

# Dexmedetomidine protects human renal tubular epithelial HK-2 cells against hypoxia/reoxygenation injury by inactivating endoplasmic reticulum stress pathway

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## Research article

**Keywords:** Dexmedetomidine, Hypoxia/Reperfusion injury, Human renal tubular epithelial HK-2 cells, Endoplasmic reticulum stress

**Posted Date:** December 3rd, 2019

**DOI:** <https://doi.org/10.21203/rs.2.17940/v1>

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# Abstract

**Background:** The study was aimed to explore the effects and potential mechanisms of Dexmedetomidine (Dex) on hypoxia/reoxygenation (H/R) injury in human renal tubular epithelial HK-2 cells.

**Methods:** Human renal tubular epithelial HK-2 cells were divided into four groups: control group, Dex group, H/R group, and Dex + H/R group. After treatment, cell viability rate and cell apoptosis rate were measured by MTT assay and flow cytometry, respectively. Afterwards, the expressions of Hypoxia-inducible factor 1 (HIF-1 $\alpha$ ), glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), caspase-12 and cleaved caspase-3 were determined by western blot. Malondialdehyde (MDA) concentration and Superoxide Dismutase (SOD) activity were determined by assay kits.

**Results:** Compared with control group, the cell viability rate was decreased and cell apoptotic was increased in H/R group. Besides, cell viability rate was increased, and cell apoptotic rate of HK-2 cells was decreased in Dex + H/R group, compared with H/R group. Western blot analysis showed that the expression of HIF-1 $\alpha$  was up-regulated, and the expressions of GRP78, CHOP, caspase-12 and cleaved caspase-3 were down-regulated in Dex + H/R group. In addition, the concentrations of MDA in Dex + H/R group and H/R group were  $1.68 \pm 0.22$  nmol/mgprot and  $0.85 \pm 0.16$  nmol/mgprot, which showed a 49.4% decrease in Dex + H/R group. However, after Dex treatment, the SOD activity was rose to  $121 \pm 11$  U/L, which was more than twice larger than that in H/R group ( $57 \pm 10$  U/L).

**Conclusions:** Dex could inhibit cell apoptosis by up-regulating the expression of HIF-1 $\alpha$ , reducing endoplasmic reticulum stress and regulating oxidative stress, thus ameliorating the H/R injury.

## Background

The ischemia/reperfusion (I/R) induced acute renal injury (ARI) is a common event in trauma, hemorrhage and resuscitation. ARI may occur more frequently during several kinds of surgeries, including aortic surgery, cardiopulmonary bypass surgery and renal transplantation (1). Compared with other parenchymal cells in the kidney, renal tubular epithelial cells are most susceptible to ischemic injury. Besides, apoptosis of tubular epithelial cells may be a main attribute of renal I/R injury. In addition, increasing evidences have indicated that anesthetics used during surgery not only showed an anesthetic action, but also had protective effects through a reduction of apoptosis during I/R (2, 3).

Dexmedetomidine (Dex), a  $\alpha_2$  adrenergic receptor agonist, exhibits a variety of effects, including sedation, anti-anxiety, analgesia, and sympatholytic properties (4, 5). Nowadays, Dex is widely used in the operating room and intensive care units, and recent evidences from animals and clinical studies showed that Dex has reno-protective effects(6–8). However, the effects and potential mechanisms of Dex on I/R injury in ARI remain to be further elucidated.

Hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) plays an important role in maintaining oxygen homeostasis, regulating the expression of a series of hypoxia-related genes, and sensing and transmitting Hypoxia signals (9). A recent study has shown that HIF-1 $\alpha$  may ameliorate brain damage during I/R by reducing cell apoptosis (10). Some treatments or drugs, such as ischemia post-treatment, may be associated with up-regulating the expression of HIF-1 $\alpha$  in the kidney of ischemia reperfusion rats, thus reducing the damage of ischemia and hypoxia to the kidney (11). It was reported that Dex has a protective effect on renal ischemia reperfusion injury (12, 13). However, the relationship between HIF-1 $\alpha$  and renal protective effects of Dex remains unknown.

