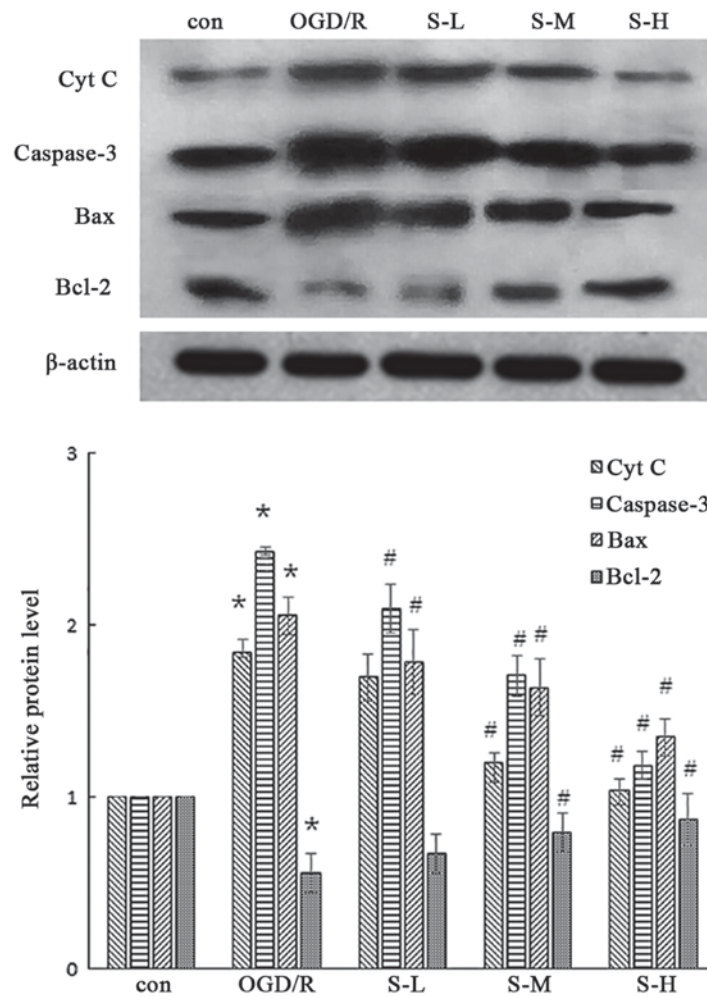


Figure 8. Effects of scoparone on the expression levels of caspase-3, Cyt C, Bcl-2 and Bax in cardiac myocytes following OGD/R injury. * $P < 0.05$ vs. the Con group; # $P < 0.05$ vs. the OGD/R group. Data are presented as the means \pm standard deviation (n=3). Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Con, control; Cyt C, cytochrome c; OGD/R, oxygen-glucose deprivation/reoxygenation; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose.

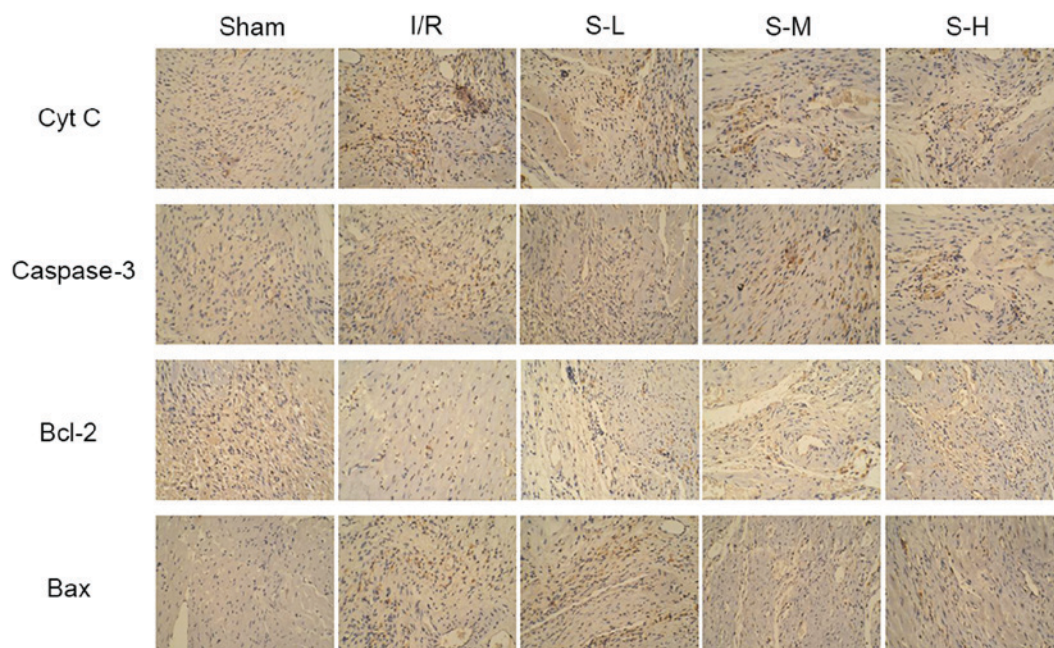


Figure 9. Effects of scoparone on the expression of caspase-3, Cyt C, Bcl-2 and Bax in rats following I/R injury (x40). Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Cyt C, cytochrome c; I/R, ischemia-reperfusion; S-H, scoparone high-dose; S-L, scoparone low-dose; S-M, scoparone mid-dose.

