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Octreotide protects doxorubicin-induced cardiac toxicity via regulating oxidative stress

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Abstract. – OBJECTIVE: To explore the role of octreotide in doxorubicin-induced (DOX) cardiac toxicity in rats, and to investigate its underlying mechanism.

MATERIALS AND METHODS: A total of 24 male Sprague Dawley (SD) rats were randomly assigned into 3 groups, including: the control group (NS group), the DOX-induced cardiac toxicity group (DOX group) and the OCT pretreatment + DOX-induced cardiac toxicity group (OCT group). Each group had 8 experimental SD rats. Electrocardiogram was performed in each rat before and after animal procedure, respectively. The serum and heart samples of each rat were collected 10 days after the surgical procedure. Cardiomyocyte apoptosis in the myocardial ischemic area of rats was determined by hematoxylin and eosin (HE) staining and Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) staining. DOX-induced oxidative stress was evaluated by detecting the activities of SOD (superoxide dismutase), MDA (malondialdehyde), GSH (glutathione), T-AOC (total antioxidant capacity) and CAT (catalase). The expression levels of nuclear factor E2 related factor-2 (Nrf2), heme oxygenase-1 (HO-1) and quinone oxidoreductase 1 (NQO1) were detected by Western blot and immunohistochemistry.

RESULTS: Compared with the NS group, heart rate and voltage of QRS wave were both significantly reduced in the DOX group, whereas Q-T interval was significantly prolonged ($p < 0.05$). Arrhythmia was even found in some rats of the DOX group. However, rats in the OCT group had significantly higher heart rate and voltage of QRS wave, as well as shorter Q-T interval when compared with those of the DOX group ($p < 0.05$). The levels of plasma CK-MB and LDH were remarkably lower in the OCT group than those of the DOX group. The activities of SOD, GSH, CAT and T-AOC in cardiac homogenate of the OCT group were higher than those of the DOX group. However, MDA activity and ROS level in cardiac homogenate were remarkably reduced in the OCT group when compared with those of the DOX group ($p < 0.05$). Cardiac pathological lesions were alleviated by OCT pretreatment. Moreover, the expression levels of Nrf2, HO-1 and NQO1 were significantly upregulated in the OCT group than those of the DOX group.

CONCLUSIONS: Octreotide improves the anti-oxidant capacity of cardiomyocytes via activating the Nrf2 pathway, thereby protecting doxorubicin-induced cardiac toxicity in rats.

Key Words:
Octreotide, Nrf2 pathway, Doxorubicin, Myocardial toxicity.

Introduction

Doxorubicin (DOX) is the most commonly used broad-spectrum and effective antibiotic in clinics nowadays. However, DOX has a significant adverse effect on heart, which severely limits its clinical application[1-3]. The accumulation of reactive oxygen species (ROS) in cardiomyocytes is the main cause of DOX-induced cardiac toxicity[4,5]. Compared with other myocytes, cardiomyocytes are highly energy-consuming cells that require abundant ATPs to maintain metabolism and biological functions[6,7]. Therefore, cardiomyocytes are very sensitive to oxidative stress damage[8,9]. Meanwhile, cardiomyocyte injury is an important feature of DOX-induced cardiac toxicity[10].

ROS and inflammatory reactions are considered as the leading causes of cardiac toxicity[11,12]. ROS is a kind of by-product of biological oxidation, including hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), peroxide radicals (ROO), hydroxyl radicals (OH^-) and others[13,14]. Once cardiac toxicity occurs, activated phagocytic cells such as neutrophils and macrophages may instantaneously consume large amounts of oxygen. Subsequently, excessive oxygen free radicals are

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produced, and the cells are further damaged[15]. Although the human body can scavenge ROS generated by cells through the anti-oxidation system, ROS is still overwhelmingly accumulated in severe myocardial damage[16, 17]. Previous studies have shown that neutrophil infiltration, abundant oxygen free radicals and inflammatory responses remain the major reasons for myocardial toxin injury[18-20]. Therefore, drug interventions have been developed based on the cellular mechanism of cardiac toxicity[21].

