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Melatonin protects against chromium(VI)-induced cardiac injury via activating the AMPK/Nrf2 pathway

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ABSTRACT

Chromium (Cr) threatens health by causing oxidative stress. However, effective therapy for cardiac damage mediated by potassium dichromate ($K_2Cr_2O_7$) still has not been defined. Melatonin (MT) possesses a number of biological activities. Our study was performed to explore the effect and mechanism of MT on Cr(VI)-induced cardiac damage by conducting both in vitro and in vivo studies. Twenty eight male Wistar rats were randomly assigned to four groups: control, MT (20 mg/kg subcutaneously), $K_2Cr_2O_7$ (4 mg/kg intraperitoneally), and $K_2Cr_2O_7$ + MT. We measured biomarkers of oxidative stress and cardiac function, and performed histopathological analysis, assay of terminal deoxynucleotidyl transferase-mediated deoxyuracil nucleoside triphosphate nick end labeling and protein levels, and the viability assay of cultured cardiomyocytes in vitro. Our results showed that MT ameliorated $K_2Cr_2O_7$-induced oxidative stress, apoptosis, and the release of inflammatory mediators in the rat heart. MT also promoted adenosine monophosphate-activated protein kinase (AMPK) phosphorylation, upregulated expression of proteins that nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1, and nicotinamide adenine dinucleotide phosphatase: quinone-acceptor 1, and inhibited nuclear factor kappa B in the heart of rats exposed to $K_2Cr_2O_7$. Furthermore, MT increased B-cell lymphoma gene 2 (Bcl-2) and B-cell lymphoma extra large protein levels and decreased cleaved caspase 3, P53, and Bcl-2-associated X protein levels. Furthermore, the experiment in vitro showed that MT increased the cells viability and protein levels of Nrf2 and phosphorylated-AMPK in H9C2 cells treated with $K_2Cr_2O_7$. Collectively, our results demonstrate that MT protects against Cr-induced cardiac damage via activating the AMPK/Nrf2 pathway.

1. Introduction

Chromium (Cr) naturally occurs in soil, and is ubiquitously present in the environment as a result of anthropogenic processes [1].

According to the investigation, the valency states of Cr are widely from −4 to +6 [2]. Among these valency states, from 0 to +6 are the oxidation states of Cr [3]. Cr with different valences has advantageous and disadvantageous properties, respectively. Cr(III), as a hard acid, combines with oxygen and donor ligands forming relatively strong complexes [4]. As an essential trace element, Cr(III) was reported to play a positive role in controlling lipid metabolism and glucose in mammals [5]. Platinum Cr bare-metal stents are used in treating coronary heart disease [6]. Even so, the potential side-effects of Cr(III) such as genotoxic deoxyribonucleic acid (DNA) lesions can not be neglected [7]. Cr(VI) may interact with nucleic acid and protein based on its stable, dominant, and highly soluble character [8]. Cr(VI) has been demonstrated to induce oxidative stress, altered gene expression and apoptotic cell death [9]. Cr(II) is unstable in the environment, and easily oxidizing to Cr(III) [10]. A large number of studies have explored the risk of occupational exposure to Cr compounds such as DNA damage [11,12].

Cr, in its various oxidation states, is often used to manufacture a large variety of chemicals [13]. However, Cr pollution poses a serious threat to animals and humans. Cr(VI) is one of the most common environmental contaminants due to the high number of industrial applications [14]. The process of transforming Cr(VI) into Cr(III) involves the generation of reactive oxygen species (ROS) [15]. Although cells produce ROS during normal metabolism process, the overproduction of ROS induced by Cr(VI) results in the imbalance of antioxidant defenses and causes cell damage [16].

It is well-known that the heart is the organ that is susceptible to heavy metal pollution [17]. Advances in cardiovascular research have

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found that oxidative stress is a significant pathophysiological pathway in developing and progressing heart damage by inducing vascular endothelial dysfunction, cardiac contraction, apoptosis, necrosis, and remodeling of extracellular matrix, ultimately resulting in severe heart dysfunction [17–22]. Althrough there are limited report on cardiac injurty induced by Cr(VI), it is important to explore the relationship between Cr poisoning and cardiac injury, and find a safe and effective drug to treat it.

Melatonin (MT), a methoxyindole synthesized and secreted mainly by the pineal gland at night, has been confirmed to modulate endocrine, neural, and immune functions in reducing inflammation and apoptosis, and to act as an antioxidant and anti-senescent agent [23–26]. Previous research has shown that MT suppresses mitochondrial oxidative stress through adenosine monophosphate-activated protein kinase (AMPK) in glycochenodeoxycholic acid-induced hepatotoxicity [27]. MT also modulates neuroinflammation by inhibiting nuclear factor kappa B (NF-κB), and alleviates oxidative stress via increasing the level of nuclear factor erythroid 2-related factor 2 (Nrf2) [28]. Recent evidence has suggested that the antioxidative and anti-inflammatory properties of MT can be used to prevent ischemia-reperfusion (IR) injury and protect against cardiomyocyte death. Moreover, several reports have explored the mechanism of action of MT-induced beneficial effects in myocardial IR injury [25]. However, the treatment potential of MT for potassium dichromate ($K_2Cr_2O_7$)-induced cardiac injury and potential mechanisms remains unclear.

Accordingly, our study designed to define the regulatory effect and mechanism of MT on $K_2Cr_2O_7$ induced cardiac injury.

2. Materials and methods

2.1. Animals and experimental protocol

Twenty-eight healthy Wistar rats (average body weight of 170 ± 10 g, male, 6–8 weeks old) were provided from Experimental Animal Centre of Harbin Medical University (Harbin, China). The rats were bred with standard pelleted rodent diet and water ad libitum under the standard laboratory conditions (22 ± 2 °C, 12-h light/dark cycle, 50 ± 3% relative humidity).

After acclimation for a week, the rats were randomly organized into four groups: control, $K_2Cr_2O_7$, $K_2Cr_2O_7$ + MT, and MT, 7 rats per group. The control and MT groups were injected intraperitoneally with physiological saline (0.9%, *w/v*). The $K_2Cr_2O_7$ and $K_2Cr_2O_7$ + MT rats were injected with $K_2Cr_2O_7$ (4 mg/kg) solution intraperitoneally. An hour later, the control and $K_2Cr_2O_7$ groups were subcutaneously injected with physiological saline (0.9%, *w/v*). The MT and $K_2Cr_2O_7$ + MT groups were subcutaneously injected with MT (20 mg/kg). All treatments lasted for 35 d. The animal experimental procedure was authorized by the Ethical Committee for Animal Experiments (Northeast Agricultural University, Harbin, China). MT (purity > 99.7%) was from Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China). $K_2Cr_2O_7$ was from Tianjin Tianli Chemical Reagent Co., Ltd. (Tianjin, China).