Endoplasmic reticulum (ER), the largest organelle in eukaryotic cells, can play important roles in maintaining homeostasis. Once cell's demand for the function of the ER exceeds its own capacity, unfolded or misfolded proteins accumulate in the ER, and the pathological state of the steady imbalance of calcium ions is called as endoplasmic reticulum stress (ERS). Studies have shown that moderate ERS is a kind of self-protection mechanism by activating unfolded protein reactions, temporarily inhibiting protein synthesis, and restore endoplasmic reticulum steady state to maintain cell survival (14, 15). However, excessive or prolonged ERS may lead to cell apoptosis or necrosis, thus resulting in tissue and organ injury(16, 17). Besides, ERS can be triggered by various stimuli, such as oxidative stress, ischemia, hypoxia, glucose starvation, and then elevated protein synthesis, which is one of the important ways to induce cell apoptosis(18, 19). Many researches have demonstrated that ERS is involved in the reperfusion injury of vital organs such as heart, brain and kidney(20, 21). However, whether ERS is involved in the kidney protection of Dex remains unclear.

The aim in this study was to explore the effects and potential mechanisms of Dex on renal tubular epithelial cells induced by hypoxia/reoxygenation (H/R), which may facilitate us to have a broader knowledge of Dex treatment in ARI.

## Materials And Methods

### Cell culture

Human renal tubular epithelial HK-2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle medium/Nutrient Mixture F12 Ham (DMEM/F12, 3:1 Mixture) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Grouping and H/R model establishment

Human renal tubular epithelial HK-2 cells were randomly divided into 4 groups using a random number table: control group, Dex group, H/R group, and Dex + H/R group. The HK-2 cells were incubated in an incubator filled with normoxia at 37°C for 28 h in control group. In Dex group, 0.1 nmol/L Dex (Sigma-

Aldrich, Merck KGaA, Darmstadt, Germany) was added to the culture medium and cells were incubated for 2 h. Thereafter, cells were incubated for 28 h in an incubator filled with normoxia at 37°C. In H/R group, cells were firstly incubated in an anaerobic chamber (Shanghai Lishen scientific instrument co. LTD, Shanghai, China) at 37°C for 24 h, and then incubated for 4 h in an incubator filled with normoxia at 37°C. In Dex + H/R group, cells were incubated for 2 h in the culture medium containing different concentrations of Dex (0.01, 0.1, 1, and 10 nmol/L). Afterwards, the cultural method of treated cells was same as the H/R group. Each experiment was repeated five times.

## **MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay**

After treated with the different concentrations of Dex, the HK-2 cells were seeded into 96-well plates at a density of 5000 cells/well, and then MTT assay was used to measure the cell viability rate. Briefly, 100 µL fresh DMEM containing 1 mg/mL MTT (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was utilized to replace 100 µL DMEM containing 10% FBS. After incubation at 37°C for 4 h, 150 µL dimethyl sulfoxide was added to dissolve the formazan crystals. Afterwards, the optical density (OD) value (absorbance) was determined at 568 nm using a microplate reader (Model ST-360, Thermo, Inc, MULTISKAN MK3, CA, USA). The whole experiment was performed in triplicate. The formula for the ratio of cell survival was as following:

$$R (\%) = A/B * 100\%$$

R: the ratio of cell survival; A: OD (experiment)–OD (blank); B: OD (control)–OD (blank).

## **Apoptosis assay**

HK-2 cells of logarithmic growth were diluted into cell suspension, and cell density was adjusted to  $5 \times 10^5$  cells/mL with DMEM medium containing 10% FBS. The cell suspension ( $1 \times 10^6$  cells) was seed in to each holes of culture plate and incubated for 24h. Subsequently, the cells were digested with pancreatic enzyme. After 3 min, the cells were harvested, washed with PBS and re-suspended in binding buffer. Then, the ratio of apoptotic cells was examined using an Annexin V-fluorescein isothiocyanate/PI kit (BD PharMingen, San Diego, CA, USA) according to the manufacturer's protocol.