Scholars[22-24] have found that free radical scavengers, antioxidants and anti-inflammatory cytokines can alleviate cardiac toxicity. As a somatostatin (SST) analog, octreotide exerts a protective role in cardiac toxicity[25]. Researches[25-28] have also confirmed that SST regulates the endocrine process *via* binding to the somatostatin receptor (SSTR). Multiple physiological functions can be regulated by octreotide, including inflammatory responses, immune functions, nervous system and retina development[29]. Animal models have already pointed out the protective effect of octreotide on important organs[30-32]. In the DOX-induced acute testicular injury model, octreotide treatment may protect apoptosis and oxidative stress in germ cells, thereby alleviating the survival of sperm[33]. However, the specific role of octreotide in DOX-induced cardiac toxicity has not been fully elucidated.

Recent studies[28-32] mainly focus on the protective effect of octreotide on toxin and ischemia-induced organ injury. In the present work, we constructed a DOX-induced cardiac toxicity model in rats. Our study aimed to provide novel directions for preventing and treating cardiac toxicity *via* octreotide intervention.

Materials and Methods

Chemicals and Reagents

Octreotide and DOX were obtained from Sinopharm Chemical Reagent (Shanghai, China). Relative commercial kits, including T-AOC (total antioxidant capacity), CAT (catalase), GSH (glutathione), SOD (superoxide dismutase), CK-MB (creatine kinase-MB) and LDH (lactate dehydrogenase) determination kit were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Coarse balance, electronic thermometer and 721 type spectrophotometer were obtained from Inesa Analytical Instrument (Shanghai, China).

Animal Procedures

A total of 24 male Sprague Dawley (SD) rats weighing 200 ± 20 g (Model Animal Research Center of Zhengzhou University, Zhengzhou, China) were randomly assigned into 3 groups, including the control group (NS group), the DOX-induced cardiac toxicity group (DOX group) and the OCT pretreatment + DOX-induced cardiac toxicity group (OCT group). Rats in the NS group received intraperitoneal injection of 10 ml/kg normal saline twice a week for 10 times. Rats in the DOX group and the OCT group were intraperitoneally injected with 2.0 mg/kg DOX twice a week for 10 times. Moreover, rats in the OCT group were additionally injected with 20 μg/kg OCT immediately after DOX administration. After two weeks of washout, rats were sacrificed, and venous blood samples and heart tissues were collected respectively. Body weight and daily activity of all rats were observed during the administration period. Animal procedures were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

Electrocardiogram Detection

Rats were first anesthetized by 10% chloral hydrate (3 mL/kg) before and after animal procedures, respectively. Then the rats were placed on the table, and corresponding electrodes were inserted into the limbs. The standard for electrocardiogram was 1 mV= 20 mm with chart speed of 20 m/s. Heart rate (HR), voltage of QRS wave (mV) and Q-T interval (ms) were recorded in each rat.

Cardiomyocyte Function Assessment

After animal procedures, 2 mL blood sample of each rat was collected, followed by centrifugation at 3500 g/min for 30 min. The serum levels of CK-MB (creatine kinase-MB) and LDH (lactate dehydrogenase) were detected by using relative commercial kits.

Histological Examination

Heart tissues collected form rats were fixed with 10% paraformaldehyde and stained with hematoxylin and eosin (HE). Histological changes were assessed by semi quantitative detection of myocardial injury and necrosis. 5 randomly selected fields of each sample were evaluated for the pathological lesions of cardiac toxicity.

Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) Assay

The apoptosis of cardiomyocytes in heart tissues was detected by TUNEL assay according to the instructions of the ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Millipore, Billerica, MA, USA). 5-μm paraffin sections were counterstained with hematoxylin, and the number of TUNEL-positive cells was counted in 10 randomly selected fields (200×).

Biochemical Measurements

Levels of MDA (malondialdehyde), T-AOC (total antioxidant capacity), CAT (catalase), GSH (glutathione), SOD (superoxide dismutase) and ROS (reactive oxygen species) in cardiac homogenate of rats were determined by using relative commercial kits.

Immunohistochemical Staining

After de-paraffin and hydration, the slices of heart tissues were incubated with the primary antibody of Proliferating Cell Nuclear Antigen (PCNA, 1:100, Abcam, Cambridge, MA, USA) at room temperature for 1 h. Subsequently, the slices were washed with PBS (phosphate buffered saline) for thee times, followed by incubation with corresponding secondary antibody at room temperature for 1 h. DAB (diaminobenzidine) (Beyotime, Shanghai, China) was then added for image exposure, and the slices were counterstained with hematoxylin for 2 min. Finally, the slices were hydrated, sealed and observed by using an inverted microscope (Nikon, Tokyo, Japan).