2.2. Sample collection

All rats were weighted and anesthetized 24 h after the last treatment. The blood samples were promptly collected and centrifuged at 4000 × *g* for 15 min. The excised cardiac tissue sample was separated to 3 pieces for different experiments. One piece was homogenated in buffer solusion (pH 7.4) for 10 min with a homogenizer; one piece was placed into 10% formalin solution for histopathology analysis; the remainder was freezed at −80 °C.

2.3. Biochemical analysis

Creatine kinase (CK), creatine kinase MB fraction (CK-MB), lactic dehydrogenase (LDH), and alpha-hydroxybutyrate dehydrogenase (HBDH) activities in serum were tested using a Uni Cel DxC Synchron chemistry system (Beckman Coulter Inc., Fulton, CA, USA).

2.4. Measurement of bio-markers

After a centrifugation at 3000 r/min for 15 min at 4 °C, superoxide dismutase (SOD) activity, content of malondialdehyde (MDA) and glutathione (GSH) in the heart were detected with the corresponding commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) respectively.

2.5. Histopathological analysis

After fixed in 4% formaldehyde for 24 h, the cardiac tissues from rats were fixed into blocks of 1–2 mm thickness, dehydrated and embedded in paraffin. Sections (3 μm) were cut, and stained using hematoxylin and eosin. Finally, a light microscope (BX-FM, Olympus Corp, Tokyo, Japan) was used to evaluate the sections.

2.6. Terminal deoxynucleotidyl transferase-mediated deoxyuracil nucleoside triphosphate nick-end labeling test

Cardiac cell apoptosis was evaluated with a commercial terminal deoxynucleotidyl transferase-mediated deoxyuracil nucleoside triphosphate nick-end labeling (TUNEL) test kit according to the manufacure's direction. Samples were evaluated under a fluorescence microscopy, and the cell apoptosis percentages of each sample were recorded.

2.7. Cell culture and viability test

H9C2 cell (from rat myocardial cell) line was purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The viability of H9C2 cell was assayed with a Cell Counting Kit-8 assay (Beyotime, Shanghai, China). After the cells were incubated for 24 h in 96-well plates, MT (1×10^{-3} μmol/L) was added to MT and $K_2Cr_2O_7$ + MT groups respectively. After 30 min, cells were treated with/without $K_2Cr_2O_7$ (5.5 μg/mL), incubated for 24 h. The viability of H9C2 cells was tested according to the instruction provided by the manufacturer. The optical density (OD) value was read at 450 nm with a Bio-Tek Epoch microplate reader (Bio-Tek, Winooski, VT, USA).

2.8. Western blot analysis

The total proteins from the cardiac tissues were extracted with a commercial kit from Beyotime Institute of Biotechnology (Shanghai, China). Nuclear proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) following the manufacturer's instruction. BCA protein assay kit (Beyotime, Shanghai, China) was used to test total protein content. Equal volume of sample (6 μL, 5 mg/mL) were added on polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking nonspecific binding sites with 5% nonfat milk in tris buffered saline tween (TBST) for 2 h, membranes were incubated for 12 h with specific primary antibodies at 4 °C. The primary antibodies P53, Nrf2, heme oxygenase-1 (HO-1), nicotinamide adenine dinucleotide phosphatase: quinone-acceptor 1 (NQO1), B-cell lymphoma gene 2 (Bcl-2), Bcl-2-associated X protein (Bax), B-cell lymphoma-extra large (Bcl-xl), and cleaved caspase 3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Interleukin - 1β (IL-1β) antibody was from Abcam commercial test (Cambridge, MA, USA). Antibodies to AMPK and nuclear factor-κB (NF-κB) were from Cell Signaling Technology Inc. (MA, USA). Nuclear protein Lamin B (Santa Cruz, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Xianzhi, Hangzhou, China) were selected as a standard controls [29].

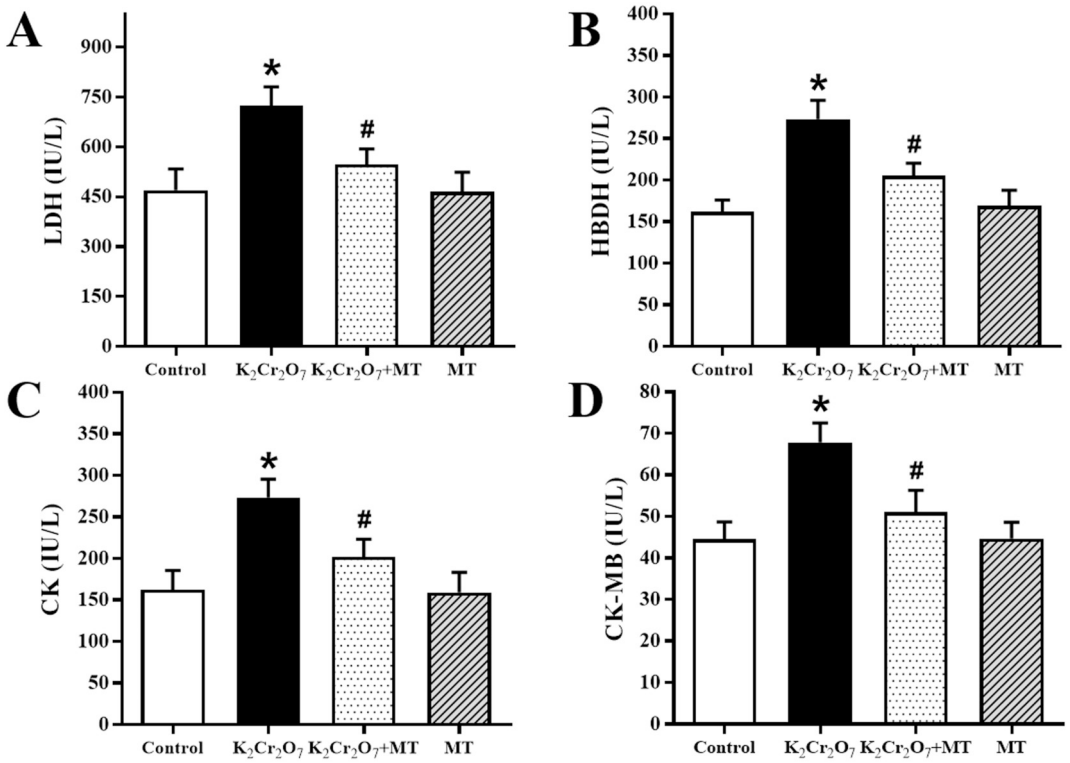

Fig. 1. Effect of MT on the $K_2Cr_2O_7$-mediated cardiac injury. (A) LDH, (B) HBDB, (C) CK, (D) CK-MB. Data are presented as mean ± SEM, $n = 7$. * Statistically different ($p < 0.05$) VS. control group. # Statistically different ($p < 0.05$) VS. $K_2Cr_2O_7$ group.