## **Western blot analysis**

Total protein was isolated using RIPA cracking liquid (Blue Skies Biotechnology Research Institute, Chengdu, Sichuan, China) and Total Cell Protein Extraction kit (EMD Millipore, Billerica, MA, USA). The lysate was centrifuged at 15,000 rpm for 30 min to obtain the supernatant. Afterwards, protein concentrations were determined using BCA Protein Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA,

USA). Subsequently, an equivalent amount of protein (30 µg/lane) from each sample was separated by 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and then transferred to PVDF (polyvinylidene difluoride) membranes (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). After blocking in 5% non-fat milk at room temperature (approximate 26°C) for 2 h, the membranes were incubated with primary antibodies at 4°C overnight as follows: rabbit anti-hypoxia inducible factor-1alpha (HIF-1α) antibody (1:1000; Abcam, Cambridge, UK), rabbit anti-glucose regulation protein 78 (GRP78) antibody (1:1000; Abcam, Cambridge, UK), rabbit anti-human C/EBP homologous protein (CHOP) antibody (1:2000; Abcam, Cambridge, UK) and rabbit anti-activation of the caspase-12 and caspase-3 one reactance antibody (1:1000; Abcam, Cambridge, UK). β-actin antibody (1:200; Abcam, Cambridge, UK) was served as the loading control. Then the incubated membranes were washed with Tris-buffered saline with 0.1% Tween-20 three times and incubated with goat anti-rabbit IgG H&L labelled with horseradish peroxidase (1:1000; Beijing Tetraphora Biotechnology co, LTD, Beijing, China) for 1 h. Finally, the protein bands were visualized with an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology Inc, CA, USA) and quantified using Quantity One software (National Institutes of Health, Bethesda, MD, U.S.).

## Measurement of Malondialdehyde (MDA) concentration and Superoxide Dismutase (SOD) activity

Five petri dishes were taken from each group, and the medium was discarded. Afterwards, the cells were digested by trypsin and re-suspended with PBS. A bilon-150y ultrasonic cell pyrolysis (Shanghai Bilang instrument co., LTD., Shanghai, China) was used to lyse cells. The concentration of MDA and SOD activity in cells were measured using assay kits for MDA (A003-1) and Total SOD (A001-1) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), respectively. The experiments were carried out according to the manufacturer's instructions.

## Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical differences between groups were determined using one-way analysis of variance followed by a Turkey's post hoc test. All statistical analysis was performed using SPSS 13.0, and  $P < 0.05$  was considered to be statistically significant.

## Results

### The effects of Dex on cell viability rate and cell apoptotic rate in HK-2 cells

Cell viability rate and cell apoptotic rate were determined via MTT assay and Apoptosis assay, respectively. The cell viability rate and cell apoptotic rate in Dex group were similar with these in control

group ( $P > 0.05$ , *Table 1, Fig. 1*). Besides, compared with control group, the cell viability rate in H/R group was significantly decreased (64%,  $P < 0.05$ ), and the cell apoptotic rate was significantly increased (19.78%,  $P < 0.05$ , *Table 1, Fig. 1*). However, after Dex treatment, the cell viability rate and cell apoptotic rate were respectively 91% and 11.79% in H/R + Dex (*Table 1, Fig. 1*), which showed that Dex could partly restore the cell growth. The results indicated that Dex could promote cell survival, and inhibit cell apoptosis of HK-2 cells to alleviate H/R injury.

