Dexmedetomidine at a dose of 1 μM attenuates H9c2 cardiomyocyte injury under 3 h of hypoxia exposure and 3 h of reoxygenation through the inhibition of endoplasmic reticulum stress

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Received July 14, 2020; Accepted October 20, 2020

DOI: 10.3892/etm.2020.9564

Abstract. Myocardial ischemia-reperfusion injury (MIRI) has been confirmed to induce endoplasmic reticulum stress (ERS) during downstream cascade reactions after the sufficient deterioration of cardiomyocyte function. However, clinically outcomes have been inconsistent with experimental findings because the mechanism has not been entirely elucidated. Dexmedetomidine (DEX), an α^2 adrenergic receptor agonist with anti-inflammatory and organ-protective activity, has been shown to attenuate IRI in the heart. The present study aimed to determine whether DEX is able to protect injured cardiomyocytes under *in vitro* hypoxia/reoxygenation (H/R) conditions and evaluate the conditions under which ERS is efficiently ameliorated. The cytotoxicity of DEX in H9c2 cells was evaluated 24 h after treatment with several different concentrations of DEX. The most appropriate H/R model parameters were determined by the assessment of cell viability and injury with Cell Counting Kit-8 and lactate dehydrogenase (LDH) release assays after incubation under hypoxic conditions for 3 h and reoxygenation conditions for 3, 6, 12 and 24 h. Additionally, the aforementioned methods were used to assess cardiomyocytes cultured with various concentrations of DEX under H/R conditions. Furthermore, the degree of apoptosis and the mRNA and protein expression levels of glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP) and caspase-12 were evaluated in all groups. The addition of 1, 5 and 10 μM DEX to the cell culture significantly increased the proliferation of H9c2 cells by >80% under normal culture conditions. In the H/R model assessment, following 3 h of anoxia exposure, H9c2 cell viability decreased to 62.67% with 3 h of reoxygenation and to 36% with 6 h of reoxygenation compared with the control. The viability of H9c2 cells subjected to hypoxia for 3 h and reoxygenation for 3 h increased by 61.3% when pretreated with 1 μM DEX, and the LDH concentration in the supernatant was effectively decreased by 13.7%. H/R significantly increased the percentage of apoptotic cells, as detected by flow cytometry, and increased the expression levels of GRP78, CHOP and caspase-12, while treatment with either DEX or 4-phenylbutyric acid (4-PBA) significantly attenuated these effects. Additionally, despite the protective effect of DEX against H/R injury, 4-PBA attenuated the changes induced by DEX and H/R. In conclusion, treatment with 1 μM DEX alleviated cell injury, apoptosis and the increases in GRP78, CHOP and caspase-12 expression levels in H9c2 cells induced by 3 h of hypoxia and 3 h of reoxygenation.

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Key words: dexmedetomidine, hypoxia/reoxygenation, endoplasmic reticulum stress, H9c2 cardiomyocytes

Introduction

Myocardial ischemia-reperfusion injury (MIRI), which usually occurs in clinical settings, leads to severe outcomes for patients if no effective strategies are applied to inhibit the downstream apoptotic cascades. However, numerous animal studies that have revealed various protective mechanisms have also confirmed the efficacy of cardioprotection in overcoming MIRI (1-6). However, the results achieved in the clinical application of these strategies have not been consistent with those achieved in experimental research (7-9). In this context, the mechanism by which the apoptotic pathway and specific key molecules are induced during MIRI, particularly pathways and molecules associated with cell death and ischemia, such as endoplasmic reticulum stress (ERS)-associated apoptosis signaling pathways, may be important. However, these factors are currently unclear.

MIRI induces severe damage to the endoplasmic reticulum. In 2016, Wu *et al* (10) suggested that ERS should be considered in the occurrence of MIRI. IRI has been indicated to be a multifactorial process that can result in multiple organ damage via ERS and the associated occurrence of apoptosis. The underlying mechanism involves excessive oxidative damage, ATP depletion and energy imbalance, calcium homeostasis and other factors. A number of studies have elucidated the effects of ameliorating ERS on the prognosis of MIRI in animal models and *in vitro* cell models (11-13). Furthermore, numerous signaling pathways, such as the miR-34a/sirtuin 1/nuclear factor erythroid 2-related factor 2 (Nrf2) (14), AMP-activated protein kinase/Nrf2 (15), PI3K/AKT (16) and Toll-like receptor 4/myeloid differentiation factor 88/NF-κB pathways (17), have been demonstrated to mediate ERS. Furthermore, in a notable review in 2019, Davidson *et al* (18) opined that multitargeted strategies are necessary to reduce MIRI, because any single approach has a limited capacity to overcome the complex state of MIRI.