Then membranes were washed and incubated with the corresponding conjugated secondary antibodies, and then washed 5 times with TBST. Finally, the densitometry assay was shown using Image Pro-Plus 6.0 software (General Electric Company, Fairfield, CT, USA). Furthermore, total and nuclear proteins from H9C2 cells were extracted by Nonidet P 40 cell lysis buffer supplement with the protease inhibitor phenylmethanesulfonyl fluoride (Beyotime, Shanghai, China). According to the above operations, AMPK, phosphorylated –AMPK (P-AMPK), and Nrf2 protein levels in H9C2 cells were tested.

2.9. Statistical analysis

Data were performed as means ± standard error of means (SEM) using SPSS22.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to finish comparisons between experimental groups. A statistical value of $p < 0.05$ was considered significant.

3. Results

3.1. MT alleciates $K_2Cr_2O_7$–induced cardiac dysfunction

$K_2Cr_2O_7$ significantly increased the activities of LDH, CK-MB, HBDH, and CK compared with the control group ($p < 0.05$, Fig. 1). However, MT significantly inhibited the activities of these enzymes in the $K_2Cr_2O_7$ + MT group ($p < 0.05$, Fig. 1).

3.2. MT alleviates $K_2Cr_2O_7$-induced histopathological changes

No clear pathological change was observed in the control group and MT-only group (Fig. 2A and D). While obvious changes including myocardial damage, irregular cellular structure, individual cell swelling, significantly hemorrhage, and infiltration of inflammatory cells were obviously noticed in the $K_2Cr_2O_7$ group (Fig. 2B). However, these damage were relieved in the $K_2Cr_2O_7$ + MT group (Fig. 2C).

3.3. MT attenuates oxidative stress in the cardiac tissue

Compared with the control group, the SOD activity and GSH concentration were significantly decreased, and the level of MDA was increased markedly in the $K_2Cr_2O_7$ group ($p < 0.05$, Fig. 3A, B and C). Meanwhile, MT significantly attenuated these change ($p < 0.05$, Fig. 3A, B and C).

3.4. MT decreases cardiomyocyte apoptosis

MT treatment significantly decreased the level of apoptosis compared with the $K_2Cr_2O_7$ group ($p < 0.05$, Fig. 4A and B).

To further prove the anti-apoptotic actions of MT, we detected a few representative apoptosis-related protein levels. The result showed that $K_2Cr_2O_7$ markedly decreased Bcl-2 and Bcl-xl protein levels, and increased the expression levels of P53, cleaved caspase-3, and Bax ($p < 0.05$, Fig. 5A and B). However, MT treatment significantly reversed these changes ($p < 0.05$, Fig. 5A and B).

3.5. MT activates the AMPK/Nrf2 pathway in MT treatment rats

Expression of P-AMPK/AMPK and Nrf2 were decreased in the $K_2Cr_2O_7$ group compared with the control group, but were increased in the $K_2Cr_2O_7$ + MT group ($p < 0.05$, Fig. 6). As downstream proteins of Nrf2, HO-1 and NQO1 had a similar expression pattern in the three groups ($p < 0.05$).

3.6. MT relieves $K_2Cr_2O_7$-induced cardiac inflammation

Compared with the control group, MT significantly decreased the protein levels of NF-κB and IL-1β ($p < 0.05$, Fig. 7A and B). However, the altered protein levels were attenuated in the $K_2Cr_2O_7$ + MT group (Fig. 7A and B).