## The effects of Dex on the expressions of HIF-1 $\alpha$ and apoptosis-related proteins

The western blot results of HIF-1 $\alpha$  showed that the expression level of HIF-1 $\alpha$  was greatly up-regulated in H/R group compared with control group ( $P < 0.05$ ), and after Dex treatment, the HIF-1 $\alpha$  expression was also increased compared with H/R group ( $P < 0.05$ , *Fig. 2A, B*). The results of apoptosis-related proteins were shown in *Fig. 2A, C-F*. Compared with control group, the expression levels of GRP78, CHOP, caspase-12, and cleaved caspase-3 were all significantly increased in H/R group ( $P < 0.05$ ). In Dex + H/R group, the levels of them were lower than those in H/R group ( $P < 0.05$ ). In addition, after treated with Dex, the expression of caspase-12 restored to the same level of control group ( $P > 0.05$ ). These results suggested that Dex could alleviate H/R injury by the up-regulating the expression of HIF-1 $\alpha$ , and inhibiting the expressions of GRP78, CHOP, caspase-12, and cleaved caspase-3.

## The effects of Dex on the concentration of MDA and SOD activity

In order to understand the mechanism of Dex relieving H/R damage, two oxidative stress related markers were identified in this study. The concentration of MDA in H/R group was obviously higher than that in control group ( $P < 0.05$ , *Fig. 3A*). The concentrations of MDA in Dex + H/R group and H/R group were severally  $1.68 \pm 0.22$  nmol/mgprot and  $0.85 \pm 0.16$  nmol/mgprot, which showed a 49.4% decrease in Dex + H/R group (*Fig. 3A*). The trend of SOD activity was opposite to the concentration of MDA. Compared with control group, the SOD activity was significantly inhibited in H/R group ( $P < 0.05$ , *Fig. 3B*). In addition, after Dex treatment, the SOD activity was rose to  $121 \pm 11$  U/L, which was more than twice larger than that in H/R group ( $57 \pm 10$  U/L, *Fig. 3B*). Taken together, Dex might regulate oxidative stress by decreasing the concentration of MDA and increasing SOD activity, thus reducing H/R injury.

## Discussion

ARI is a rapid decline in renal function caused by a variety of etiologies, seriously affecting people's health and life (22). Dex, a kind of sedatives, has been reported to have the protective effect on renal injury (23, 24). In the present study, the effects and potential mechanism of Dex on H/R injury in human renal tubular epithelial HK-2 cells were explored. [The results showed that](#) after HK-2 cell hypoxia for 24

h and reoxygenation for 4 h, cell viability rate was significantly decreased, while cell apoptotic rate was increased compared to control group. After Dex treatment, the cell viability rate and apoptotic rate were restored to some extent. Dex was found to alleviate H/R injury by up-regulating HIF-1 $\alpha$  and inhibiting the expressions of GRP78, CHOP, caspase-12, and cleaved caspase-3. In addition, Dex could also improve H/R injury by regulating the oxidative stress response. All results indicated that Dex may have a protective effect on HK-2 cells after H/R injury.

In this research, HIF-1 $\alpha$  protein expression was up-regulated after H/R in HK-2 cells, indicating that HIF-1 $\alpha$  was activated as an endogenous protective factor after H/R. After administering with Dex, the expression of HIF-1 $\alpha$  was further increased, suggesting that the protective role of Dex on improving H/R injury in human renal tubular epithelial cells may be related to the up-regulation of HIF-1 $\alpha$  expression. Several studies have shown that HIF-1 $\alpha$  plays an important role in the regulation of hypoxia-induced apoptosis and promotes cell survival by mediating cell adaptation to hypoxia (25). A study of Zhang et al. has reported that berberine protected renal tubular epithelial cells from hypoxia/high glucose-induced apoptosis by activating HIF-1 $\alpha$  expression (26). Therefore, we speculated that Dex also played a protective role in H/R injury by upregulation of HIF-1 $\alpha$  and promoting cell apoptosis. However, the specific mechanism of up-regulation of HIF-1 $\alpha$  by Dex remains to be further investigated.