The highly selective α^2 adrenergic receptor agonist dexmedetomidine (DEX) is frequently used clinically, especially to provide protection to the heart and other organs during surgery (19-22). Currently, most of the functions of DEX appear to be associated with its anti-inflammatory activity and ability to inhibit IRI. However, the effects of DEX on ERS and the resulting apoptosis have not yet been thoroughly elucidated. In previous studies, DEX exhibited a protective role in the hearts of diabetic mice by interfering with ERS or autophagy, thereby suppressing IRI (6,23); however, the results were partially attributed to the diabetes context. Furthermore, researchers have focused on the study of cells other than cardiomyocytes, such as endothelial cells, under IRI or hypoxia/reoxygenation (H/R) conditions (24,25), and have examined several crucial ERS chaperones, proteins and apoptosis indicators that are produced by organs other than the heart under IRI or H/R conditions (6,26-30). These studies have shown that DEX effectively regulates the function of non-cardiomyocytes and interferes with the ERS signaling pathway under these conditions. In addition, a couple of studies have explored the function of DEX in preventing the injury of H9c2 cardiomyocytes under H/R conditions (31,32). In both studies, DEX was used to precondition the H/R H9c2 cell model, and a significant alleviation of H/R injury was achieved; the study by Wang *et al* (31) indicated that this was achieved through the increased expression of mediator of RNA polymerase II transcription subunit 13, while that by Yuan *et al* (32) demonstrated the involvement of increased FK506 binding protein 1B expression. However, the exact regulatory effect of DEX on ERS and the appropriate experimental conditions for the evaluation of its regulatory effects on H/R remain unknown. Furthermore, if DEX protects against IRI through the inhibition of ERS alone or whether other functions are also involved is unclear.

In the present study, we hypothesized that DEX protects cardiomyocytes against H/R injury by mechanisms in addition to its interference with ERS. The aims of the study were to verify the capacity of DEX to protect injured cardiomyocytes under *in vitro* H/R conditions and to optimize suitable experimental conditions for future research.

Materials and methods

Cell culture. H9c2 embryonic rat cardiomyocytes were obtained from the cell bank of the Central Experimental Laboratory of the Second Hospital of Jiaxing University. The cells were cultured in DMEM (Corning Inc.) containing 4.5 g/l glucose and supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100 g/ml streptomycin and 100 U/ml penicillin (Beijing Solarbio Science & Technology Co., Ltd.). All cells used for experiments were cultured in a 37˚C incubator containing 95% air and 5% CO_2.

Toxicity testing. The original DEX solution was prepared by dissolving DEX hydrochloride powder (SML0956; Sigma-Aldrich; Merck KGaA) in DMSO and then diluting the solution >1,000-fold in DMEM to achieve media with final DEX concentrations of 0, 1, 5 and 10 nM, and 0.1, 1, 5 and 10 μM. The H9c2 cells were incubated in 96-well plates under normal conditions, and the medium was replaced with fresh medium containing DEX 24 h prior to the cell viability assay.

Establishment of the H/R injury model. To establish an optimal *in vitro* H/R model, methods similar to those used by Wang *et al* (31) and Yuan *et al* (32) were used. A single-cell suspension of H9c2 cells (~5x10^5 cells/ml) was prepared and 5x10^3 cells/well were seeded in 96-well plates. The cells were cultured in two plates, namely a control plate and an experimental plate, and the cells in each plate were randomly divided into four groups: The H3/R3, H3/R6, H3/R12 and H3/R24 groups. The control plate was incubated in a cell incubator at 37˚C in 5% CO_2. The experimental plates underwent a 3-h exposure to anoxia after replacement of the medium with serum-free DMEM. The medium in the control plate was not replaced with serum-free DMEM at the same time point. At the end of the anoxic culture step, the cell groups in the experimental plates were cultured under normoxic conditions upon the initiation of reoxygenation. The experimental H9c2 cells in the H3/R3, H3/R6, H3/R12 and H3/R24 groups underwent 3, 6, 12 and 24 h of reoxygenation, respectively, in a cell incubator at 37˚C and 5% CO_2. The parameters of the group in which the most severe damages were observed, according to the results of cell viability and injury assays were considered the optimal conditions for H/R and were used for the H/R groups in subsequent experiments.

To analyse the effect of DEX on the viability and injury of H9c2 cells under H/R conditions, the cells were divided into a control group, H/R group and H/R + DEX group. The H/R + DEX group included 7 subgroups, to which 7 different concentrations of DEX (1, 5 and 10 nM; 0.1, 1, 5 and 10 μM) were added 1 h prior to the initiation of hypoxia. Cells in the H/R and H/R + DEX groups were incubated for 3 h in a hypoxia chamber filled with 5% CO_2 and 95% N_2 and were then subjected to 3 h of reoxygenation under normoxic conditions. Correspondingly, the control group was incubated under normoxic conditions for 6 h.

Experimental protocols. After the above procedures were complete, the optimal concentration of DEX for intervention and experimental conditions for H/R were identified and used in the following experiments. To verify that the selected concentration of DEX (1 μM) was effective for attenuating H/R injury in H9c2 cells and evaluate whether the experiment could be completed under the selected experimental conditions (3 h hypoxia and 3 h regeneration), cells cultured in 96- or 6-well plates were divided into 7 groups, namely the control, DEX, H/R, H/R + DEX, 4-phenylbutyrate (4-PBA; P-21005; Sigma-Aldrich; Merck KGaA), H/R + 4-PBA and H/R + DEX + 4-PBA groups. A total of 1 mM 4-PBA, an effective ERS inhibitor, was used to treat the cells 24 h prior to H/R.

In addition to cell viability and injury assays, flow cytometry assays were conducted to evaluate apoptosis in the groups. The protein and mRNA expression levels of glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP) and caspase-12 were also measured via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, as described below.

Cell viability and injury assays. According to the instructions of the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.), H9c2 cells (~$5x10^3$ cells/well) were seeded in a 96-well plate. For viability testing, the cells in each well were covered with 10 μl CCK-8 solution for 1 h and incubated at 37˚C. Then, the optical density was measured at 450 nm using a microplate reader (Molecular Devices, LLC) and the cell viability (%) was calculated. In addition, cell injury was assessed with a lactate dehydrogenase (LDH) kit (cat. no. C0017; Beyotime Institute of Biotechnology) according to the manufacturer's instructions, based on the amount of LDH released into the supernatant.