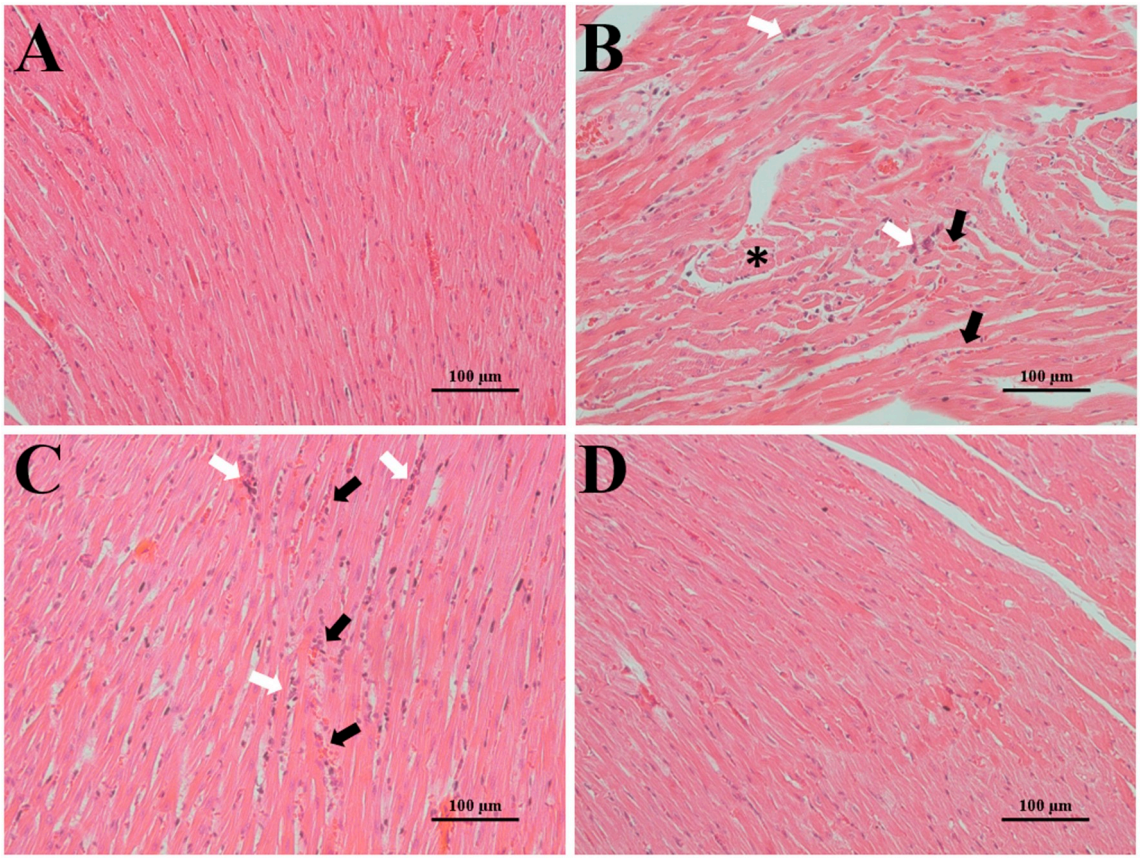

Fig. 2. Effect of MT on $K_2Cr_2O_7$-induced histopathological damage. (A) control, (B) $K_2Cr_2O_7$, (C) $K_2Cr_2O_7$ + MT, (D) MT. Paraffin sections of cardiac tissues were stained with hematoxylin and eosin and examined (200×, bar: 100 μm). White arrow: inflammatory cell infiltration; Black arrow: hemorrhage; *: individual cell swelling.

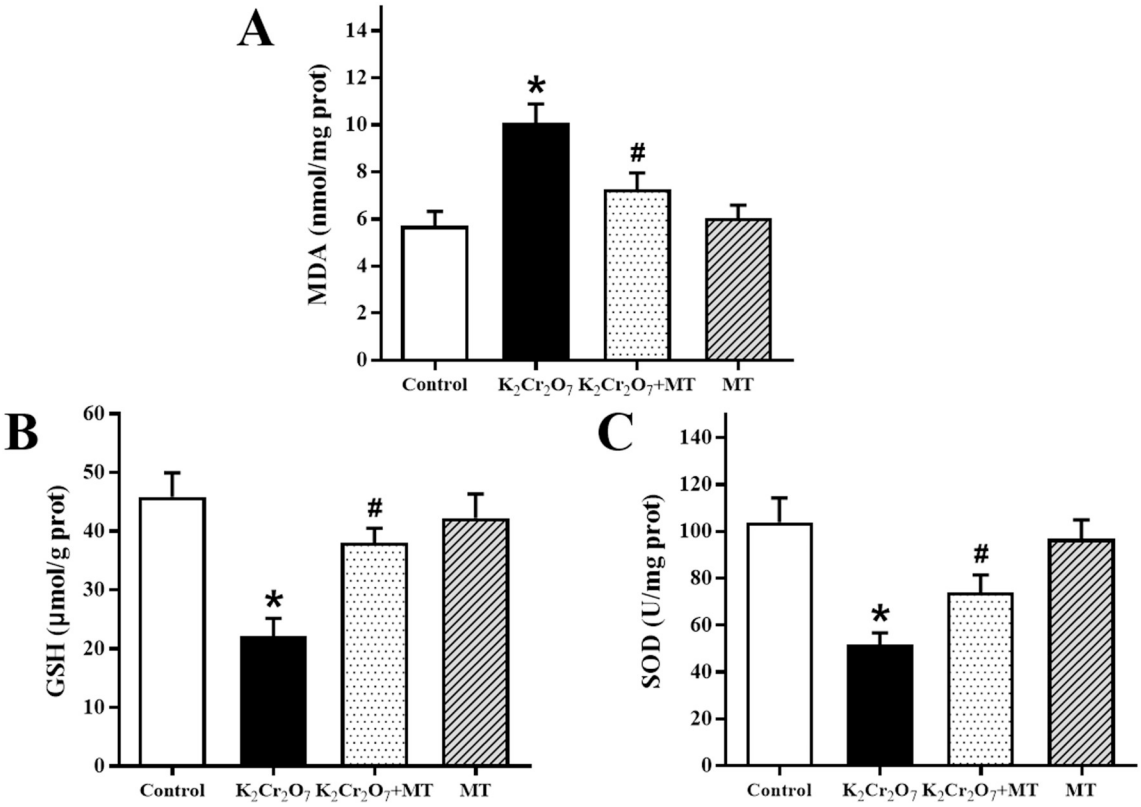

Fig. 3. Effect of MT on $K_2Cr_2O_7$-induced oxidative stress in heart tissues. MDA level (A), GSH content (B), and SOD activity (C) were determined. Data are presented as mean ± SEM, n = 7. * Statistically different ($p < 0.05$) VS. control group. # Statistically different ($p < 0.05$) VS. $K_2Cr_2O_7$ group.