Western Blot

The RIPA (radio-immunoprecipitation assay) protein lysate (Beyotime, Shanghai, China) was used to extract total protein of tissues in each group. The concentrations of protein samples were quantified by the BCA (bicinchoninic acid) method (Pierce, Rockford, IL, USA). Extracted proteins were then separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody at 4°C overnight. After washing with the buffer solution (TBST), the membranes were then incubated with corresponding secondary antibody at room temperature for 1 h. Chemiluminescence was used to expose the protein bands.

Statistical Analysis

SPSS22.0 (Statistical Product and Service Solutions) Software Package (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as $\bar{x}\pm s$. *t*-test was used to analyze the difference between two groups. One-way ANOVA was applied to compare the differences among different groups, followed by post-hoc LSD (Least-Significant Difference). $p < 0.05$ was considered statistically significant.

Results

Octreotide Improved Heart Function in DOX-Induced Myocardial Injury in Rats

No significant differences in heart rate, voltage of QRS wave and Q-T interval were found among the three groups ($p>0.05$). After animal procedures, rats in the DOX group exhibited significantly reduced heart rate, decreased voltage of QRS wave and prolonged Q-T interval when compared with the NS group ($p<0.05$). Arrhythmia was even occurred in some rats of the DOX group. Meanwhile, compared with the DOX group, heart rate and voltage of QRS wave of the OCT group was significantly higher, whereas the Q-T interval was significantly shorter($p<0.05$, Table I).

To evaluate DOX-induced cardiac toxicity, the plasma levels of CK-MB and LDH were detected in rats. We found that the levels of CK-MB and LDH was remarkably decreased in the DOX group when compared with the NS group, indicating the successful construc-

Table I. The effect of OCT on the electrocardiogram of the model rats induced by adriamycin ($\bar{x}\pm s$, n = 8).

Group	Heart rate/min	QRS/mv	Q-T interval/ms
NS	444.00 ± 18.52	0.660 ± 0.103	60.20 ± 6.69
DOX	384.00 ± 41.42*	0.412 ± 0.085*	71.60 ± 4.78*
OCT	433.80 ± 33.75#	0.610 ± 0.089#	55.20 ± 5.72#

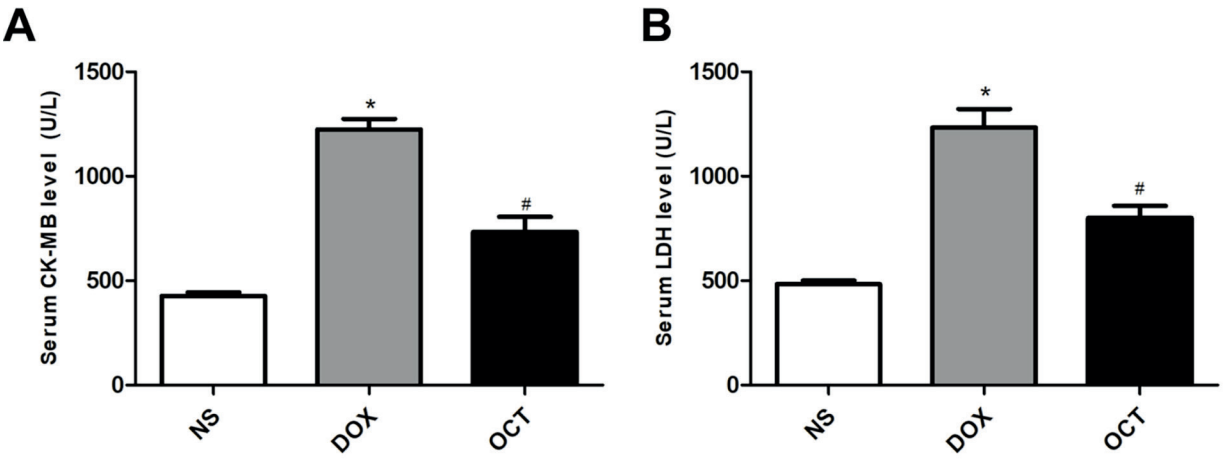

Figure 1. Octreotide conserved myocardial function in DOX-induced myocardial injury. ***A,*** Serum levels of CK-MB in the NS group (n=8), the DOX group (n=8) and the OCT group (n=8); ***B,*** Serum levels of LDH in the NS group, the DOX group and the OCT group. Data were presented as mean ± SD, *Significant difference *vs.* the NS group ($p < 0.05$); #Significant difference *vs.* the DOX group ($p < 0.05$).