Apoptosis assay. Apoptosis of the H9c2 cells in each group was evaluated by flow cytometry with an Annexin V-PE/7-AAD kit (cat. no. CT1030; Beijing Solarbio Science & Technology Co., Ltd.). In brief, after digestion with 0.25% trypsin without EDTA and 3,000 x g centrifugation for 5 min at room temperature, cells were washed twice with PBS, resuspended in binding buffer, and incubated with 5 μl Annexin V-PE and 10 μl 7-AAD for 20 min at room temperature. The apoptotic cells were detected using a flow cytometer (BD Biosciences), and early and late apoptosis were presented in the lower and upper right quadrants of the plots for each group, respectively. The apoptosis rate was calculated with FlowJo X (Tree Star, Inc.).

RT-qPCR. The primers used to amplify GRP78, caspase-12, CHOP and β-actin were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.), and the sequences are presented in Table I. Following the aforementioned treatments, total RNA was extracted from the H9c2 cells with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the purity of the RNA from each group was determined. RNA (500 ng/sample) was reverse transcribed to cDNA using a reverse transcription kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. Subsequently, qPCR was conducted with 2 μl cDNA and other necessary reagents according to the instructions of the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.), and the final amplification reaction (25 μl) was conducted in an ABI Prism 7500 system (Thermo Fisher Scientific, Inc.). The following thermal cycling conditions were used for amplification: Initial denaturation at 95˚C for 3 min followed by 40 amplification cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 20 sec and elongation at 72˚C for 20 sec. mRNA expression levels were calculated as the ratio of the target gene expression level to that of β-actin using the $2^{-\Delta\Delta Cq}$ method (33).

Western blot analysis. Following the aforementioned treatments, H9c2 cells were washed thoroughly with ice-cold PBS solution, and RIPA buffer (Beyotime Institute of Biotechnology) was then added to the wells and incubated for 30 min on ice. Protein was quantified using a BCA assay. A total of 30 μg proteins/lane were separated by 8% SDS-PAGE and transferred to PVDF membranes at 4˚C and 200 mA for 2 h. The membranes were blocked by 5% skimmed milk powder in TBS with Tween-20 (TBS-T) solution for 2 h at room temperature and then incubated at 4˚C overnight with the following primary antibodies: Anti-CHOP (1:1,000; cat. no. DF6025; Affinity Biosciences), anti-GRP78 (1:1,000; cat. no. AF5366; Affinity Biosciences), anti-caspase-12 (1:1,000; cat. no. AF5199; Affinity Biosciences) and mouse anti-β-actin (1:1,000; cat. no. T0022; Affinity Biosciences). A horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. 111-095-003; Jackson ImmunoResearch Laboratories, Inc.) was then added for 2 h at room temperature after the membranes were washed five times with TBS-T buffer. Finally, the membranes were washed with TBST, and signals were visualized with an enhanced chemiluminescence detection kit (Beyotime Institute of Biotechnology). The protein band densities were quantified with ImageQuant TL software (version 7.0; Cytiva).

Statistical analysis. All data are expressed as the mean ± standard deviation (SD). The significance of differences among multiple groups was evaluated by one-way analysis of variance followed by Tukey's post hoc test in SPSS 19.0 software (IBM Corp.). GraphPad Prism (GraphPad Software, Inc.) was used to generate the graphs. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DEX promotes the proliferation of H9c2 cells. DEX caused no obvious cytotoxicity to H9c2 cells; instead, the viability of the DEX-treated cells was increased compared with that of the control cells. Moreover, DEX concentrations of ≥1 μM resulted in significantly increased viability, with increases of 81, 89 and 80% in cells treated with 1, 5 and 10 μM, respectively, compared with the viability of the control cells (Fig. 1).

Optimal experimental level of hypoxia is achieved by 3 h of hypoxia/3 h of reoxygenation. To establish the optimal H/R conditions that met subsequent experimental requirements, several model conditions were selected: 3 h hypoxia/3 h reoxygenation, 3 h hypoxia/6 h reoxygenation, 3 h hypoxia/12 h reoxygenation and 3 h hypoxia/24 h reoxygenation (Fig. 2). As shown in Fig. 2A, the viability of the hypoxic cells exhibited a decreasing trend after 3 h of reoxygenation and remained relatively low until 24 h after reoxygenation. The cell viability in the H3/R3, H3/R6, H3/R12 and H3/R24 groups was decreased significantly to 62.67, 36, 53.33 and 58%, respectively, of that in the control group ($P<0.05$).

In addition, as shown in Fig. 2B, H/R significantly increased the level of LDH in the supernatant. The significant increase in LDH level started at 3 h of reoxygenation, at which time the mean LDH level was 480% of the control ($P<0.01$), and maintained high levels throughout the rest 24 h, as and peaked at 6 h of reoxygenation.

Table I. Primer sequences.

Gene	Forward (5'-3')	Reverse (5'-3')
GRP78	ACTGGAATCCCTCCTGCTC	CAAACTTCTCGGCGTCAT
CHOP	TGCCTTTCGCCTTTGAGAC	GCTTTGGGAGGTGCTTGTG
Caspase-12	GGGATAGCCACTGCTGATA	GCCACTCTTGCCTACCTTC
β-actin	TGAGAGGGAAATCGTGCGTG	TTGCTGATCCACATCTGCTGG

GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein.

