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Original Article

Berberine protects human and rat cardiomyocytes from hypoxia/reoxygenation-triggered apoptosis

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Abstract: Berberine (BBR) confers potential cardioprotective effects. However, the relevant mechanisms underlying its regulation of cardiomyocyte survival following hypoxia/reoxygenation (H/R) treatment remain unknown. The present study investigated whether BBR could protect H/R by suppressing apoptosis and explored how TGF-β/Smad4 signaling pathway influenced H/R *in vitro*. Two cardiomyocyte cell lines-AC16 and H9c2- were treated with H/R and BBR. The survival and apoptosis of these two cell lines were assessed using the MTT and BrdU assays and western blotting (WB) and flow cytometry. Mitochondrial reactive oxygen species (ROS) and caspase (Cas)-3, Cas-8, and Cas-9 activation were evaluated using enzyme-linked immunosorbent assay as well as WB. Compared to the control group, H/R resulted in notable cell apoptosis, whereas BBR treatment evidently counteracted the process. BBR also markedly suppressed H/R-triggered excessive mitochondrial ROS generation and inhibited Smad4 expression. Overexpressing Smad4 in BBR-treated H/R-exposed cardiomyocytes reversed the effect of BBR treatment on apoptosis. Therefore, BBR protects H/R-treated cardiomyocytes from apoptosis by inhibiting the TGF-β/Smad4 signaling pathway.

Keywords: BBR, cardiomyocytes, hypoxia/reoxygenation, apoptosis, TGF-β/smad4 pathway

Introduction

Myocardial infarction (MI)-the principal trigger for heart disease-related mortality worldwide-is largely caused by coronary heart diseases [1]. Currently, severe MI is a major public health concern. In acute MI, decreased blood supply results in sudden myocardial ischemia, which consequently triggers heart impairment [2, 3]. Hypoxic heart failure is characterized by the failure of the blood supply rate to meet metabolic demands [4, 5].

Hypoxia contributes to reactive oxygen species (ROS) production and oxidative stress (OS), resulting in cell death [6, 7]. Mitochondria can regulate hypoxia/reoxygenation (H/R)-induced myocardial apoptosis.

Berberine (BBR) is extracted from herbs such as *Cortex phellodendri* and *Hydrastis canadensis* [8]. BBR treatment has positive effects in diverse disease models [9-11]. BBR has been found to improve cardiovascular performance [12-16]. Chang et al. found that mice administered 0.1 g/kg/day BBR for 2 weeks exhibited cardiac protection against ischemia/reperfusion (I/R) injury [17]. Likewise, BBR could prevent cardiac I/R injury by mitigating oxidative stress in endoplasmic reticulum through JAK2/STAT3 signaling [18]. Furthermore, by suppressing autophagy, BBR treatment could decrease cardiac I/R injury [19]. A previous study demonstrated that TGF-β/Smad4 signaling pathway could lead to the apoptosis [20]. Another study indicated that Smad4 increases the infarct size and induces apoptosis by regulating c-fos and c-JUN [21]. However, to the best of our knowledge, whether BBR attenuates cardiac I/R injury remains to be determined. Some studies found that BBR also pre-

served mitochondrial function in pathological conditions [22, 23]. Loss of mitochondrial function is associated with suppression of apoptosis; hence, BBR treatment is believed to confer a protective effect by repressing I/R-triggered immoderate autophagy or cell death.

To test this presumption, an *in vitro* human and rat model of MI using cardiomyocytes under H/R exposure was established. BBR was administered to mitigate apoptosis and mitochondrial ROS triggered by I/R injury, and how BBR influenced TGF-β/Smad4 signaling was evaluated.

Material and methods

Cells

The human cardiomyocyte cell line AC16 and rat cardiomyocyte cell line H9c2 were obtained from BeNa Culture Collection and American Type Culture Collection, respectively. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) and DMEM in this research was added with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.). Additionally, these cells were incubated in a 5% CO_2 atmosphere at 37°C.

Cardiomyocytes were also freshly isolated from 4-day-old Wistar rats. Briefly, cartilage tissue was isolated and sliced (0.5 × 0.5 × 0.5 cm) and then digested with 0.1% w/v trypsin (Roche) for 30 min. Following that, 0.15% w/v collagenase II (Roche) was utilized to digest the tissue at 37°C. The tissue suspension was filtered via 100-μm nylon cell strainer (BD Falcon) and then it was cultured in T-75 culture flasks. After these cells were passaged a few times, they would be used for further research.

H/R treatment and grouping

The following were our experimental groups: control group, H/R group, H/R + BBR (10, 33, and 100 μM BBR) group, H/R + BBR + pcDNA3-NC group, and H/R + BBR + pcDNA3-Smad4 group. In the H/R group, cells were first seeded in 24-well plates and the culture medium was switched to low-carbohydrate DMEM. Cells were then subjected to hypoxia under 95% N_2 and 5% CO_2 for approximately 3 h. After these cells were treated with hypoxia, these cells were re-oxygenated with 95% O_2 and 5% CO_2. In the control group, cells were not subjected to any treatment. In the H/R + BBR group, cells were treated with BBR (10, 33, and 100 μM) for 2 h before H/R exposure. In the H/R + BBR + Smad4/NC group, cells were first transfected with pcDNA3-Smad4 or pcDNA3-NC for 48 h and then incubated with 100 μM BBR for 2 h before exposure to H/R.

Plasmid construction

Smad4 cDNA was amplified. The following are the forward and reverse primers: 5'-GAACGCTACCTTCGTTCAGACTTC-3' and 5'-CTACTCGTCATAGCCACCGTGAGCATA-3'. Following that, pcDNA3 vector (Invitrogen, USA) was used to subclone into PTCSC3 to construct Smad4 plasmids, which was confirmed by polymerase chain reaction (PCR).

MTT assay

The cell viability was detected through MTT assay (Merck, USA). First, cells were treated with H/R and/or BBR (10, 33, and 100 μM). At 24 h after H/R treatment, MTT was added into each sample. After 2 h incubation, the absorbance value was measured at 570 nm through microplate reader.