In addition, some studies have revealed that ER was a major contributor to cellular apoptosis and damage after hypoxia (27, 28). Our study showed that incubation of HK-2 cells in hypoxia condition increased the expressions of GRP78, CHOP, caspase-12 and cleaved caspase-3. However, after Dex administration, their expressions were down-regulated compared with H/R group. GRP78, the molecular chaperone protein of ER, is one of the classical markers for the stress of ER by feeling the aggregation of misfolded proteins in the mesh cavity to promote proper folding of proteins (29). When the ERS occurs, the intracellular non-foldable protein response pathway is activated (30), thus stimulating the transcription and synthesis of the marker protein CHOP of cell apoptosis (31). CHOP is the specific transcription factor of ERS. Oyadomari et al. reported that the knockout of CHOP gene could enhance the resistance of cells to ERS induced apoptosis, and suggested that CHOP played an important role of promoting apoptosis in the ERS (32). Besides, a study has shown that the expression of CHOP would increase substantially when severe ERS occurred, and eventually inducing cell apoptosis (33). Caspase family is the cell apoptotic participant and the mediator. The research of Zhao et al. showed that LipoxinA4 could protect myocardial ischemia/reperfusion injury via a mechanism related to down-regulation of caspase-12 and inhibition of apoptosis (34). Cleaved caspase-3 is produced after caspase-3 shearing and is regarded as a sign of apoptosis (35). Damarla et al. demonstrated that the cleaved caspase-3 was activated in acute lung injury induced by lipopolysaccharide (36). Combined with our results, ERS was overexpressed during apoptosis of renal tubular epithelial cells, and Dex may protect the renal by inhibiting excessive ERS response, so as to reduce apoptosis of renal tubular epithelial cells caused by H/R.

It has been reported that oxidative stress mediated by reactive oxygen species (ROS) is involved in the pathogenesis of I/R injury (37). Oxygen free radical, one of the important factors that induced renal I/R

injury, can interact with lipid peroxidation of unsaturated fatty acids to produce MDA (38). The concentration of MDA can often reflect the degree of lipid peroxidation in the body and indirectly reflect the severity of the cells from free radicals attack(39). SOD, an antioxidant enzyme that scavenges oxygen free radical, plays an important role in protecting cells from oxidative damage (40). In addition, oxygen free radicals also can participate in cell signal transduction and regulate apoptosis(41). In our study, the MDA concentration in Dex + H/R groups was decreased and the SOD activity was increased, compared with H/R group. The results indicated that Dex could regulate the oxidative stress reaction to alleviate acute renal injury induced by H/R.

## Conclusions

In conclusion, we demonstrated that Dex can protect acute renal injury by inhibiting cell apoptosis, excessive ERS response and regulating oxidative stress reaction. These results provide a theoretical basis for the possibility that Dex may be a potentially effective treatment strategy for patients undergoing kidney surgery.

## Declarations

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Availability of data and materials

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

## Competing interests

The authors declare that they have no competing interests.

## Funding

There is no financial support in this study.

# Authors' contributions

J. and Z. M. Y. designed the manuscript. Z. M. Y. and K. F. did the experiment and drafted the manuscript. H. M. M. and H. X. acquired and analyzed the data. Y. C. W. did the statistical analysis and revised the manuscript. L. J. obtained the funding, and provided administrative, technical, material support and supervision. All authors have read and approved to submit the manuscript.

# Acknowledgements

Not applicable.