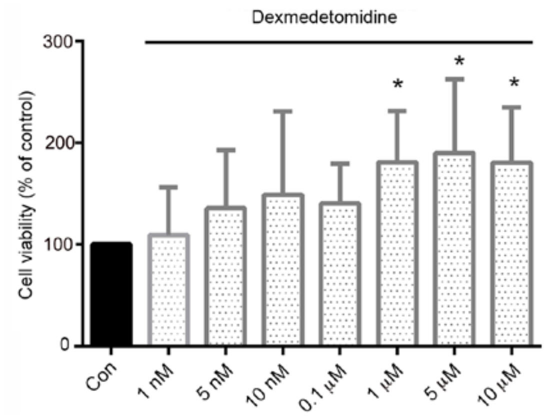

Figure 1. Effects of different concentrations of dexmedetomidine on the viability of H9c2 cardiomyocytes cultured under normal conditions. Results are expressed as the mean ± standard deviation. *P<0.05 vs. Con. Con, control.

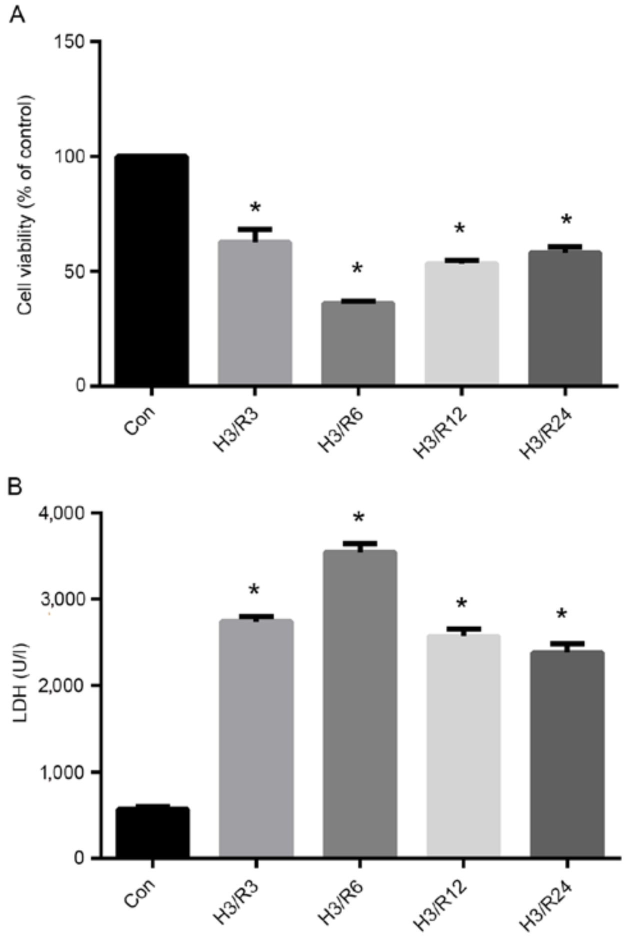

Figure 2. Levels of myocardial cell injury induced by hypoxia/reoxygenation under different conditions. (A) Cell viability percentages and (B) LDH release. Results are expressed as the mean ± standard deviation. *P<0.05 vs. Con. LDH, lactate dehydrogenase; H3, 3 h hypoxia; R3, 3 h reoxygenation; R6, 6 h reoxygenation; R12, 12 h reoxygenation; R24, 24 h reoxygenation; Con, control.

DEX (1 µM) effectively protects H9c2 cells from H/R injury. H9c2 cell viability and LDH release were evaluated following H/R with DEX at the concentrations shown to most effectively promote proliferation: 1, 5 and 10 µM. As shown in Fig. 3, treatment with DEX at all three concentrations increased the viability of H/R-exposed H9c2 cells compared with control group, although no significant differences were found in 5 and 10 µM groups. Furthermore, the effect appeared to gradually reduce from 1 to 10 µM. Concentrations of 1 µM exhibited the greatest effect compared with control treatment. Similarly, compared with the control, pretreatment with 1 µM DEX significantly inhibited the release of LDH from H9c2 cells by 13.7% (P<0.05).

DEX reduces the apoptosis of H9c2 cells. To further investigate the role of DEX in H9c2 cells during H/R, several different treatments were applied (Figs. 4 and 5). Cell viability and LDH release in the control group, the DEX pretreatment group incubated under normal conditions and the 4-PBA pretreatment group incubated under normal conditions exhibited similar and comparable results (P>0.05). However, the H/R group exhibited significantly decreased cell viability and increased LDH release compared with the control group (P<0.05). The 1 µM DEX + H/R group exhibited an attenuation of cellular injury, and 4-PBA pretreatment successfully reversed the protective effect of DEX against H/R injury in terms of cell viability and LDH release (Fig. 4).

In the cell apoptosis assay, the percentage of apoptotic cells increased by 10% in the H/R group (mean, 16%) compared with the control group (mean, 6%) (Fig. 5). However, apoptosis was reduced in the DEX + H/R (mean, 9%) and 4-PBA + H/R (mean, 10%) pretreatment groups, which was significantly attenuated by 7 and 6%, respectively, compared with that in the H/R group (P<0.05). Additionally, 4-PBA reversed the anti-apoptotic effect of DEX and resulted in a significant increase of ~4% in the 4-PBA + DEX + H/R group (mean, 13%) (Fig. 5).