tion of the DOX-induced cardiac toxicity rat model (Figure 1A). Moreover, elevated CK-MB and LDH levels were found in the OCT group, suggesting that octreotide had a protective effect on DOX-induced myocardial injury (Figure 1B).

Octreotide Preserved Myocardial Histologic Structure and Inhibited Neutrophil Infiltration

The infarct area of the DOX group was significantly larger than that of the NS group. On the contrary, the OCT group showed smaller infarct area than the DOX group ($p < 0.05$, Figure 2A). HE-staining showed that myocardial cells were well ordered with regular structure in the NS group and only a small amount of focal infiltration of inflammatory cells was found. However, a large amount of inflammatory cells, disordered myocardial cells and pink protein mucus exudation were observed in the DOX group. These results indicated that octreotide pretreatment remarkably alleviated the pathological damage of cardiomyocytes (Figure 2A).

Octreotide Decreased Cardiomyocyte Apoptosis and Enhanced Cell Proliferation After DOX-Induced Cardiomyocyte Injury

TUNEL staining indicated that the number of TUNEL-positive cells in the DOX group was significantly higher than that of the NS group. However, TUNEL-positive cells in the heart tissues of the OCT group were significantly less than the DOX group ($p < 0.05$, Figure 2B). It's known to all that PCNA is an indicator of cell proliferation. Results of TUNEL staining also demonstrated that the number of PCNA-positive cells in the OCT group was much more than the DOX group ($p < 0.05$, Figure 2C).

Octreotide Decreased ROS Production and Tissue Impairment by Enhancing Antioxidant Capacity

To evaluate the oxidative stress induced by DOX administration in rats, the activities of relative oxidation indicators were detected, respectively. Results indicated that compared with the DOX group, the activity of MDA was decreased, whereas the activities of SOD, CAT and T-AOC were significantly increased in the OCT group (Figure 3A-3D). Besides, lower ROS level was found in the myocardium of rats in the OCT group when compared with the DOX group (Figure 3E).

Octreotide Upregulated the Expression of Nrf2 and Downstream Genes

Immunohistochemistry results demonstrated that the ratio of Nrf2-positive cells in the OCT group was significantly higher than that of the DOX group. However, NF-κB staining showed