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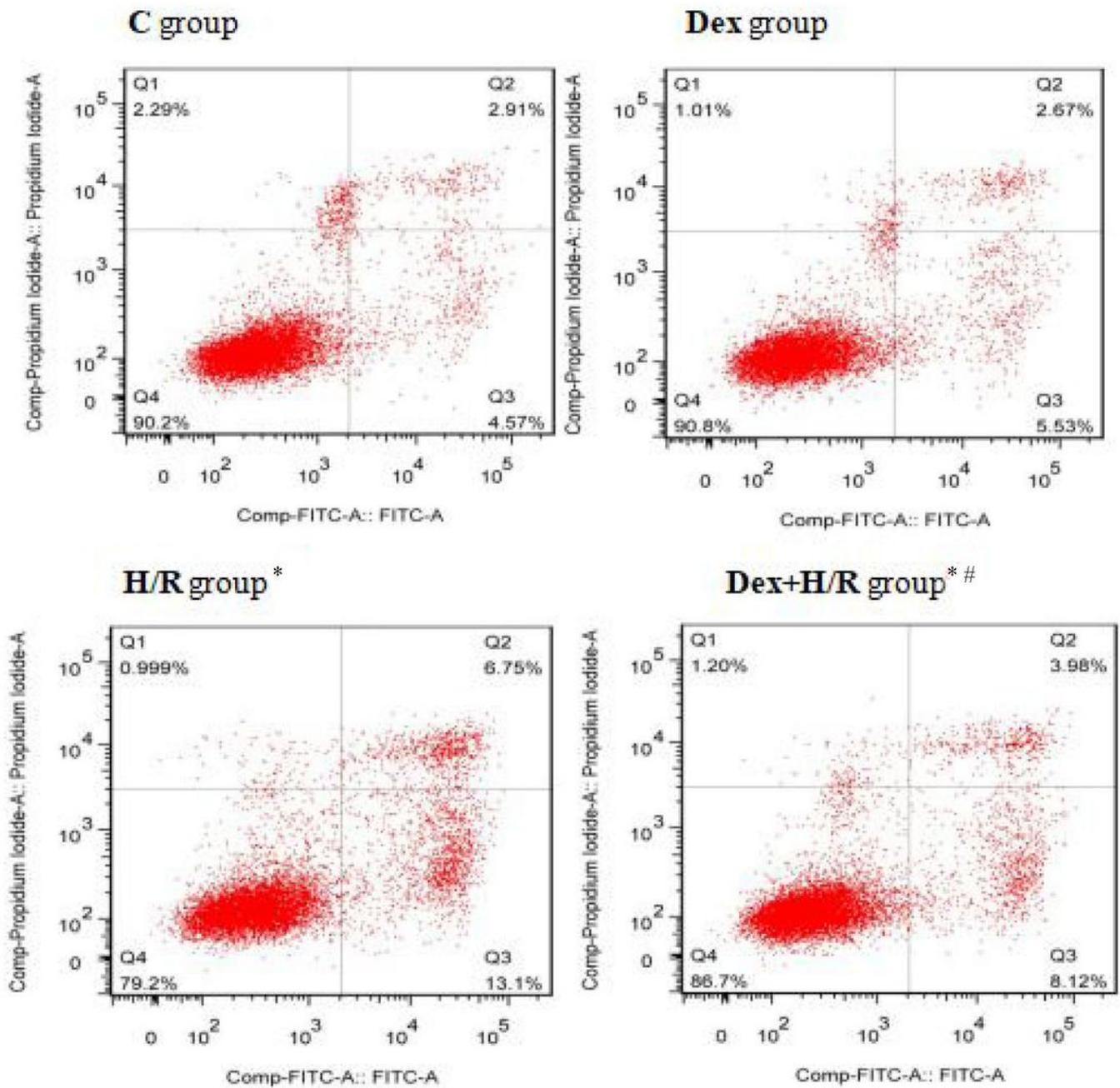
## Table

**Table 1** Cell viability rate and cell apoptotic rate in each group

Group	Cell viability rate	Cell apoptotic rate
Control group	100 ± 1.30%	9.42 ± 1.31%
Dex group	100 ± 5.21%	9.91 ± 1.14%
H/R group	64 ± 4.51% <sup>a</sup>	19.78 ± 1.56% <sup>a</sup>
Dex + H/R group	91 ± 6.13% <sup>b</sup>	11.79 ± 0.58% <sup>b</sup>