BrdU assay

Intracellular DNA synthesis was detected using bromodeoxyuridine (BrdU, B23151, Thermo Fisher). Cells were inoculated in 96-well plates and subjected with H/R and/or BBR treatment. BrdU assays were conducted at day 1 following BBR treatment. BrdU was added into each well and incubated for 120 min. After that, cells were fixed at 25°C for 0.5 h, incubated with peroxidase-conjugated anti-BrdU antibody (B35128, Thermo Fisher) for 1 h at 25°C, and cultured with a peroxidase substrate for 30 min. Then, absorbance of each sample was read at 490 nm. Cells not treated with BrdU or incubated with anti-BrdU antibody served as blank controls.

Colony formation assay

Cells were inoculated in 12-well plates and treated with H/R and/or BBR. At 24 h after H/R treatment, cells were transferred into the top agar layer (0.4%, 8 mm), and seeded into 12-well plates containing the bottom agar layer

Table 1. Sequences of primers in RT-qPCR

Primers	Sequences
Bcl-2 forward	5'-AAG CCG GCG ACG ACT TCT-3'
Bcl-2 reverse	5'-GGT GCC GGT TCA GGT ACT CA-3'
Bax forward	5'-ATG GAC GGG TCC GGG GAG-3'
Bax reverse	5'-ATC CAG CCC AAC AGC CGC-3'
TGF-beta forward	5'-TAC CTG AAC CCG TGT TG-3'
TGF-beta reverse	5'-GTT GCT GAG GTA TCG CC-3'
Smad4 forward	5'-CGC TTT TGT TTG GGT CAA CT-3'
Smad4 reverse	5'-CCC AAA CAT CAC CTT CAC CT-3'
GAPDH forward	5'-ACC ACA GTC CAT GCC ATC AC-3'
GAPDH reverse	5'-TCC ACC CTG TTG CTG TA-3'

(0.5%, 0.5 ml). After approximately 4 days, four areas on each plate were randomly selected, and the number of colonies was calculated.

Real-time quantitative PCR (RT-qPCR)

With TRIzol reagent (Sigma-Aldrich), total RNA was extracted and RNA was transcribed into cDNA. On ABI 7300 real-time PCR system (Applied Biosystems), RT-qPCR was carried out using SYBR-Green (Roche). The following run protocol was used: initial activation step at 95°C for 15 min followed by 3 step cycling of denaturation (at 94°C for 15 s), annealing (at 55°C for 30 s) and extension (at 70°C for 30 s). GAPDH was used as an internal control. Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method, and the formula ΔΔCt, where ΔΔCt = Ct(gene) - Ct(*GAPDH*), was used to calculate the relative mRNA level. The primers used were shown in **Table 1**.

Western blotting (WB)

Proteins in lysed cells were isolated by RIPA buffer (Beyotechonology, Shanghai, China) added with protease inhibitors (Beyotechonology, Shanghai, China). BCA kit (Sigma-Aldrich, USA) was employed to measure the concentration of protein. Protein was loaded onto SDS-PAGE gel and then onto polyvinylidene difluoride membranes following electrophoresis. What followed was that these membranes were inoculated overnight with primary antibodies against SMAD4 (1:1000, ab236321, Abcam), TGF-β (1:1000, ab92486, Abcam) and actin (1:3000, ab8227, Abcam) at 4°C respectively. After being incubated with primary antibodies, these membranes were then incubated with secondary antibodies at room temperature for approximately 1 h. Blots in these membranes were visualized with ECL detection substrate (Merck, USA). The density of each blot was measured by Gel-Pro Analyzer software (version 6.3; Media Cybernetics, Inc., Rockville, MD, USA) and the internal references in this research include Actin, Lamin B1 and HSP70.

Immunofluorescence assay (IFA)

Cells were placed onto 96-well plates with cover slips (0.5 ml/well). After washing with PBS once for 60 s, cells were fixed in 4% paraformaldehyde for 10-15 min, washed with PBS twice for 60 s, permeated with 0.1% Triton X-100 for 300 s, and then blocked with 10% goat serum for 60 min. Following these procedures, plates were incubated with anti-Smad4 antibody and the appropriate secondary antibody. Following incubation, cells were washed with PBS for 60 s several times at 24°C and 4',6-diamidino-2-phenylindole (DAPI) was employed to stain their nuclei. Images were obtained through a 63 × oil immersion lens using an SP8 Leica microscope equipped with DAPI and TRITC fluorescent excitation filters. For each group, more than 200 cells were classified as predominantly nuclear (N) and total (T) cells.

Flow cytometry

Cells were dispersed first and then stained with Annexin V-FITC and propidium iodide (Annexin V-FITC Apoptosis Detection kit; Beyotime Institute of Biotechnology) at 25°C for 15 min in the dark. Stained cells were subsequently loaded onto BD FACSCalibur™ flow cytometer (BD Biosciences) and the data was analyzed with FACS Diva version 6.0 software (BD Biosciences).

Intracellular and mitochondrial ROS production

Cellular ROS levels were detected using DCFH-DA. DCFH-DA was added into each sample and then each sample was incubated for 20 min at 37°C. The ROS level was determined through microplate reader (PlateReader M5, SpectraMax) at respective excitation and emission spectra of 495 nm and 529 nm.

In order to measure mitochondrial ROS, cells were exposed to 5 μM MitoSOX RED at 37°C