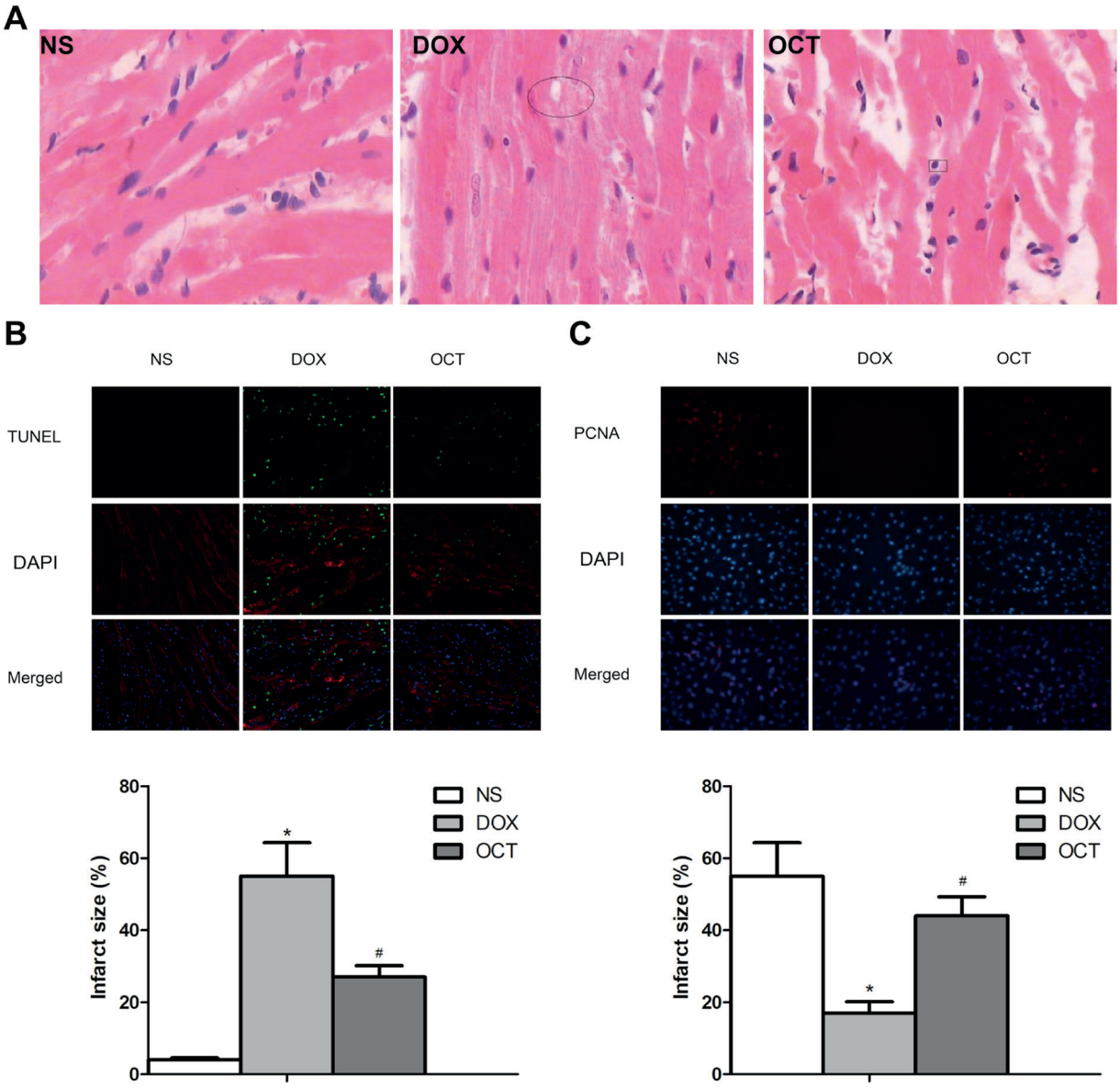

Figure 2. Octreotide prevented DOX-induced myocardial injury in cardiac morphology. ***A,*** Myocardial sections were stained with hematoxylin and eosin, and examined by using a light microscope (200×). HE-staining of myocardial tissues in the NS group, the DOX group, and the DOX group. ***B,*** Representative images (200×) of TUNEL assay in myocardial ischemia reperfusion injury. The number of TUNEL-positive cells was quantified by the average number of 5 HPF in different groups. ***C,*** Representative images (200×) of PCNA immunostaining in myocardial ischemia reperfusion injury. The number of PCNA-positive cells was quantified by the average number of 5 HPF in different groups. Data were expressed as mean ± SD. *Significant difference *vs.* the NS group ($p < 0.05$); #Significant difference *vs.* the DOX group ($p < 0.05$).

the opposite findings (Figure 4A-4C). Subsequently, we extracted the cytoplasm and nucleus of myocardium in each group, respectively. Western blot results indicated that the cytoplasmic levels of Nrf2 and its downstream factors were all upregulated than the nuclear levels, whereas the cytoplasmic level of NF-κB was downregulated (Figure 4D-4H).