DEX reduces the expression levels of GRP78, CHOP and caspase-12 in H9c2 cells after H/R. To investigate the role

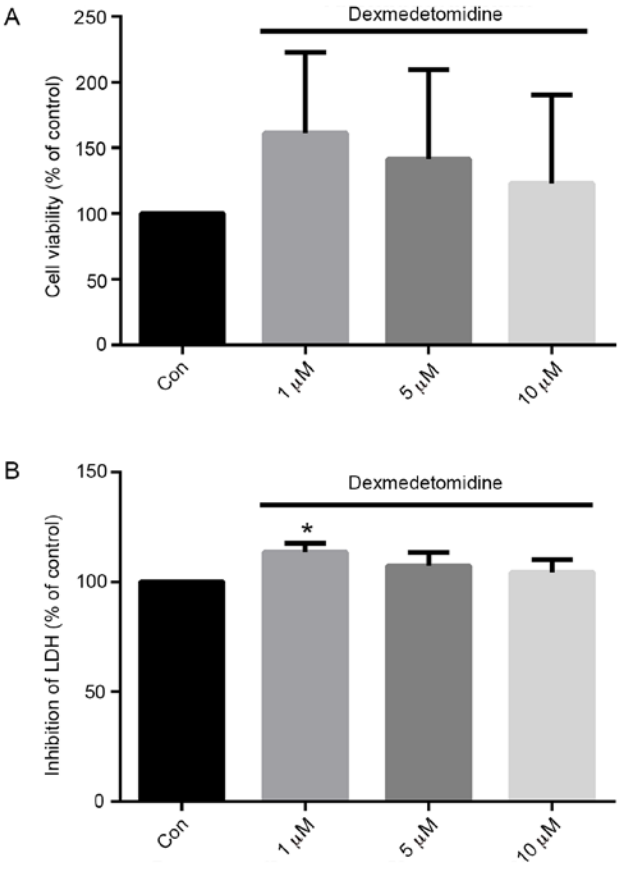

Figure 3. Effects of different concentrations of dexmedetomidine on the levels of myocardial cell injury induced by hypoxia/reoxygenation. (A) Cell viability percentages and (B) inhibition of LDH release. Results are expressed as the mean ± standard deviation. *P<0.05 vs. Con. LDH, lactate dehydrogenase; Con, control.

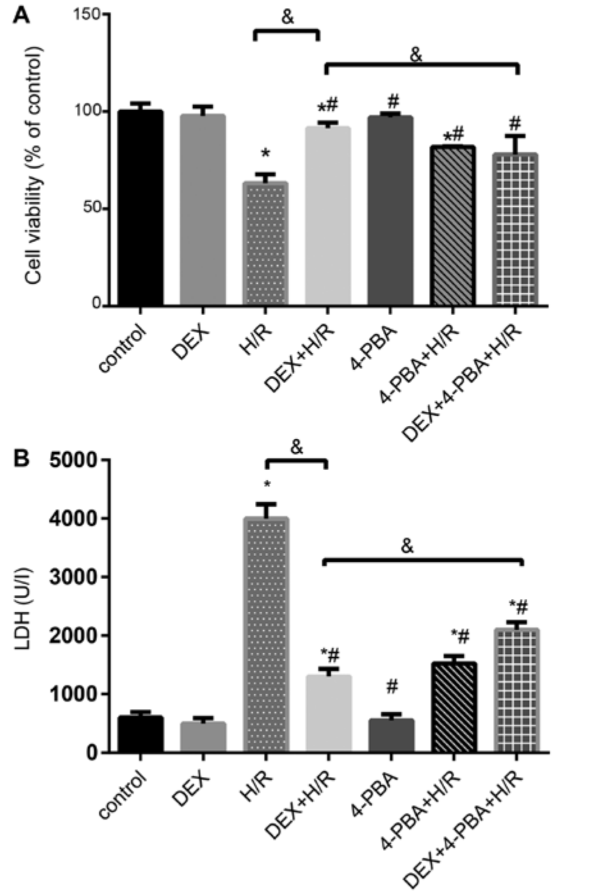

Figure 4. Effects of DEX on the levels of myocardial cell injury induced by H/R under different conditions. (A) Cell viability percentages and (B) LDH release. Results are expressed as the mean ± standard deviation. *P<0.05 vs. Con; #P<0.05 vs. the H/R group; &P<0.05 vs. the DEX + H/R group. Con, control group; 1, normoxic incubation with DEX; 2, H/R incubation; 3, H/R incubation with DEX; 4, normoxic incubation with 4-PBA; 5, H/R incubation with 4-PBA; 6, H/R incubation with DEX and 4-PBA; DEX, dexmedetomidine; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase.

of DEX, three molecules mediating ERS and the associated signaling pathways were examined (Fig. 6). Compared with the control group, the H/R group exhibited the marked and significant upregulation of indicators of ERS and apoptosis. Pretreatment with either DEX or 4-PBA significantly reduced the expression of GRP78, CHOP and caspase-12 in response to H/R (P<0.05). Moreover, these reductions, which are indicative of the alleviation of ERS and apoptosis, observed in the DEX + H/R group were significantly attenuated by 4-PBA, as seen in the DEX + H/R + 4-PBA group.

Discussion

DEX has been demonstrated to exert protective effects on the myocardium under IRI or H/R conditions. The present study aimed to determine whether DEX treatment affects ERS in the complex state of H/R and to identify the optimal experimental conditions for analyzing this. The results confirmed that DEX attenuated H/R-induced myocardial damage through the downregulation of several key molecules associated with ERS and apoptosis, and indicated that its cardioprotective effects might be connected with the regulation of ERS.