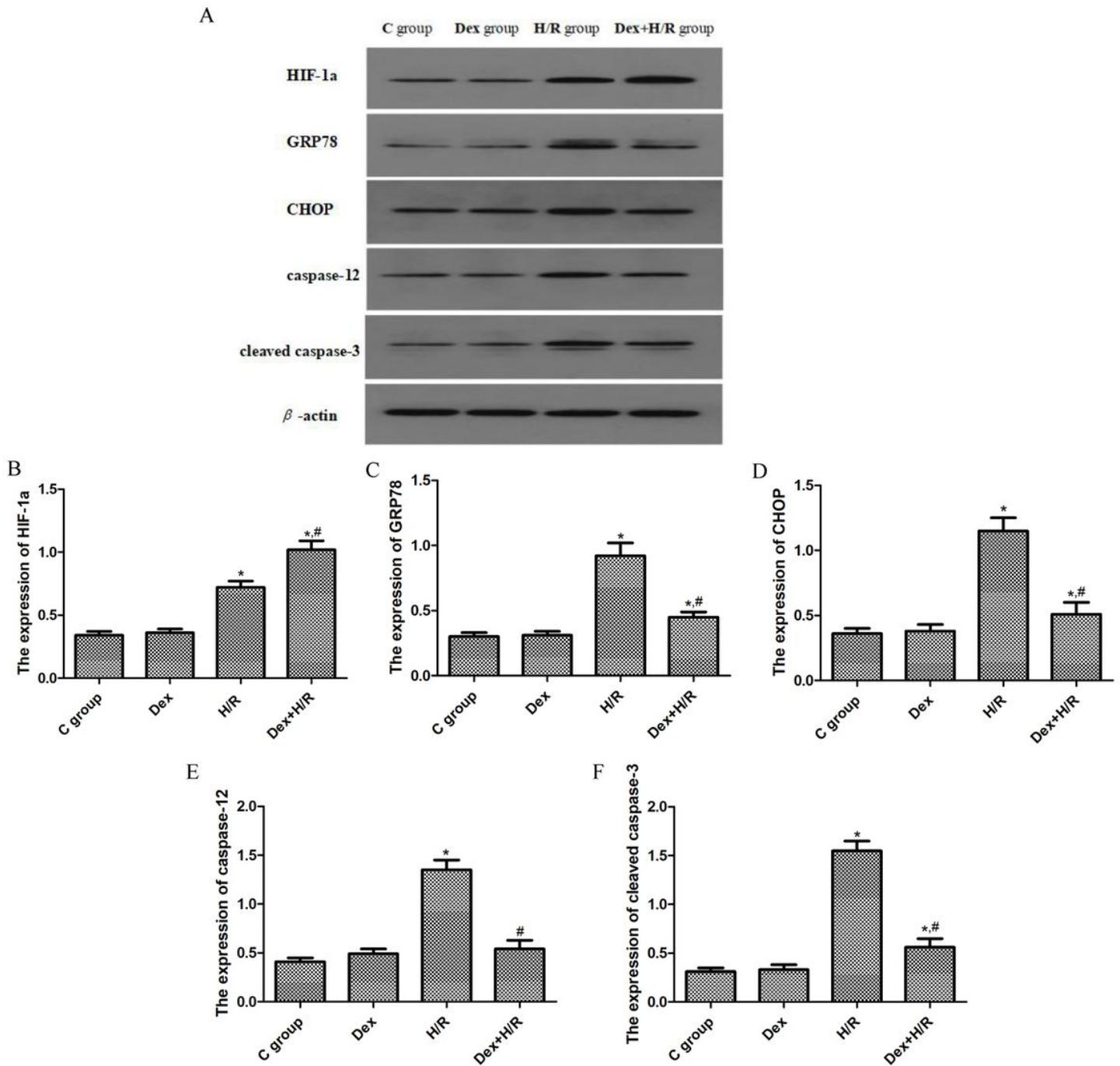
<sup>a</sup>  $P < 0.05$  vs. Control group; <sup>b</sup>  $P < 0.05$  vs. H/R group.