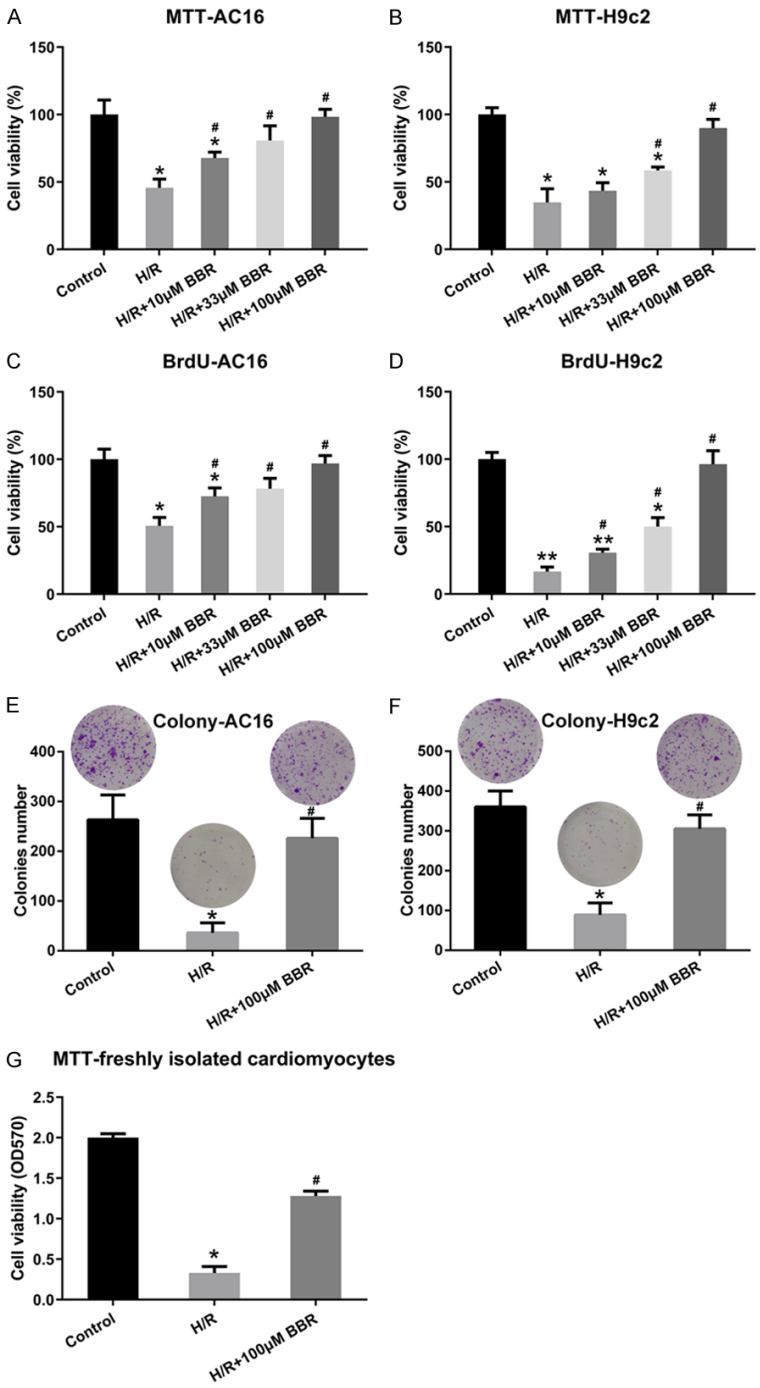


Figure 1. Effect of BBR treatment on cardiomyocyte viability following exposure to H/R. Human and rat cardiomyocytes were pretreated with various concentrations of BBR (0, 10, 33, and 100 μM) and then subjected to H/R treatment. A, B. MTT assay was performed to assess cell survival following different treatments. C, D. BrdU assay was performed to examine cell proliferation following different treatments. E, F. Colony formation assay was performed to assess cell growth following different treatments. G. Non-proliferating cardiomyocytes from 4 day-old Wistar rats were isolated, cultured, pretreated with 100 μM BBR, and subjected to H/R treatment. MTT assay was performed to assess cell survival following the treatments. Bars indicate mean (from three parallel experiments) ± SD. *P < 0.05, **P < 0.01 *vs.* the control group; #P < 0.05, ##P < 0.01 *vs.* the H/R group.

for 10 min and subsequently analyzed via microplate reader (PlateReader M5, SpectraMax) at respective excitation and emission spectra of 510 nm and 580 nm.

Subcellular fractionation

Cells (1 × 10^6/ml) plated on 6-cm dishes were harvested via cell lysis buffer (Thermo Fischer Inc.). Then, they were centrifuged at 7,500 rpm for 5 min and pellets were resuspended. After a few cycles of centrifugation and resuspension, pure nuclei were extracted.

Statistical analysis

Data are shown in the form as the mean ± standard deviation (SD). The t-test and one-way analysis of variance followed by Tukey's post hoc test were applied to compare differences between two groups and among multiple groups respectively. Two-sided $P < 0.05$ was chosen as the cutoff value which carried statistical significance.

Results

BBR treatment enhanced H/R-triggered cardiomyocyte viability

Human and rat cardiomyocytes were pretreated with different concentration of BBR. Then they were subjected to H/R treatment. MTT assay results showed that the viability of human and rat cardiomyocytes were suppressed following H/R exposure, whereas BBR treatment promoted cell survival (**Figure 1A**, **1B**). Furthermore, the proliferative rates of AC16 and H9c2 cells were impaired due to