Discussion

Acute myocardial infarction (AMI) is a condition of acute myocardial necrosis resulted from persistent and severe myocardial ischemia. AMI is manifested as chest pain, acute circulatory dysfunction, as well as ECG abnormalities reflecting injury, ischemia and necrosis[34]. Studies have

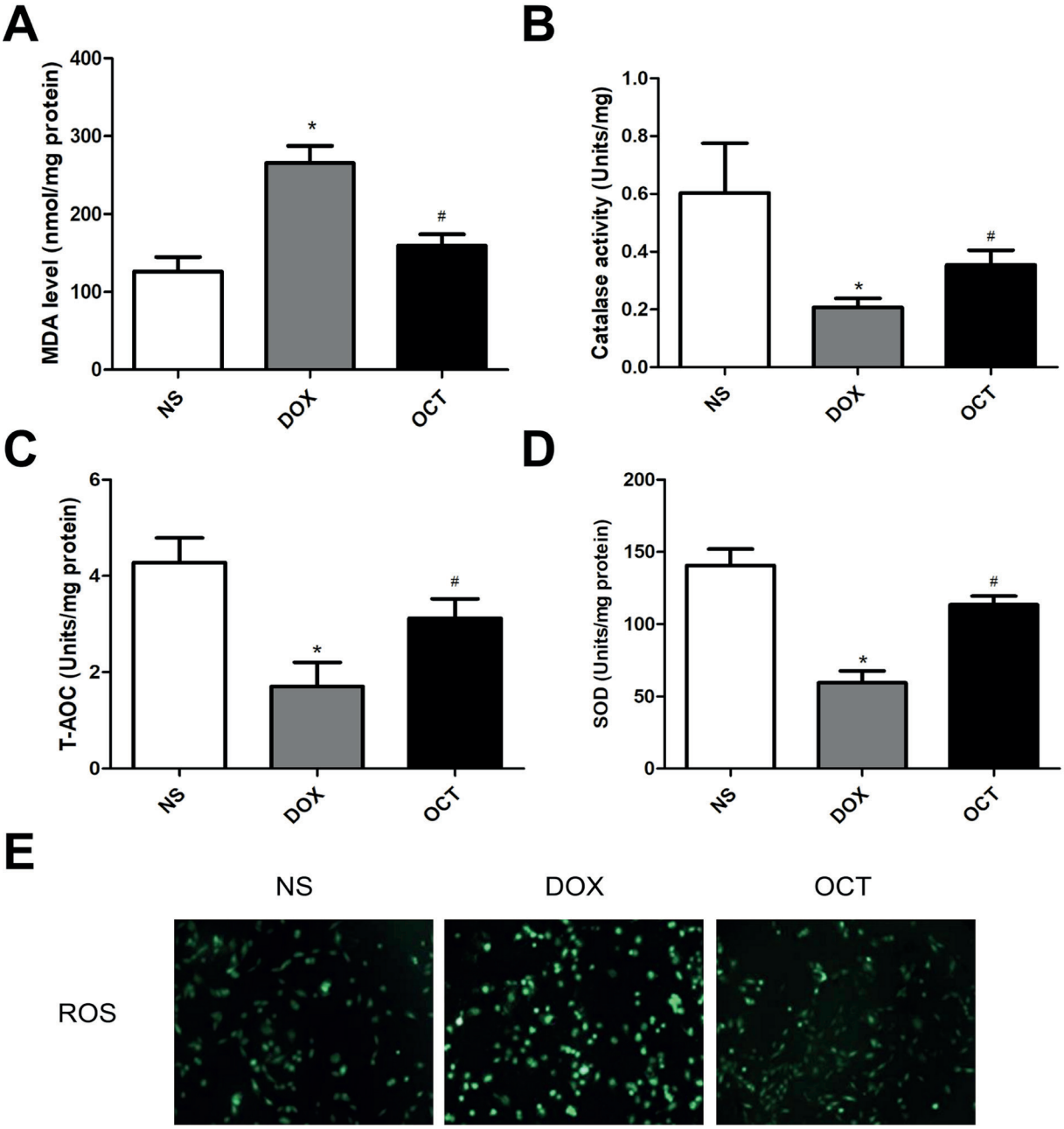

Figure 3. Octreotide attenuated oxidative stress injury by evaluating biochemical parameters. ***A,*** The level of MDA in myocardial tissues. ***B,*** The level of CAT in myocardial tissues. ***C,*** The level of T-AOC in myocardial tissues. ***D,*** The level of SOD in myocardial tissues. ***E,*** The level of ROS was reported as an arbitrary unit per millimeter square field. Data were expressed as mean ± SD. *Significant difference *vs.* the NS group ($p < 0.05$); #Significant difference *vs.* the DOX group ($p < 0.05$).

found that cardiac toxicity is a crucial reason for AMI. A large number of free oxygen radicals are produced after cardiac macrovascular surgery or shock[35]. Meanwhile, studies have shown that Nrf2 is an important nuclear transcription factor in cell defense against oxidative stress. As a effective Nrf2 inducer, octreotide exerts a protective role in preventing oxidative stress and cardiomyocyte apoptosis[28]. Currently, AMI is still a severe disease with high morbidity in clinics. Therefore, it is of great significance to develop novel targets for the treatment of AMI.