## Figures



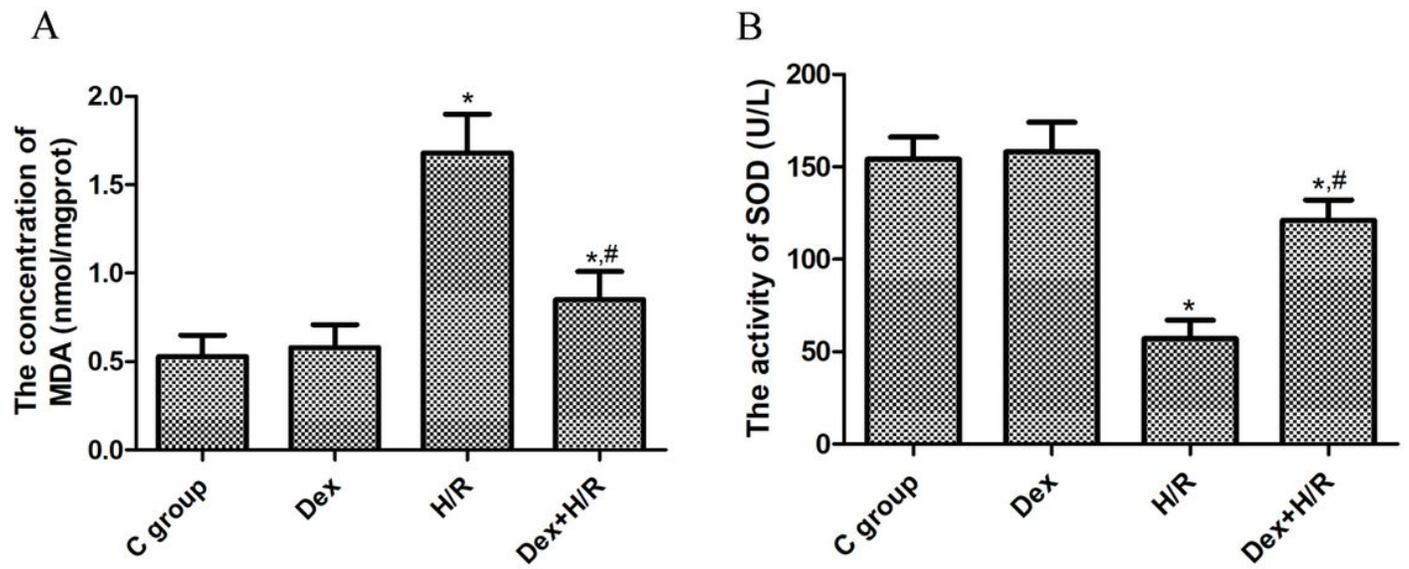
**Figure 1**

Representative flow cytometry images. Compared with control group, the cell apoptotic rate increased in H/R and Dex + H/R groups. Compared with H/R group the cell apoptotic rate was decreased in Dex+ H/R group. \* P < 0.05 vs. control group; # P < 0.05 vs. H/R group. C group: control group.



**Figure 2**

(A) The expression levels of HIF-1 $\alpha$ , GRP78, CHOP, caspase-12 and cleaved caspase-3 were assessed by western blot. (B) The expression level of HIF-1 $\alpha$  by gray analysis of western bolt. (C) The expression level of GRP78 by gray analysis of western bolt. (D) The expression level of CHOP by gray analysis of western bolt. (E) The expression level of caspase-12 by gray analysis of western bolt. (F) The expression level of cleaved caspase-3 by gray analysis of western bolt. \*  $P < 0.05$  vs. control group, #  $P < 0.05$  vs. H/R group. C group: control group.



**Figure 3**

(A) The concentrations of MDA and (B) the activity of SOD. \*  $P < 0.05$  vs. control group; #  $P < 0.05$  vs. H/R group. C group: control group.