As a common clinical phenomenon, MIRI is associated with a variety of processes. To date, no clinical therapy has been effective in ameliorating MIRI, including therapies that were successful in experimental studies, such as cyclosporine A (34). The limitations of animal experiments, with the exception of those in large animals, underlie this discrepancy due to numerous differences between animal and human cardiac physiology. Further clarification of the protective mechanisms of numerous approaches is necessary (35), and this could be accomplished by the establishment of well-designed *in vitro* models with isolated cardiomyocytes that allow the independent control of external factors (36). Thus, it is crucial to use validated *in vitro* models to draw conclusions and clarify the important mechanisms.

Among previous studies, the durations of hypoxia and reoxygenation used vary, which has led to disagreement regarding the experimental strategy. In addition, the composition of the culture medium, including the nutrients, extracellular pH and calcium concentration, at the time of reoxygenation is a major factor that requires consideration (36). A previous study indicated that 30 min is sufficient to establish IRI in animal experiments (37); however, no consistent duration has been established for cardiomyocytes because of their different levels of maturity and oxygen dependency based on the cell source used. For example, Xie *et al* (38) determined that an ischemic period ranging from 2 to 5 h was optimal in an I/R model of primary adult rat ventricular myocytes.

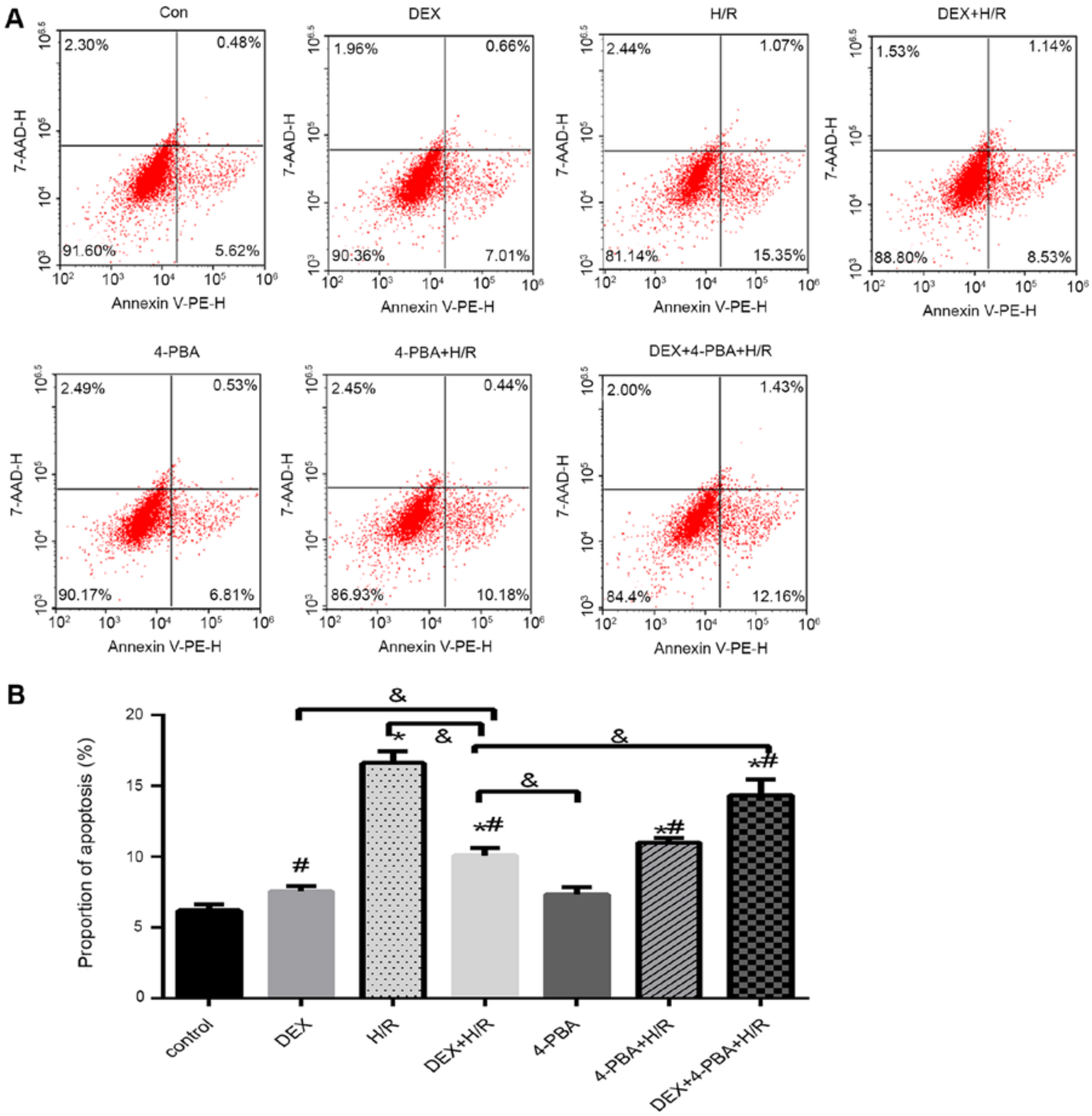

Figure 5. H9c2 cardiomyocyte apoptosis under different conditions. (A) Representative flow cytometry plots and (B) percentages of apoptotic H9c2 cardiomyocytes. Results are expressed as the mean ± standard deviation. *P<0.05 vs. Con; #P<0.05 vs. the H/R group; &P<0.05 vs. the DEX+H/R group. Con, control group; 1, normoxic incubation with DEX; 2, H/R incubation; 3, H/R incubation with DEX; 4, normoxic incubation with 4-PBA; 5, H/R incubation with 4-PBA; 6, H/R incubation with DEX and 4-PBA; DEX, dexmedetomidine; H/R, hypoxia/reoxygenation; 4-PBA, 4-phenylbutyric acid.