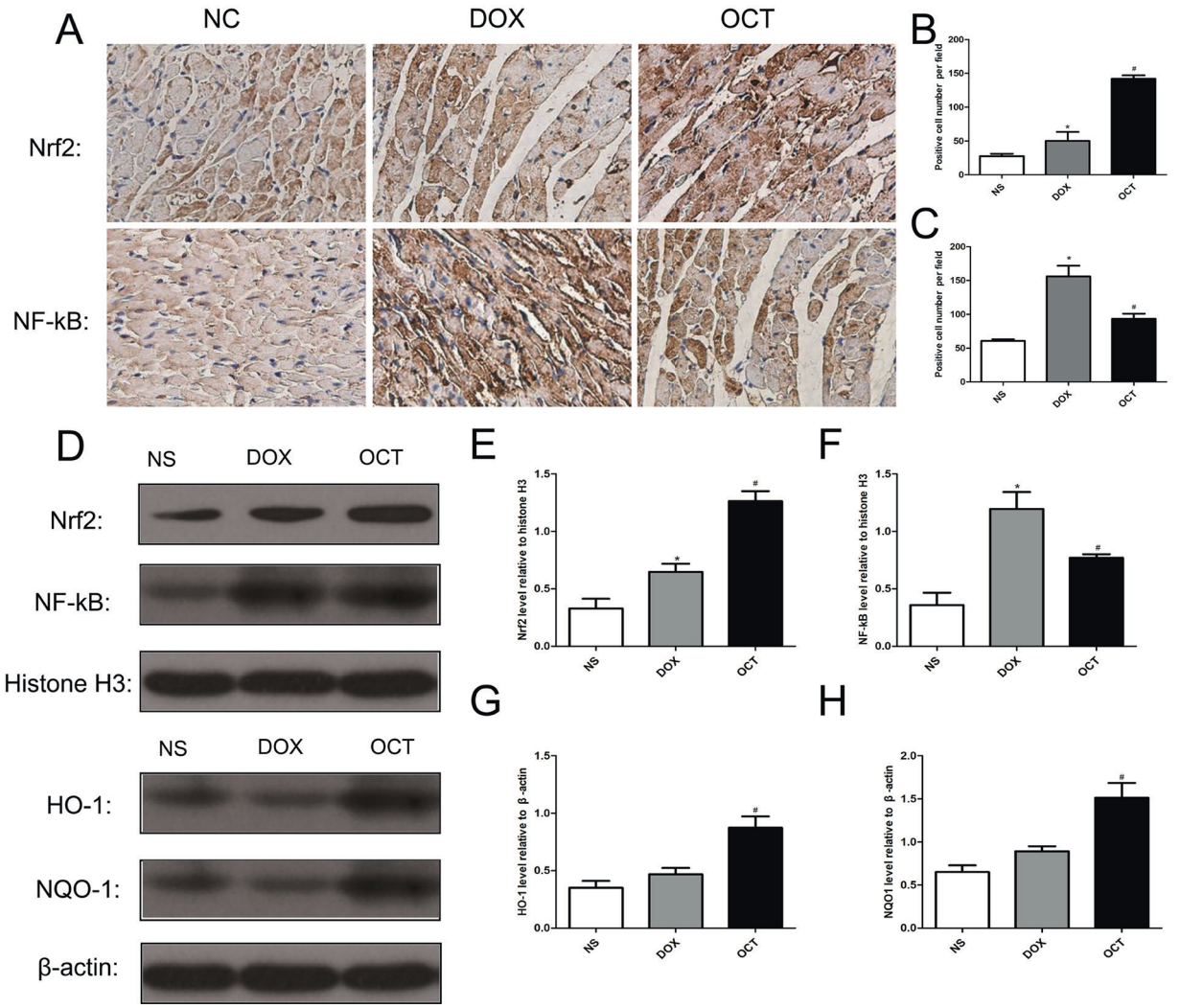

Figure 4. Octreotide supplementation enhanced Nrf2 nuclear translocation and increased the protein expressions of HO-1 and NQO-1. **A,** The expression levels of Nrf2 and NF-κB in the NS group, the DOX group and the OCT group, respectively. ***B,*** Statistical analysis of IHC results in Nrf2 expression. ***C,*** Statistical analysis of IHC results in NF-κB expression. ***D,*** Protein expressions of Nrf2, HO-1, and NQO-1 in different groups. Histone H3 was used as an internal control to normalize the volume of protein expression. ***E-F,*** Protein levels were determined by optical density analysis and normalized to the Histone H3 signal. ***G-H,*** Protein expressions of HO-1 and NQO1 in different groups. β-actin was used as an internal control to normalize the volume of protein expression. Data were expressed as mean ± SD. *Significant difference *vs.* the NS group ($p < 0.05$); #Significant difference *vs.* the DOX group ($p < 0.05$).

Oxidative stress caused by pathological ROS overproduction is closely related to the development of AMI[11,12]. Under normal physiological conditions, ROS is produced by dynamic balance of the mitochondrial respiratory chain[13-15]. However, under pathological conditions, abnormal activation of the xanthine oxidase pathway may result in ROS accumulation, eventually leading to oxidative stress in human body[18]. Oxidative stress is a known as key pathogenic factor for cardiac toxicity[20,21]. Studies have found that lipid is extremely sensitive to oxidative stress. Since the constituent components of the cardiomyocyte membrane are mainly polyunsaturated fatty acids (PUFAs), oxidative stress can severely damage cardiomyocytes. In addition, excess ROS can induce inflammasome formation, autoimmune activation, and platelet activation, which may further impair cardiomyocytes[15-17]. Meanwhile, ROS can induce chronic cardiomyopathy by enhancing fibrosis[19-21]. Hence, anti-oxidation is a preferred option for treating cardiac toxicity.