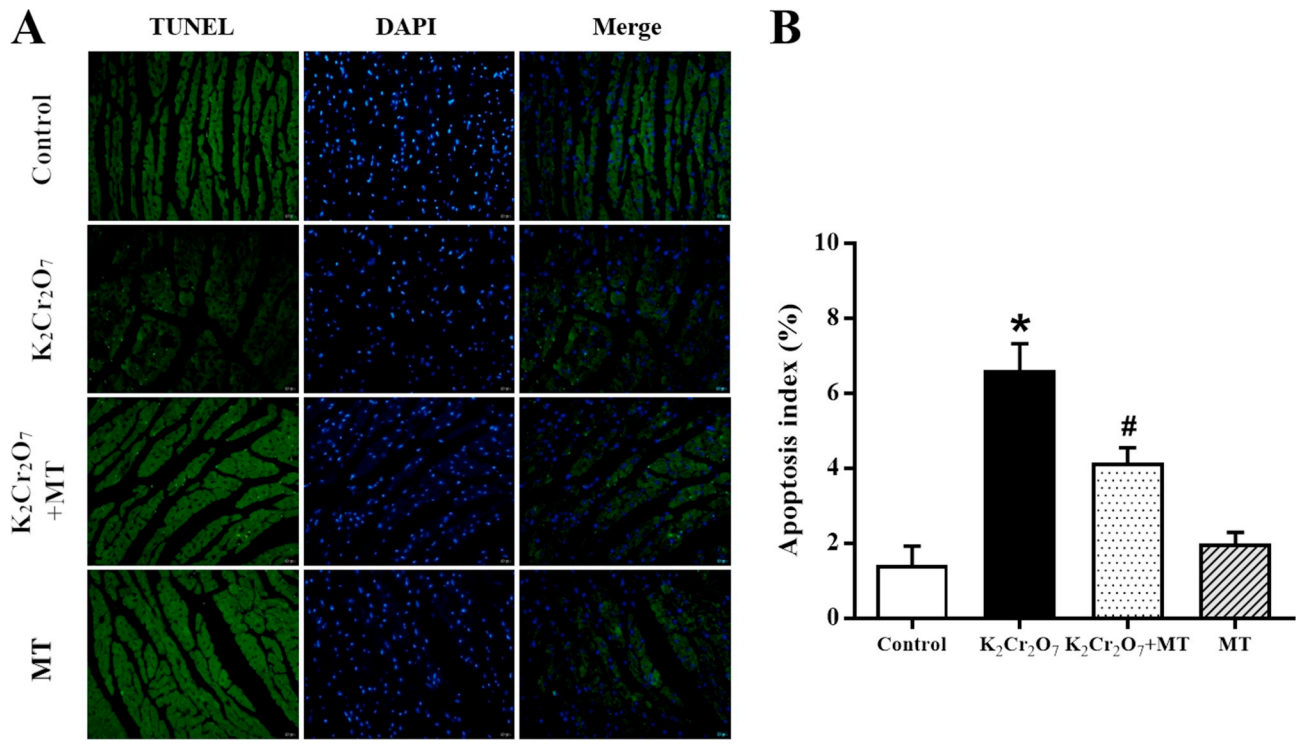

Fig. 4. Effect of MT on $K_2Cr_2O_7$-induced myocardial apoptosis. (A) Apoptosis (400 ×). (B) Values of quantitative analysis. Data are presented as mean ± SEM ($n = 3$). * Statistically different ($p < 0.05$) VS. control group. # Statistically different ($p < 0.05$) VS. $K_2Cr_2O_7$ group.

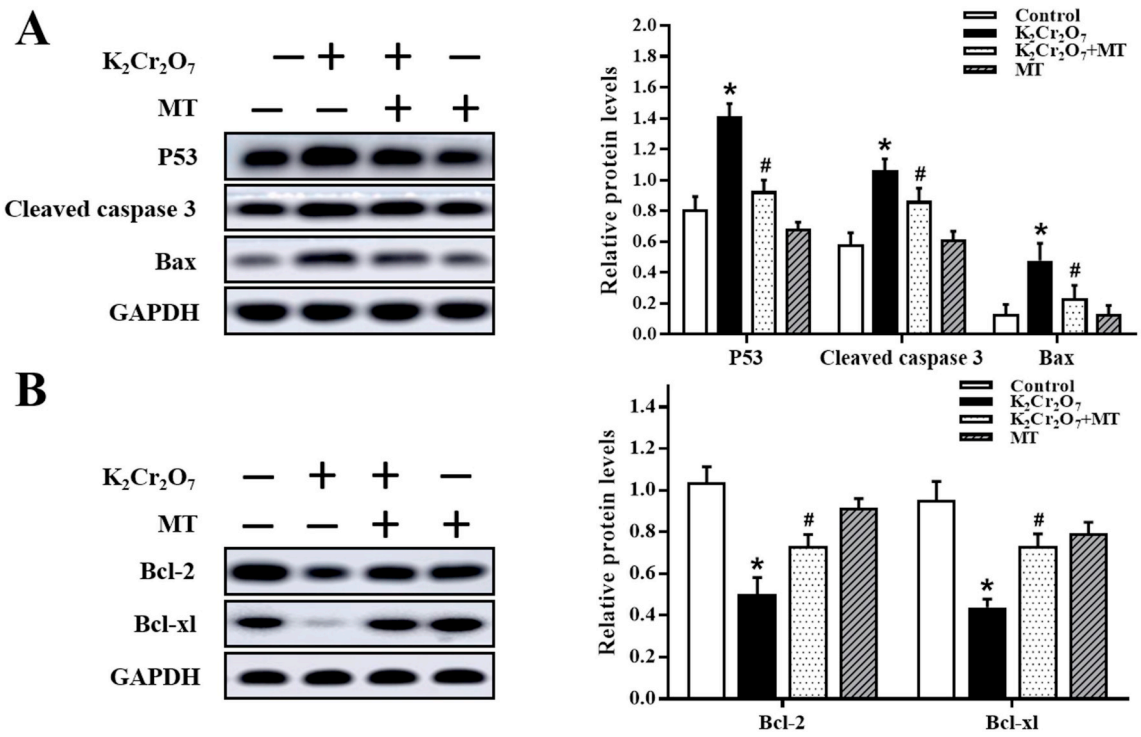

Fig. 5. Effect of MT on apoptosis related pathway in $K_2Cr_2O_7$-induced rats. (A) The relative protein levels of P53, cleaved caspase 3, and Bax in heart. (B) The relative protein levels of Bcl-2, and Bcl-xl. Values are presented as mean ± SEM (n = 3). * Statistically different ($p < 0.05$) VS. control group. # Statistically different ($p < 0.05$) VS. $K_2Cr_2O_7$ group.