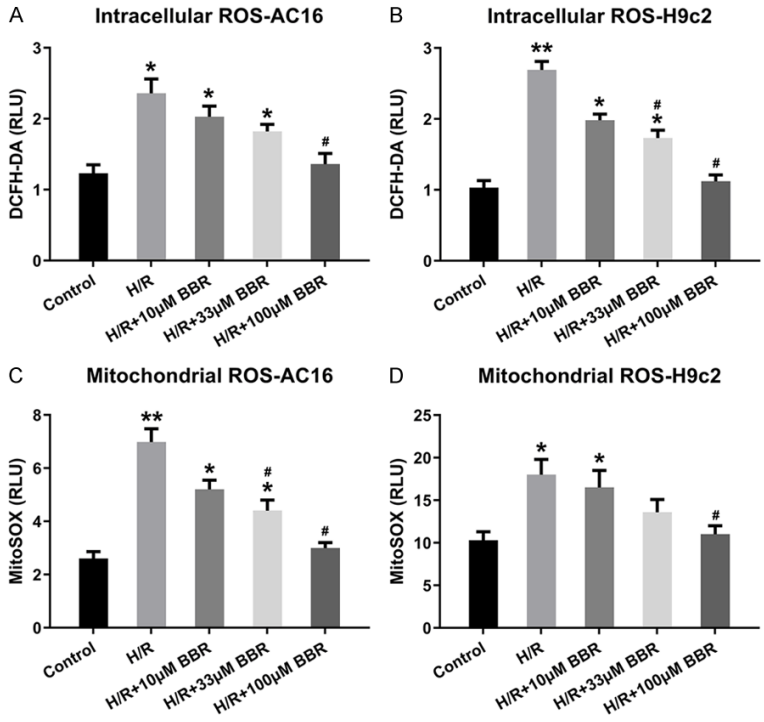


Figure 2. Effect of BBR treatment on ROS production. Human and rat cardiomyocytes were pretreated with various concentrations of BBR (0, 10, 33, and 100 μM) and then subjected to H/R treatment. A, B. ROS levels in AC16 and H9c2 cells were measured via DCFH-DA for 20 min following BBR incubation and H/R exposure. C, D. Mitochondrial ROS exhibited MitoSOX RED fluorescence, as determined using a microplate reader. Bars indicate mean ± SD. *P < 0.05, **P < 0.01 *vs.* the control group; #P < 0.05, ##P < 0.01 *vs.* the H/R group.

H/R injury, whereas BBR treatment mitigated this damage with its dosage increasing (**Figure 1C**, **1D**). Also, H/R exposure decreased the number of colonies in two cell types, whereas treatment with 100 μM BBR reversed this effect (**Figure 1E**, **1F**). To explore the effect of BBR on cardiomyocytes, freshly isolated cardiomyocytes from 4 day-old Wistar rats were subjected to treatment with BBR and H/R. H/R treatment considerably impaired the viability of freshly isolated cardiomyocytes, whereas BBR treatment prior to incubation partially restored cell viability (**Figure 1G**).

BBR treatment inhibited H/R-induced intracellular and mitochondrial ROS production

OS plays an essential role in H/R injury. Following exposure to H/R, intracellular and mitochondrial ROS production considerably increased in both human and rat cardiomyocytes (**Figure 2A**, **2B**). Following BBR treatment, ROS levels were reduced with its dosage increasing (**Figure 2C**, **2D**).

BBR treatment reduced H/R-triggered cardiomyocyte apoptosis

The apoptotic rates of human and rat cardiomyocytes raised following H/R exposure (**Figure 3A-D**), whereas their apoptosis decreased following BBR treatment. Furthermore, H/R injury resulted in a marked increase of Bax and Cas-3 expression and Cas-3 cleavage and decrease in Bcl-2 expression. However, BBR treatment reversed this effect (**Figure 3E**, **3F**). Also, H/R exposure led to apoptosis of AC16 and H9c2 cells, whereas BBR treatment mitigated this effect (**Figure 3G**, **3H**).

Cardiac fibrosis accompanies I/R injury, and galectin 3 (Gal3) is a well-known cardiac fibrosis marker [24]. We therefore tested Gal3 expression after BBR treatment to assess whether BBR also exerted protective effects on cardiac fibrosis in coupled with I/R injury. Moreover, I/R treatment increased Gal3 expression, whereas BBR treatment clearly decreased Gal3 expression in the treated cells (**Figure 3I**, **3J**).

BBR inhibited the TGF-β/Smad4 signaling pathway.

TGF-β/Smad4 signaling has been found to trigger cell death [20]. Thus, we evaluated whether BBR exerted its role through modulating the TGF-β/Smad4 signaling. RT-qPCR showed that H/R exposure evidently increased TGF-β expression in human and rat cardiomyocytes, whereas BBR treatment showed no effect on its expression (**Figure 4A**, **4B**). WB results further confirmed this observation (**Figure 4E**, **4F**). At the levels of mRNA and protein, H/R injury did not influence Smad4 expression, whereas BBR treatment suppressed its ex-