myocardial infarction and an indicator for myocardial tissue injury. An increase in serum levels of CK indicates that the myocardial cell biological membrane is damaged. Myocardial hypoxia produces a large amount of ROS, which oxidize unsaturated fatty acids, leading to damaged cell membrane structure and sarcoplasmic reticulum calcium pump function, thus inducing extracellular Ca^{2+} internal flow and calcium overload. Calcium overload accelerates ROS generation and causes myocardial cell damage (19). ROS also damage the mitochondrial membrane system, oxidize Cyt C and reduce the activity of ATP synthetase, thus resulting in mitochondrial dysfunction, which can lead to cell apoptosis (20). MDA is formed by lipid peroxidation, and is often used to quantify the extent of lipid peroxidation and reflects the damage caused by ROS (14,21). SOD is an important antioxidant, which has exhibits ROS scavenging properties. During tissue ischemia, SOD is consumed in large quantities, and SOD synthesis of SOD is suppressed, resulting in a decrease in SOD levels. The serum levels of SOD are able to reflect the ability to clear ROS.

It has previously been reported that Bcl-2 and Bax are involved in the process of myocardial cell apoptosis (22), and the ratio of Bcl-2 to Bax may be a critical factor for apoptosis (23). The caspase cascade also serves a key role in apoptosis (24); caspase-3 typically functions downstream of other caspases and directly activates enzymes that are responsible for DNA fragmentation in the intrinsic apoptosis pathway (25).

Scoparone is a major component of the shoot of *Artemisiae Scopariae* Herba. In the present study, scoparone was revealed to exert a protective effect on I/R-induced myocardial injury.

The results of the present study demonstrated that I/R leads to an increase in LDH levels, MDA and CK content, ROS generation, cell apoptosis and myocardial IA, these alterations are accompanied by reductions in cell viability and SOD activity. However, pretreatment with scoparone prior to I/R injury, significantly decreased LDH levels, MDA production, CK levels and ROS generation, and increased SOD activity. These results indicated that scoparone may reduce ROS-induced cell lipid peroxidation. In addition, scoparone was able to increase cell viability, and decrease cell apoptosis and myocardial IA in a dose-dependent manner following I/R. Furthermore, there was a marked reduction in the expression levels of Bax, caspase-3 and Cyt C, alongside a significant increase in the expression levels of Bcl-2 in scoparone-treated groups compared with in the I/R group. Myocardial cell apoptosis is induced by various factors and involves two main signaling pathways: The death receptor pathway and the mitochondrial signaling pathway, during which the mitochondria receive various apoptosis-stimulating signals. Apoptosis-stimulating signal, including ROS, attack the mitochondrial membrane, which is rich in polyunsaturated fatty acids, thus causing mitochondrial swelling, decreases in membrane fluidity, opening of the mitochondrial membrane permeability transition pore and Cyt C release. Eventually, the caspase protease cascade is activated; caspase-9 is activated first, which further activates the downstream effector molecule caspase-3, thus leading to cell apoptosis. The Bcl-2 family serves an important role in the mitochondrial-dependent apoptotic pathway, and includes proapoptotic and anti-apoptotic proteins. The Bcl-2 protein is distributed in the outer mitochondrial membrane; Bcl-2 has an anti-apoptotic role via inhibition of Cyt C release, thus

suppressing activation of the downstream caspase cascade. Bax is a proapoptotic protein that belongs to the Bcl-2 family; Bax is mainly located in the cytoplasm, and it can transfer to the outer mitochondrial membrane in response to stimulation by apoptotic signals. Bax can induce cell apoptosis by increasing permeability of the mitochondrial outer membrane to promote the release of Cyt C (26). The results of the present study suggested that scoparone inhibited cell apoptosis by influencing the aforementioned pathway.

In conclusion, scoparone may significantly reduce the formation of MDA, enhance SOD activity, decrease LDH and CK levels, and attenuate the myocardial IA. In addition, scoparone is able to inhibit cell apoptosis, upregulate Bcl-2 expression, and downregulate Bax, caspase-3 and Cyt C protein expression. These findings suggested that scoparone can scavenge ROS, reduce oxidative stress, protect mitochondria, improve myocardial dysfunction and inhibit cell apoptosis induced by I/R via the mitochondrial pathway.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to further research but are available from the corresponding author on reasonable request.

Authors' contributions

CW and XG participated in the research design. CW, YW and JM conducted experiments. CW and YW performed data analysis. CW was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hebei Medical University (Shijiazhuang, China).

Consent for publication

Not applicable.

Conflicts of interest

The authors declare that they have no competing interests.

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