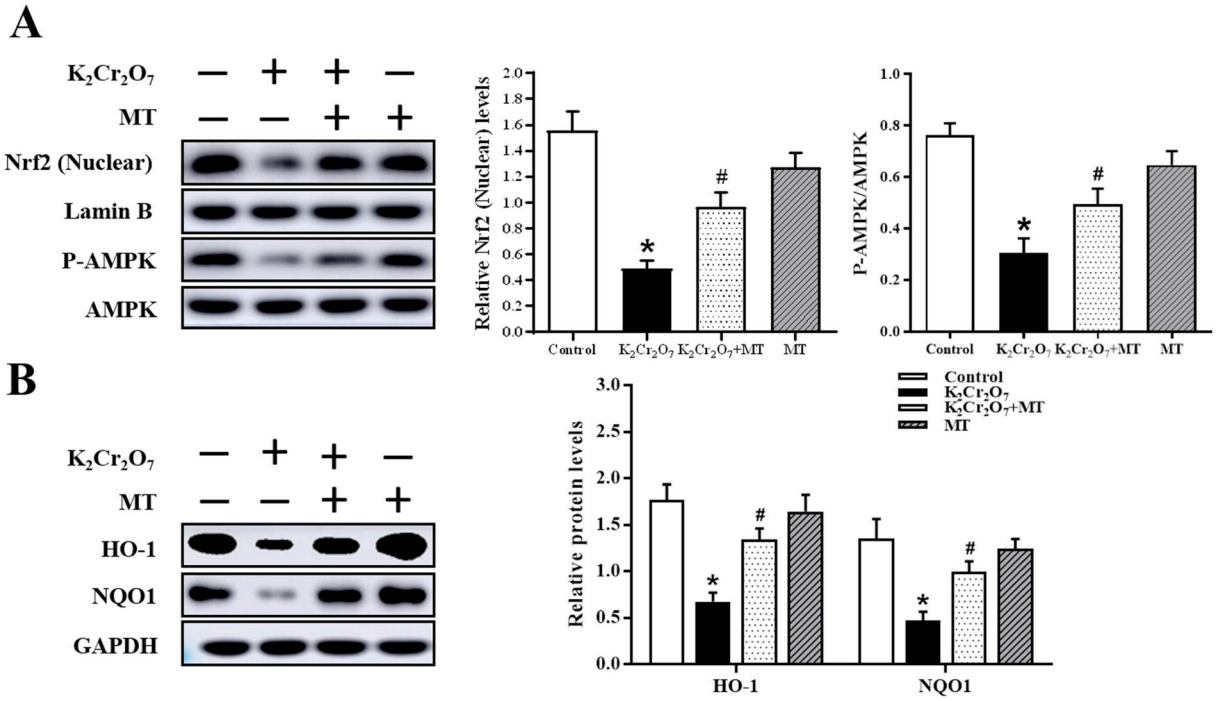

Fig. 6. Effect of MT on oxidative stress pathway in $K_2Cr_2O_7$-treated heart. (A) The relative protein levels of Nrf2 (nuclear) and P-AMPK/AMPK in heart. (B) The relative protein levels of HO-1 and NQO1. Data are presented as mean ± SEM, n = 7. * Statistically different ($p < 0.05$) VS. control group. # Statistically different ($p < 0.05$) VS. $K_2Cr_2O_7$ group.

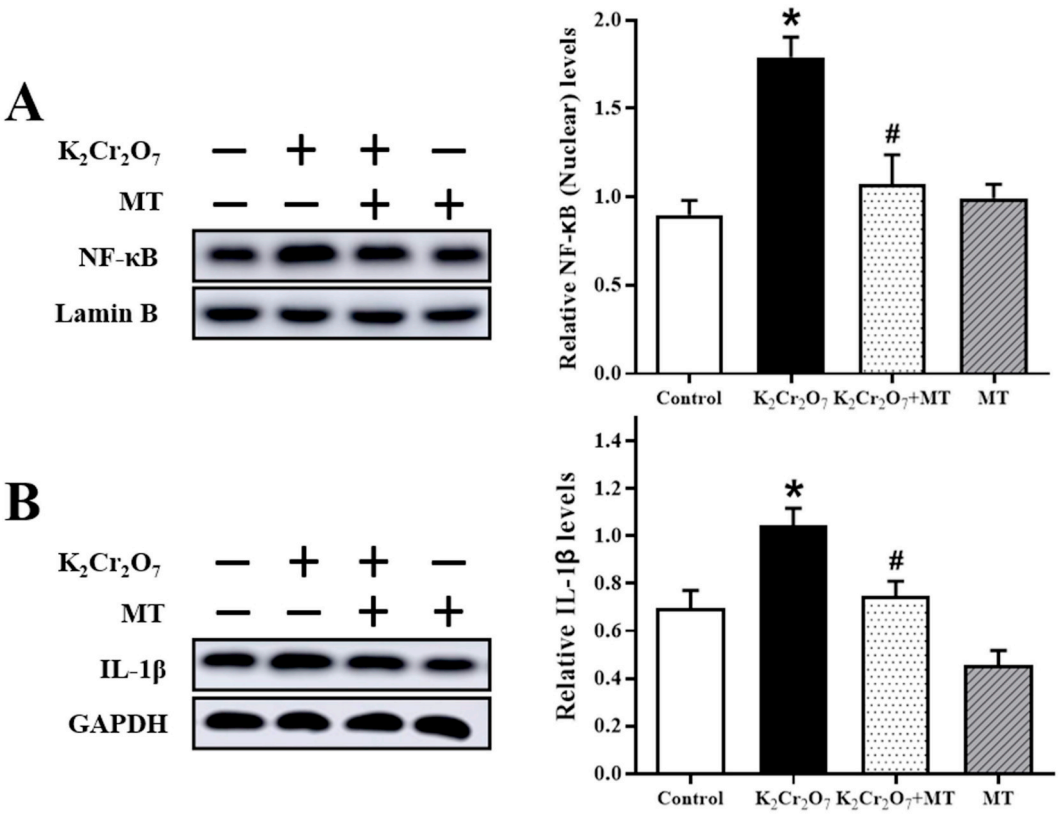

Fig. 7. Effect of MT on inflammatory mediators in $K_2Cr_2O_7$-treated heart. (A) The relative Nuclear NF-κB protein levels. (B) The relative protein levels of IL-1β. Values are presented as mean ± SEM (n = 3). * Statistically different ($p < 0.05$) VS. control group. # Statistically different ($p < 0.05$) VS. $K_2Cr_2O_7$ group.