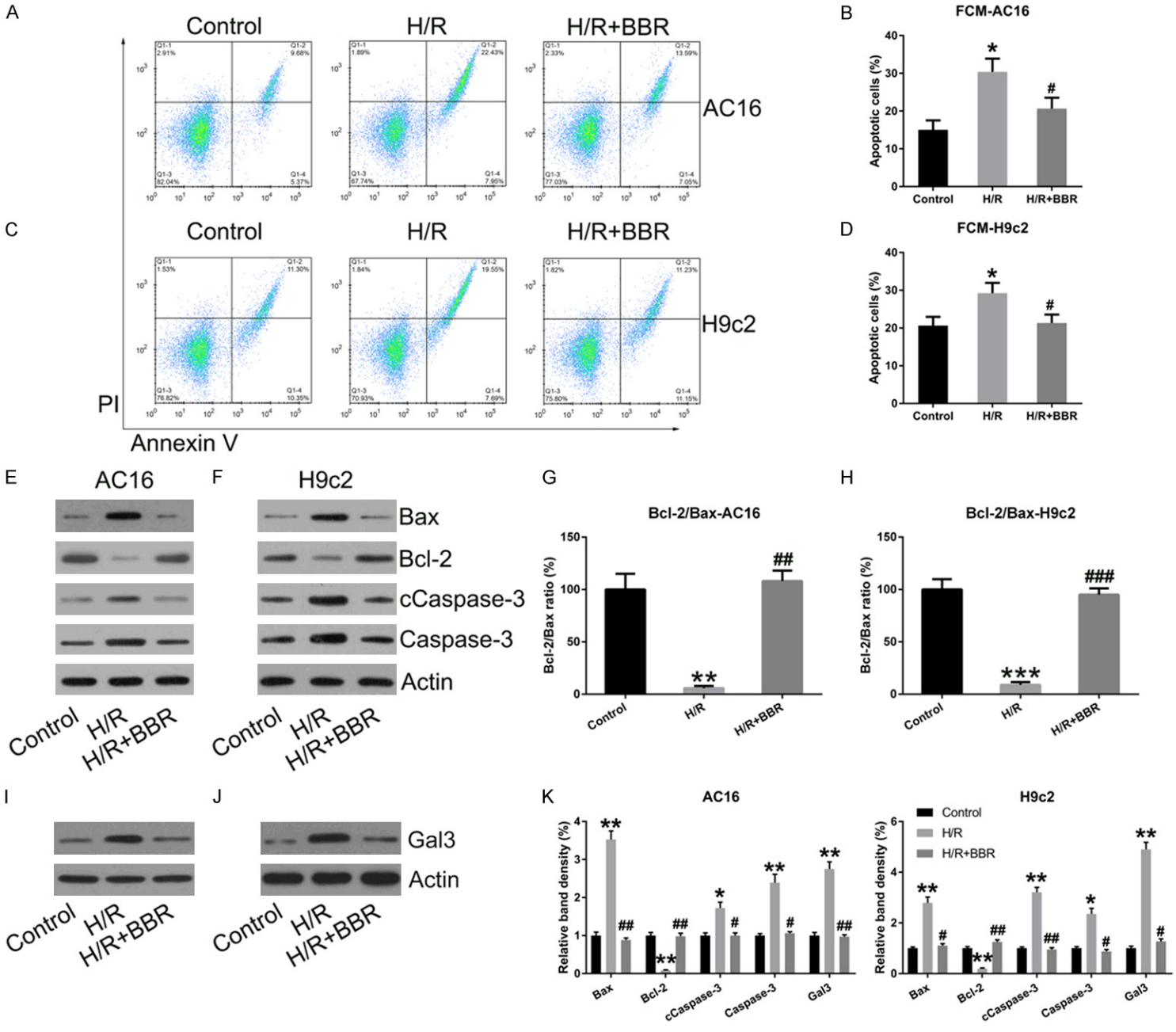

A
Control
H/R
H/R+BBR
AC16
C
H9c2
PI
Annexin V
B
FCM-AC16
Apoptotic cells (%)
Control
H/R
H/R+BBR
D
FCM-H9c2
E
AC16
F
H9c2
Bax
Bcl-2
cCaspase-3
Caspase-3
Actin
G
Bcl-2/Bax-AC16
Bcl-2/Bax ratio (%)
H
Bcl-2/Bax-H9c2
I
J
Gal3
K
Relative band density (%)
Control
H/R
H/R+BBR

Figure 3. Effects of BBR treatment on H/R-triggered cardiomyocyte apoptosis. Human and rat cardiomyocytes were pretreated with BBR (100 μM) and then subjected to H/R treatment. (A, C) Flow cytometry was performed to determine the extent of apoptosis in human and rat cardiomyocytes following different treatments. (B, D) Histograms show the level of cardiomyocyte apoptosis following different treatments. (E, F) WB was performed to assess the expression levels of Bcl-2, Bax, and Cas-3 and its cleaved form. (G, H) Bcl-2 and Bax bands were quantified to measure the Bcl-2/Bax ratio. (I, J) WB was performed to assess the expression level of Gal3. (K) Quantification of WB bands of (G-J). Bars indicate mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* the control group; #P < 0.05, ##P < 0.01, ###P < 0.001 *vs.* the H/R group.

pression (**Figure 4C-F**). Furthermore, IFA results revealed the cellular localization of Smad4 following different treatments, indicating TGF-β/Smad4 pathway activation. Exposure to H/R led to evident nuclear accumulation of Smad4, whereas BBR treatment led to decreased Smad4 expression, although its localization was unaffected (**Figure 4G**, **4H**). These findings suggest that BBR treatment inhibited TGF-β/Smad4 signaling by suppressing Smad4 activity.

Smad4 overexpression counteracted the effect of BBR in H/R-triggered cardiomyocytes

To further confirm its involvement in BBR-treated cells, Smad4 was overexpressed in BBR-treated H/R-triggered cardiomyocytes. RT-qPCR and WB analyses showed that Smad4 expression was upregulated following pcDNA3-Smad4 transfection (**Figure 5A-D**). Furthermore, IFA results confirmed that the fluorescence intensity of Smad4 increased following transfection, although its nuclear localization remained unaltered (**Figure 5E**, **5F**).

Next, we analyzed cell apoptosis and viability of BBR-treated H/R-induced human and rat cardiomyocytes following Smad4 overexpression. Flow cytometry results indicated that Smad4 overexpression significantly restored the BBR-downregulated apoptosis of H/R-induced AC16 and H9c2 cells (**Figure 6A-D**). Moreover, MTT assay results showed that Smad4 upregulation impaired the viability of BBR-treated H/R-induced human and rat cardiomyocytes (**Figure 6E**, **6F**). Hence, Smad4 gets involved in BBR-mediated apoptosis and viability of H/R-exposed human and rat cardiomyocytes.

Discussion

I/R injury can result in the impairment and dysfunction of organs [25], and it is regulated by a series of complicated events, including energy substrate loss [26], change in ionic homeostasis [27], ROS production [28], and cell death [29]. This study investigated how BBR affected the survival of rat and human cardiomyocytes following H/R treatment, which can simulate I/R injury *in vitro*. The findings indicated that (1) BBR treatment dramatically repressed apoptosis in H/R-triggered cardiomyocytes; (2) BBR treatment prevented excessive mitochondrial ROS generation, mitigated TGF-β/Smad4 signal transduction, and deactivated Cas-3 expression; and (3) Smad4 overexpression abrogated the effects of BBR on cardiomyocyte apoptosis.

Several studies have reported that elevated levels of pro-apoptotic Bax family proteins and reduced levels of anti-apoptotic Bcl-2 family proteins are involved in apoptosis [30]. Therefore, the Bc1-2/Bax ratio is potentially an essential factor for cell apoptotic threshold [31]. Caspase cleavage also plays an important role in apoptosis [32]. Cas-3 serves as a downstream factor for other caspases and directly activates enzymes that stimulate DNA fragmentation [32]. In this study, H/R exposure reduced the Bcl-2/Bax ratio and enhanced Cas-3 cleavage, whereas BBR treatment reversed this effect, indicating that BBR mitigated H/R-triggered cardiomyocyte apoptosis, which contributed to the cytoprotective effect of BBR on myocardial I/R injury, as reported by previous studies [12-16].