The H9c2 cell line, as an immortalized cell line, is currently considered to be the most suitable cardiomyocyte line for IRI and toxicity experiments if no cellular contraction is necessary (39,40). Additionally, the European Society of Cardiology Working Group Cellular Biology of the Heart Position Paper has clarified that the optimal duration for combined ischemic and reperfusion is that which results in 50% cell death (41) but is not too long to affect the possible intervention effect.

Based on all of the above considerations, H9c2 cells were used in the present study to conduct experiments with exposure to hypoxia for 3 h, a duration that has previously been used by other researchers (31,42), and several reoxygenation durations were evaluated. A cell death rate of ~50% was achieved with 3 h of reoxygenation, although cell death peaked at 6 h, with a reduction of 64%.

A number of studies have been conducted to test the cardioprotective function of DEX in the context of pretreatment or postconditioning (1,6,24,32). Several animal studies have identified that DEX exerts a protective effect on the myocardium by reducing ERS after myocardial IRI or by regulating myocardial apoptosis, which proceeds via intrinsic and extrinsic apoptotic pathways (1,6,24,43). To further understand the underlying mechanism of IRI, some cell-level experiments (17,31,32,44) have been carried out with different cardiomyocytes or H/R protocols in which DEX was infused 1-2 h prior to the H/R procedure, as in the present study. With the exception of Wang *et al* (31) and Yuan *et al* (32), who reoxygenated cells for 2-3 h, all other researchers cultured cells in high-glucose medium for >12 h after hypoxia, which is much longer than the duration of reoxygenation used in the present study. From the aforementioned studies of IRI mechanism, it was concluded that DEX affects, for example, calcium overload, small non-coding RNAs and inflammation.