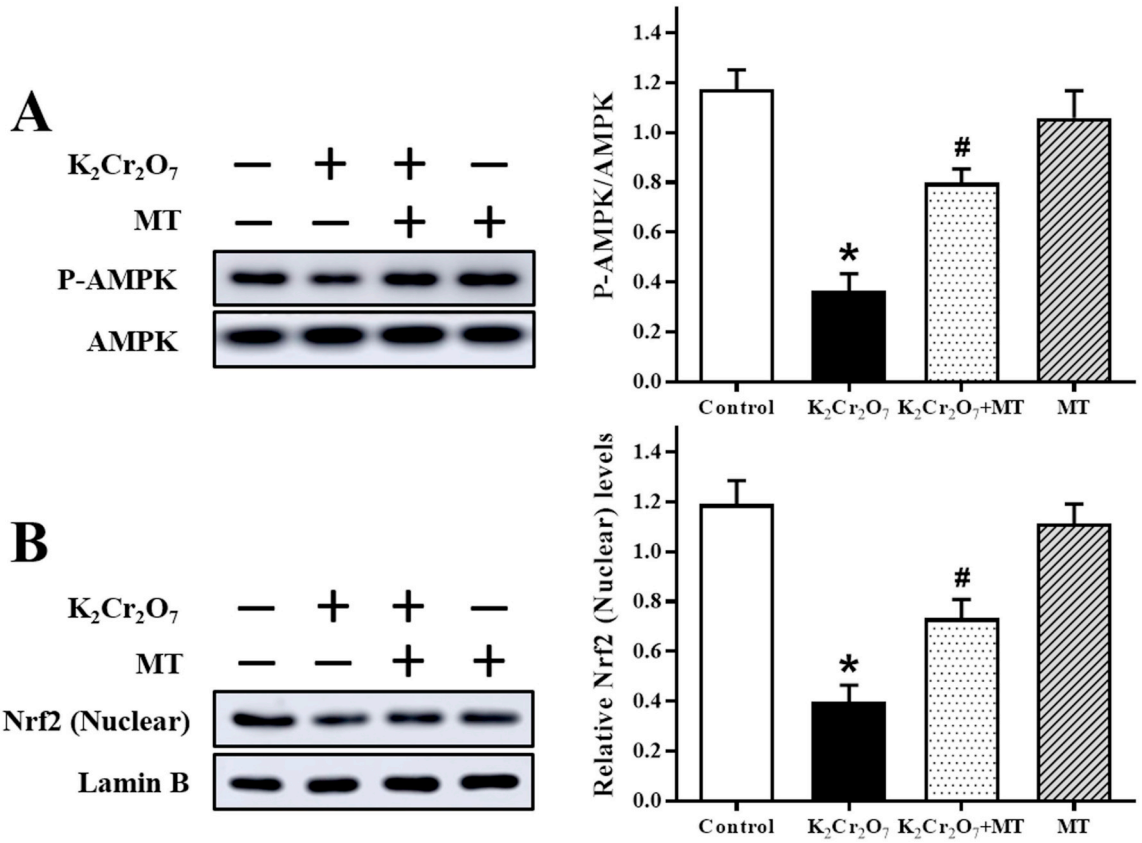

Fig. 8. Effect of MT on the protein levels of Nrf2 (nuclear) and P-AMPK/AMPK in $K_2Cr_2O_7$-treated H9C2 cells. (A) The relative P-AMPK/AMPK protein levels. (B) The relative protein levels of Nuclear Nrf2. Values are presented as mean ± SEM (n = 3). * Statistically different ($p < 0.05$) VS. control group. # Statistically different ($p < 0.05$) VS. $K_2Cr_2O_7$ group.

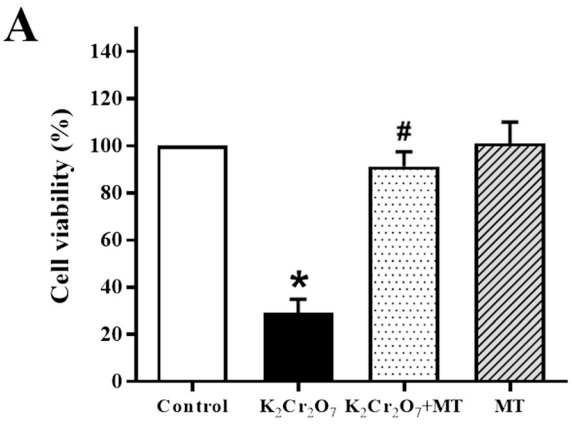

Fig. 9. Effect of MT on $K_2Cr_2O_7$-treated H9C2 cell viability. Values are presented as mean ± SEM (n = 7). * Statistically different ($p < 0.05$) VS. control group. # Statistically different ($p < 0.05$) VS. $K_2Cr_2O_7$ group.

3.7. MT increased the P-AMPK and Nrf2 protein levels in H9C2 cells treated by $K_2Cr_2O_7$

Compared with the control group, the expression of P-AMPK/AMPK and Nrf2 were decreased in the $K_2Cr_2O_7$ group, but were increased in the $K_2Cr_2O_7$ + MT group ($p < 0.05$, Fig. 8).

3.8. MT attenuated the viability inhibition $K_2Cr_2O_7$-induced in H9C2 cells

As shown in Fig. 9, the viability of myocardial cell was decreased in the $K_2Cr_2O_7$ group compared with the control group ($p < 0.05$). However, treatment with MT significantly increased cell viability ($p < 0.05$).

4. Discussion

Although MT has been shown to have positive effect on various biological functions, the benefits of MT for the cure of cardiac injury are unclear. Cr(VI) promoted the overproduction of free radicals and ROS [30]. Free radicals attack the cell membrane, increase lipid peroxidation, and result in the production of MDA [16,31,32]. Meanwhile, the antioxidant defense system enzyme, SOD, and the non-enzymatic antioxidant, GSH, are consumed in large quantities [16,33]. Our results has shown that MT effectively reduces MDA levels, and increases GSH concentration and SOD activity after $K_2Cr_2O_7$ treatment. Furthermore, in this study, MT attenuates the viability inhibition $K_2Cr_2O_7$-induced in H9C2 cells. These results indicate that MT mitigates the oxidative damage in $K_2Cr_2O_7$-induced cardiac injury. It is worth mentioning that the metabolic pathways of Cr(VI) in body may be partly related to the form of Cr(V)-GSH species [34]. However, there is no evidence that MT may react directly with Cr, so whether MT takes part in this process still needs to be furtherly investigated.