The key roles played by mitochondria in BBR-induced cardioprotective effect have been reported previously [33]. BBR can protect cardiomyocytes by ameliorating post-ischemic cardiac function and increasing SIRT1 levels. Similarly, BBR treatment was shown to inhibit myocardial superoxide production and gp9 expression and reduce levels of malondialdehyde and cardiac inflammatory markers but increase levels of myocardial superoxide dismutase [33]. Another study demonstrated that BBR alleviates cardiac I/R injury by in-

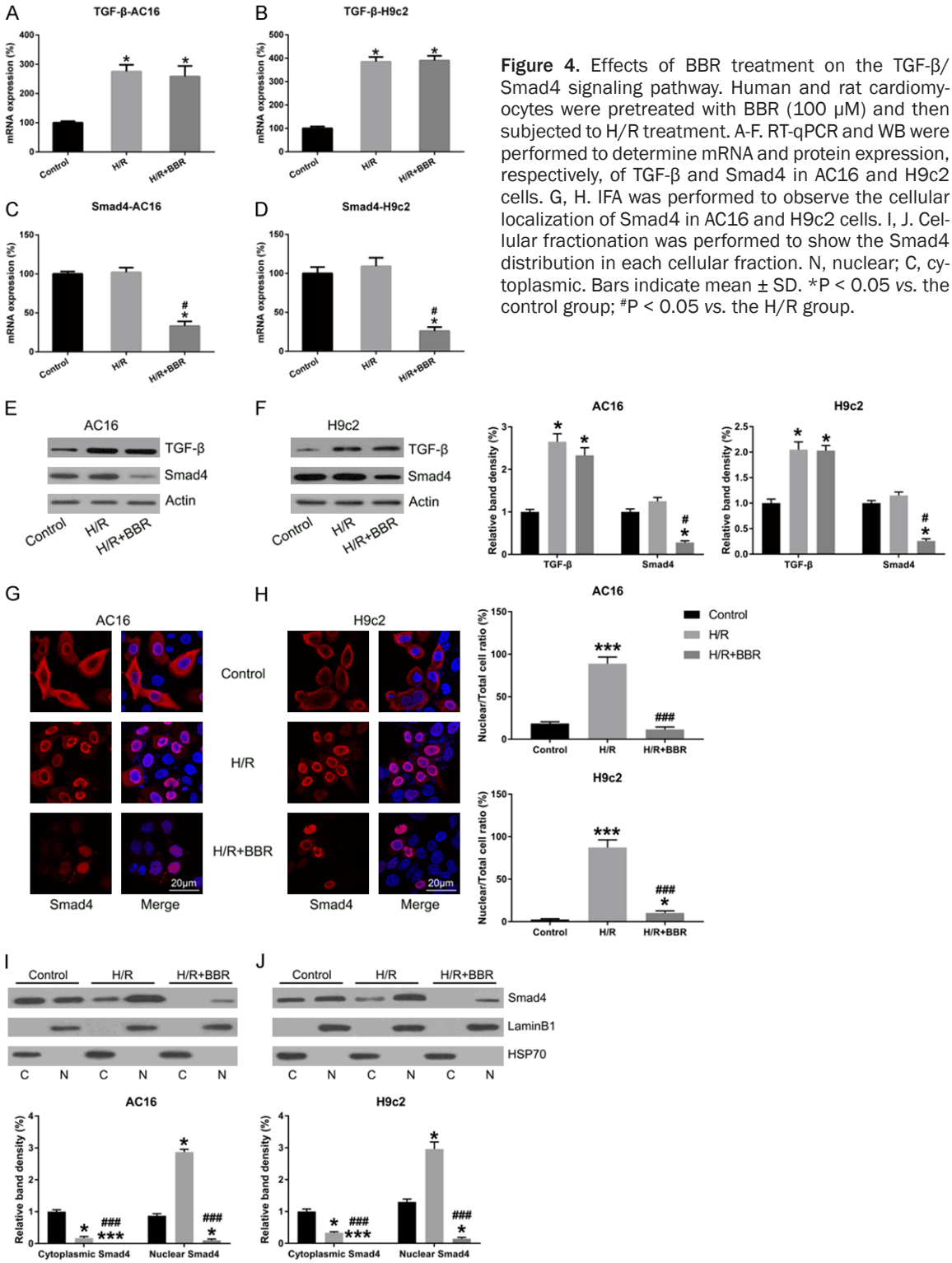
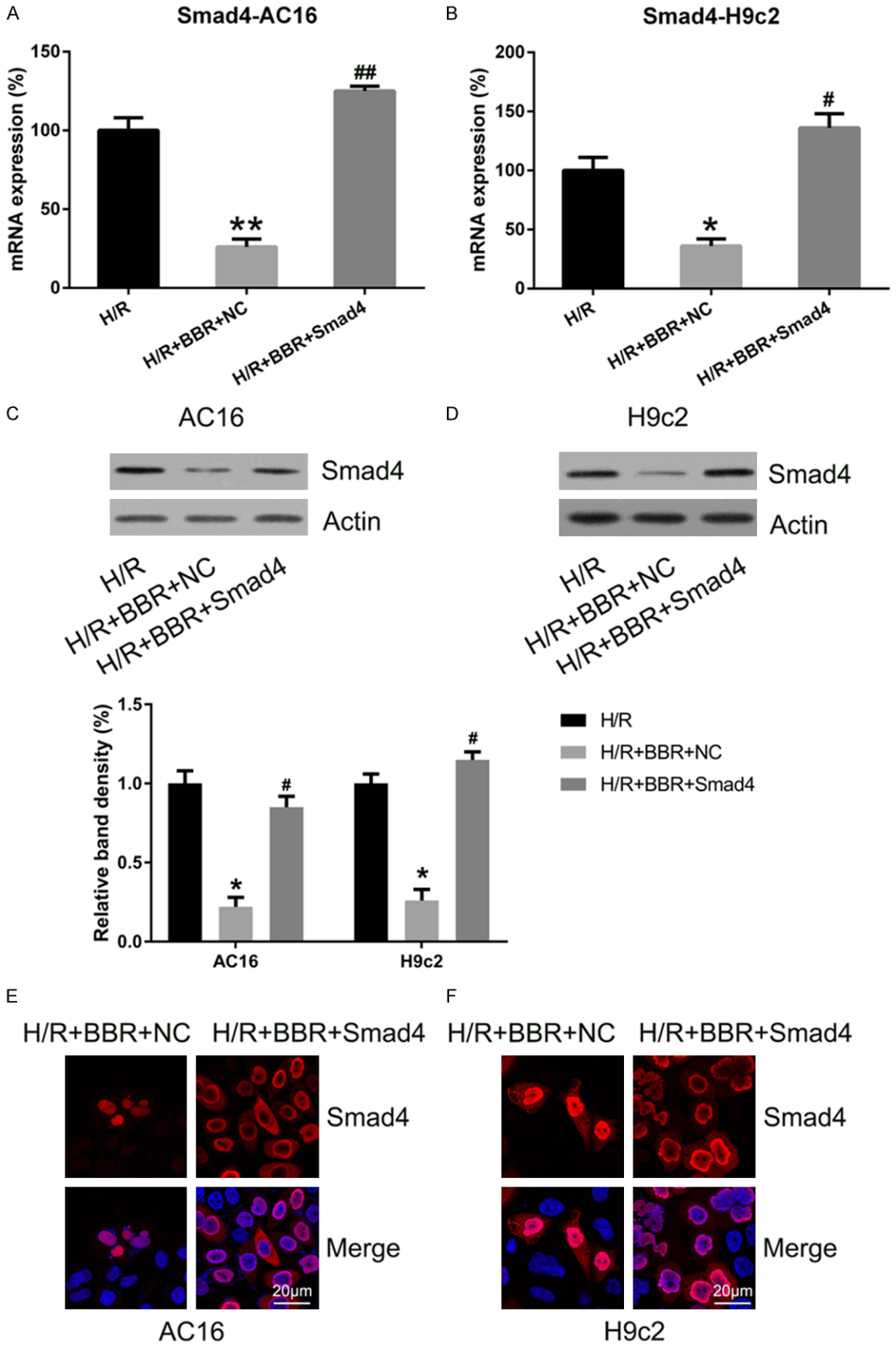