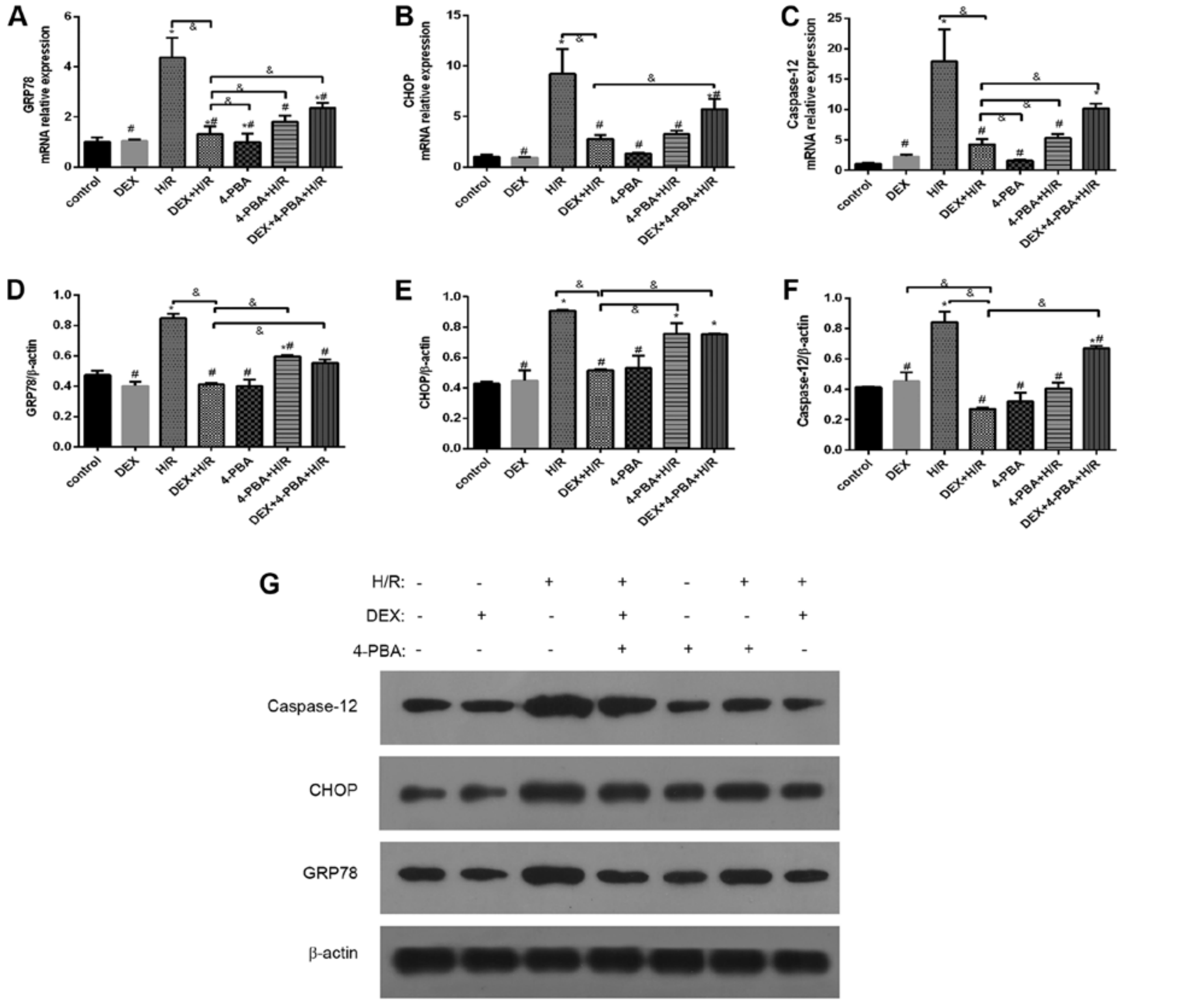

Figure 6. Effects of DEX on the expression of GRP78, CHOP and caspase-12 in myocardial cells treated under different conditions. mRNA expression levels of (A) GRP78, (B) CHOP and (C) caspase-12, and protein expression levels of (D) GRP78, (E) CHOP and (F) caspase-12. (G) Representative western blots. Results are expressed as the mean ± standard deviation. $^{*}P<0.05$ vs. Con; $^{\#}P<0.05$ vs. the H/R group; $^{\&}P<0.05$ vs. the DEX+H/R group. Con, control group; 1, normoxic incubation with DEX; 2, H/R incubation; 3, H/R incubation with dexmedetomidine; 4, normoxic incubation with 4-PBA; 5, H/R incubation with 4-PBA; 6, H/R incubation with DEX and 4-PBA; DEX, dexmedetomidine; GRP78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; H/R, hypoxia/reoxygenation; 4-PBA, 4-phenylbutyric acid.

In the present research, a wide range of concentrations of DEX (1 nM to 10 μM) was adopted for pretreatment to determine the optimal concentration, and the effect of DEX on certain indicators of ERS, namely GRP78, CHOP and caspase-12, was tested. To the best of our knowledge, this study is the first to examine the protective function of DEX over such a broad range of concentrations. According to a study by Peng *et al* (44), concentrations of DEX >30 μM can reduce the viability of cardiomyocytes and induce cytotoxicity. Thus, 10 μM was selected as the highest concentration of DEX to study. The results indicated that DEX was not cytotoxic to H9c2 cells at any concentration ≤10 μM; indeed, a significant increase in cell proliferation was observed at the higher concentrations of 1, 5 and 10 μM. Furthermore, 1 μM DEX attenuated the H/R-induced injury of H9c2 cells. This result is similar to that of other studies conducted by Yuan *et al* (32) and Gao *et al* (17), who used the same dose of DEX but examined the involvement of non-ERS pathways.

MIRI can lead to severe ERS, which is associated with GRP78 upregulation (45,46). If ERS increases, apoptotic cascades are considered an underlying mechanism of MIRI, and the transcription of specific molecules, such as CHOP and caspase-12, is upregulated in an ERS-dependent manner. Most researchers concur that these molecules, as downstream markers of the ERS signaling pathway, are able to represent the ERS status and even the developmental direction of cell survival (47-49).

In the present *in vitro* study, these three molecules were highly expressed under H/R conditions. However, the increases in their expression levels were strongly reduced by DEX, and 4-PBA attenuated this effect of DEX. These findings indicate that DEX attenuates ERS-associated apoptosis and regulates ERS via an unknown mechanism that is inhibited by 4-PBA, as reflected by the decreased expression of CHOP, GRP78 and caspase-12 at the mRNA and protein levels. Consistent results were observed in a study by Liu *et al* (26), in which DEX intervention led to a significant reduction in the expression level of

GRP78, a marker of ERS, and to ER-phagy, while treatment with 4-PBA successfully elevated the expression of GRP78. In a study by Liu *et al* (30), DEX exerted a similar effect in an animal model of cerebral ischemia-reperfusion injury, decreasing the levels of CHOP and GRP78. Additionally, in the present study, DEX exerted stronger protective effects against ERS-related apoptosis than can be accounted for by the inhibition of ERS in the DEX + H/R + 4-PBA group compared with the H/R+4-PBA group, which exhibited lower expression levels of CHOP, GRP78 and caspase-12. It is possible that DEX intervenes in mitochondria-dependent or death receptor-dependent apoptosis in addition to the ERS-associated apoptotic signaling pathway. Considering this possibility, the findings of the present study are compatible with those of Davidson *et al* (18), who found that both higher and lower concentrations of DEX might perform multiple functions to alleviate IRI. Future studies should be conducted to focus on other functions of DEX potentially involved in this process.

Undoubtedly, several limitations of the present study require consideration. First, this study was a pretreatment experiment that did not establish a connection with any signaling pathway, which is a clear direction for future fundamental research. Second, the purpose of any *in vitro* H/R model is to mimic clinical IRI, and condition-dependent models may vary. Furthermore, human cells or stem cells that can more accurately reflect the clinical scenario were not investigated in the present study. Therefore, it is necessary to test the hypothesis of the present study using different types of cardiomyocytes, such as primary cells. Finally, the study investigated only the effects of DEX pretreatment, and the effects of post-event treatments as applied in clinical trials were not investigated. In addition, the precise mechanisms identified in this study merit further investigation.

In conclusion, 1 μM DEX was confirmed at the cellular level to protect H9c2 cells against injury induced by 3 h of hypoxia and 3 h of reoxygenation. Furthermore, these results indicate that the effects of DEX were mediated via intervention with ERS and subsequent apoptosis.

Acknowledgements

Not applicable.

Funding

This research was supported by the Zhejiang Provincial Natural Science Foundation of China (grant no. 2017C33185) and the Project of the Health Commission of Zhejiang Province (grant no. 2019ZD053).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZZ designed the protocol and prepared and revised the manuscript. XL acquired the data and revised the manuscript. HZ performed experiments and analyzed the data. CZ interpreted the data and plotted the graphs. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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