Apoptosis, as a kind of programmed cell death, can be triggered by much environmental or chemical irritant, such as ROS [35–37]. Oxidative stress promotes the activation of P53, displacement of the anti-apoptotic proteins such as Bcl-2 and Bcl-xl [38], and the expression of Bax which is a pro-apoptotic protein [39]. Subsequently, excessive cytochrome *c* release, and activates caspase-3 resulting in cleaved caspase-3 [40]. We have shown that MT inhibits the reduction of Bcl-2 and Bcl-xl levels, and decreases the levels of P53, caspase-3, and Bax. Moreover, TUNEL assay results confirm that the myocardial apoptosis induced by $K_2Cr_2O_7$ is significantly blocked by MT. Hence, the results

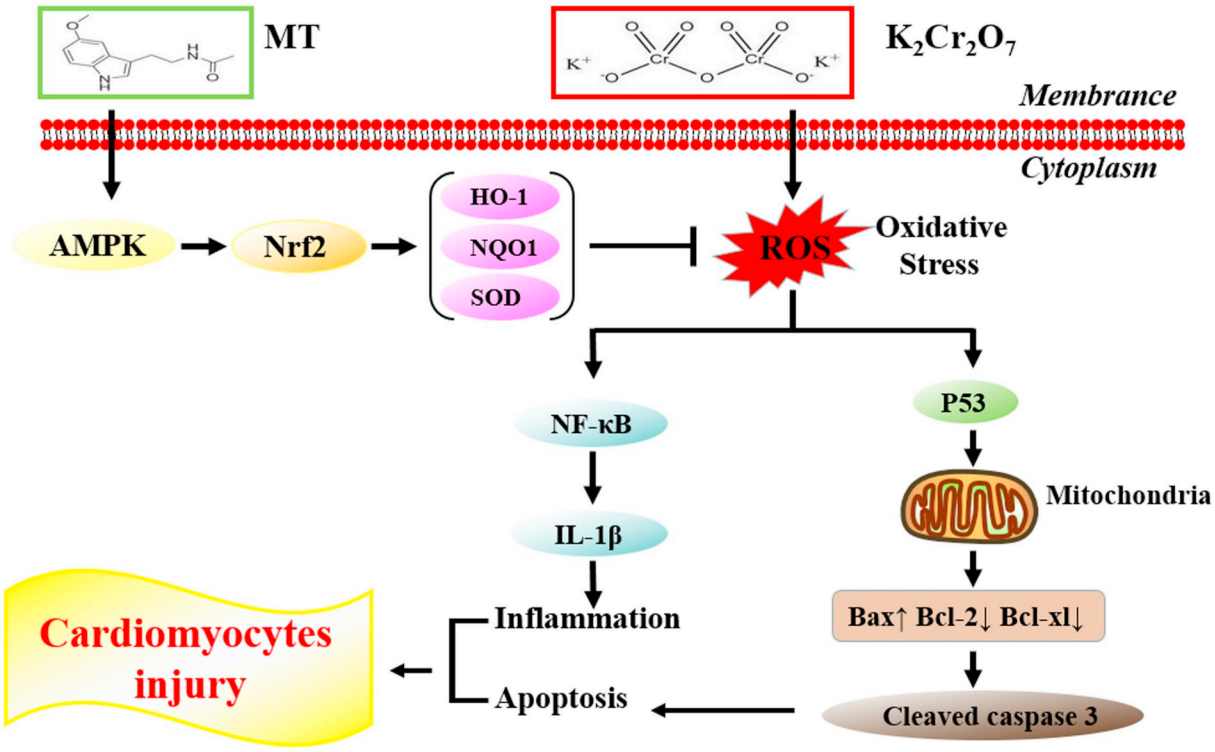

Fig. 10. Schematic representation of proposed mechanism of MT on the prevention of $K_2Cr_2O_7$-induced cardiac injury. MT attenuated $K_2Cr_2O_7$-induced cardiac injury by mitigating oxidative damage, inhibiting apoptosis, and attenuating inflammatory response via AMPK/Nrf2 pathway.

suggest that MT inhibits $K_2Cr_2O_7$-induced myocardial apoptosis.

As a potent inflammatory mediator, NF-κB can be activated by oxidative stress [41–43]. Under the stimulation of pro-inflammatory cytokines, NF-κB is activated and transfers to the nucleus, then accumulates and accelerates the production of IL-1β which is a pro-inflammatory cytokines [41,44]. Due to the adverse impact of oxidative stress, the cell upregulates genes involved in inflammation to activate or amplify the inflammatory response [36,45]. Our results show that nuclear translocation of NF-κB and the increase in IL-1β induced by $K_2Cr_2O_7$ are substantially suppressed by MT. Thereby, MT may protect against $K_2Cr_2O_7$-induced cardiac injury by reducing the expression of inflammatory cytokines to weaken the inflammatory response.

Nrf2 is a key nuclear transcription factor for maintaining redox homeostasis and detoxification under many heavy metals exposure, and plays a significant role in preventing cell injury resulting from oxidative stress [46–49]. When body under oxidative stress status, Nrf2 starts the antioxidant response element, and enables downstream antioxidant processes to activate detoxifying genes. NQO1 and HO-1, combined with Nrf2, inhibit $K_2Cr_2O_7$-induced ROS production [50–53]. AMPK has been shown to promote mitochondrial health, and several recently discovered AMPK targets have been shown to be involved in mitochondrial homeostasis [54]. Research has shown that AMPK may play a role in Nrf2 activation and promote the expression of downstream antioxidant enzymes [55]. In the present study, the decreases of AMPK, Nrf2, NQO1, and HO-1 induced by $K_2Cr_2O_7$ in the cardiac tissues were attenuated by MT. In addition, our results have shown that MT increases the P-AMPK and Nrf2 protein levels in H9C2 cells treated by $K_2Cr_2O_7$. Therefore, the results suggest that AMPK/Nrf2 pathway activation is the key link in the protective mechanism of MT on cardiac damage mediated by $K_2Cr_2O_7$.

5. Conclusion

In conclusion, our results demonstrate that MT attenuates Cr-induced cardiac damage via activating the AMPK/Nrf2 pathway (Fig. 10). This study provides a beneficial proof for the use of MT in treatment of $K_2Cr_2O_7$-induced cardiac injury.

Abbreviations

AMPK	Adenosine 5′-monophosphate-activated protein kinase
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma gene 2
Bcl-xL	B-cell lymphoma-extra large
CK	Creatine kinase
CK-MB	Creatine kinase-MB
Cr	Chromium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
GSH	Glutathione
HBDB	Alpha-hydroxybutyrate dehydrogenase
HO-1	Heme oxygenase-1
IL-1β	Interleukin-1β
LDH	Lactic dehydrogenase
MDA	Malondialdehyde
MIR	Myocardial ischemia reperfusion
MT	Melatonin
NF-κB	Nuclear factor kappa B
NQO1	Nicotinamide adenine dinucleotide phosphatase: quinoneacceptor 1
Nrf2	Nuclear factor-erythroid-2-related factor 2
OD	Optical density
PDH	Glyceraldehyde-3-phosphate dehydrogenase
P-AMPK	phosphorylated–AMPK
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBST	Tris buffered saline tween
TUNEL	Terminal deoxynucleotidyl transferase-mediated deoxyuracil nucleoside triphosphate nick-end labeling

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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