Nrf2 (nuclear factor E2 related factor 2) is a central regulatory factor in the antioxidant system[36], which is highly expressed in liver, myocardium, skin, lung and digest tract[36, 37]. Meanwhile, Nrf2 is the most active transcriptional regulator in the CNC (cap "n" collar) transcription factor family and contains six different conserved functional regions (Nehl-Neh6). The Neh1 region contains a C-terminal leucine zipper bZIP that binds to small Maf protein, allowing Nrf2 to recognize and bind to the antioxidant response element (ARE). The Neh2 region contains a high-affinity ETGE motif and a low-affinity DLG motif, both of which can bind to Keap1. Neh3 is necessary for the activation of Nrf2 transcription, while Neh4 and Neh5 contribute to the negative regulation of Nrf2 in a non-redox-dependent manner[38]. Meanwhile, Nrf2 normally maintains low expression in cells because it is rapidly ubiquitinated by Kelch-like ECH-related protein 1 (Keap1) and subsequently degraded by proteasome[39]. Accumulated ROS may lead to the conformational changes of Keap1, thus dissociating Nrf2 and Keap1. Subsequently, Nrf2 is translocated into nucleus to bind to ARE, thereby activating the Nrf2/ARE pathway[40]. A series of phase II detoxification enzymes and antioxidant enzyme genes are activated, including catalase (CAT), superoxide dismutase (SOD), quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1)[41].

The specific pharmacological effect of octreotide has not been fully elucidated[25]. It has been found that SST can inhibit the release of growth hormone (GH), thyroid stimulating hormone (TSH), insulin, serotonin, and calcitonin via decreasing Ca^{2+} influx[26, 27]. However, the role of octreotide in cardiac toxicity has not been reported. Our study showed that DOX might induce significant pathological damage and oxidative stress in myocardium, which could also be alleviated by octreotide pretreatment. In the present study, higher expression of Nrf2 and lower expression of NF-κB were found in the OCT group, suggesting that octreotide could activate Nrf2 and inhibit the NF-κB pathway in DOX-induced cardiac toxicity.

Conclusions

We showed that octreotide improves the anti-oxidation capacity of cardiomyocytes via activating the Nrf2 pathway, thereby protecting doxorubicin-induced cardiac toxicity in rats.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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