Figure 4. Effects of BBR treatment on the TGF-β/Smad4 signaling pathway. Human and rat cardiomyocytes were pretreated with BBR (100 μM) and then subjected to H/R treatment. A-F. RT-qPCR and WB were performed to determine mRNA and protein expression, respectively, of TGF-β and Smad4 in AC16 and H9c2 cells. G, H. IFA was performed to observe the cellular localization of Smad4 in AC16 and H9c2 cells. I, J. Cellular fractionation was performed to show the Smad4 distribution in each cellular fraction. N, nuclear; C, cytoplasmic. Bars indicate mean ± SD. *P < 0.05 *vs.* the control group; #P < 0.05 *vs.* the H/R group.

creasing the activity of JAK2/STAT3 signaling and alleviating oxidative stress in endoplasmic reticulum [34] and even regulating Notch1/Hes1-PTEN/Akt signaling [35]. Mitochondria are the principal sites for ROS production, and ROS production is closely associated with mitochondrial impairment, further eliciting apoptotic pathways [36]. This study confirmed the sup-


A
Smad4-AC16
mRNA expression (%)
150
100
50
0
**
##
H/R
H/R+BBR+NC
H/R+BBR+Smad4
B
Smad4-H9c2
mRNA expression (%)
200
150
100
50
0
*
#
H/R
H/R+BBR+NC
H/R+BBR+Smad4
C
AC16
Smad4
Actin
H/R
H/R+BBR+NC
H/R+BBR+Smad4
D
H9c2
Smad4
Actin
H/R
H/R+BBR+NC
H/R+BBR+Smad4
Relative band density (%)
1.5
1.0
0.5
0.0
*
#
*
#
AC16
H9c2
H/R
H/R+BBR+NC
H/R+BBR+Smad4
E
H/R+BBR+NC
H/R+BBR+Smad4
Smad4
Merge
20μm
AC16
F
H/R+BBR+NC
H/R+BBR+Smad4
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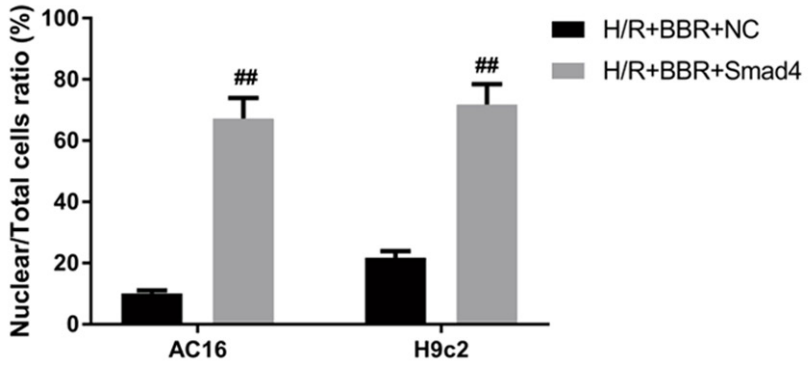

Figure 5. Smad4 overexpression in H/R-triggered BBR-treated cardiomyocytes. Human and rat cardiomyocytes were pretreated with BBR (100 μM) and then subjected to H/R treatment. Human and rat cardiomyocytes were pretreated with BBR and then transfected with pcDNA3-Smad4/NC following H/R exposure. A-D. RT-qPCR and WB were performed to determine the mRNA and protein expression levels, respectively, of TGF-β and Smad4 in AC16 and H9c2 cells. E, F. IFA was performed to observe the cellular localization of Smad4 in AC16 and H9c2 cells. Bars indicate mean ± SD. *P < 0.05, **P < 0.01 *vs.* the control group; #P < 0.05, ##P < 0.01 *vs.* the H/R group.

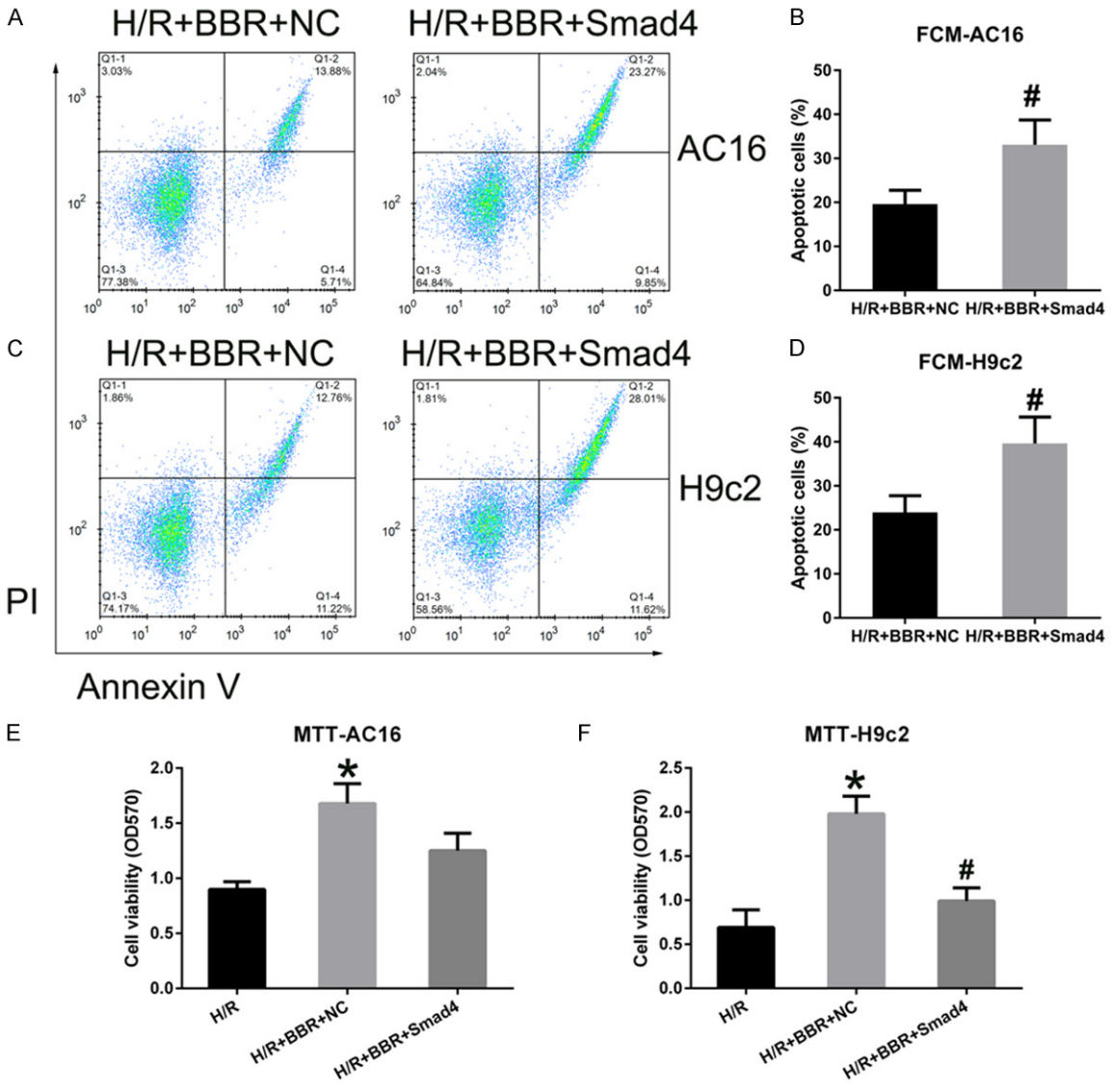

Figure 6. Effects of Smad4 overexpression on cardiomyocyte apoptosis and viability. Human and rat cardiomyocytes were pretreated with BBR (100 μM) and then transfected with pcDNA3-Smad4/NC following H/R exposure. A-D. Flow cytometry was performed to assess the extent of apoptosis in human and rat cardiomyocytes following different treatments. E, F. MTT assay was performed to assess cell survival following different treatments. Bars indicate mean ± SD. *P < 0.05 *vs.* the control group; #P < 0.05 *vs.* the H/R group.

pressive dose-dependent effects of BBR on H/R-triggered cellular and mitochondrial ROS generation [33]. Interestingly, in this study, the suppressive effects of BBR on ROS production may be associated with the TGF-β/Smad4 signaling pathway. Meanwhile, ROS production following BBR treatment rescued cell viability to various extents *in vitro*. Collectively, these data suggest that the decrease in ROS production to a normal level over the course of H/R is involved in the cytoprotective effects of BBR.

Smad4 is an important signal transducer in the TGF-β pathway [37]. Smad4 protein can be activated by TGF-β receptors and then translocated to nucleus to modulate gene transcription [38]. A previous study reported that Smad4 overexpression increased apoptosis of cancer cells, implying that Smad4 may trigger apoptosis [39]. Di et al. evaluated miR-146b/Smad4 axis during cardiac I/R injury; miR-146b was shown to reduce infarct size and apoptosis rates by acting on Smad4 and regulating c-fos and c-JUN expression [21]. Thus, Smad4 may facilitate H/R-injured cardiomyocyte apoptosis. In this study, Smad4 expression in human and rat cardiomyocytes following H/R-triggered apoptosis decreased following BBR treatment. Therefore, the role of Smad4 in BBR-treated H/R-triggered cardiomyocytes was further assessed. Smad4 overexpression restored apoptosis and impaired cell viability of BBR-treated cardiomyocytes. These results suggest that Smad4 is involved in BBR-regulated H/R injury in human and rat cardiomyocytes through modulating ROS production and inducing apoptosis.

This study has some limitations. First, the experiments were limited to *in vitro* settings. To further explore the mechanism of BBR, I/R rat models are required. Second, the effects of BBR on H/R-triggered apoptosis and mitochondrial ROS production were determined; however, the interplay between apoptotic protein expression and mitochondrial stress remains to be studied. Third, we did not assess the mechanism of how Smad4 overexpression interferes with the protective activity of BBR in detail, and this should be investigated in the future.

To conclude, this research confirmed that BBR prevents H/R-triggered OS and apoptosis in human and rat cardiomyocytes. The underlying mechanisms are associated with mitochondrial damage and the TGF-β/Smad4 pathway. Therefore, BBR is a promising candidate to prevent myocardial I/R injury. However, further investigations are warranted prior to its clinical application.

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Disclosure of conflict of interest

None.

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