Am J Transl Res 2025;17(11):8803-8814
www.ajtr.org /ISSN:1943-8141/AJTR0168328

Original Article

Klotho mitigates diquat-induced myocardial injury in rats by activating Nrf2/ARE-mediated suppression of oxidative stress

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Received August 25, 2025; Accepted October 27, 2025; Epub November 15, 2025; Published November 30, 2025

Abstract: Objectives: Diquat (DQ) induces severe cardiotoxicity through oxidative stress, yet no specific antidote is currently available. Klotho, a known agonist of nuclear factor erythroid 2-related factor 2 (Nrf2), exerts antioxidative effects in various diseases. However, its role in DQ-induced myocardial injury remains undefined. Methods: Acute myocardial injury was induced in rats by intragastric DQ administration, followed by treatment with recombinant Klotho protein. Myocardial histopathology, oxidative stress markers, and components of the Nrf2/antioxidant response elements (ARE) pathway - including Nrf2, heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase 1 (NQO1) - were evaluated. Additionally, DQ-exposed H9c2 cardiomyocytes were treated with Klotho or the Nrf2 inhibitor ML385. cell viability, apoptosis, oxidative stress markers, and Nrf2 pathway protein expression were evaluated. Results: DQ administration induced significant oxidative stress and upregulated mRNA levels (2-3-fold; all $P<0.0001$) and protein expression (1.3-1.4-fold; $P=0.0018$, $P=0.0039$, $P=0.0030$) of Nrf2, HO-1, NQO1 in rat myocardial tissues. Klotho treatment effectively preserved H9c2 cell viability (by 60%; $P<0.0001$), reduced apoptosis (by 43%; $P<0.0001$), and alleviated oxidative stress (all $P<0.0001$). Klotho also enhanced Nrf2, HO-1, and NQO1 protein expression (2.5-3-fold; all $P<0.0001$). ML385 abrogated the protective effects of Klotho against DQ-induced oxidative stress (all $P<0.0001$) and apoptosis (all $P<0.0001$) in H9c2 cells. *In vivo*, recombinant Klotho administration attenuated myocardial histopathologic damage, normalized oxidative stress markers (all $P<0.0001$), and increased Nrf2, HO-1, NQO1 protein levels (1.8-2-fold; all $P<0.0001$). Conclusion: Klotho mitigates DQ-induced myocardial injury in rats by activating Nrf2/ARE signaling pathway and suppressing oxidative stress.

Keywords: Diquat, Klotho, myocardial injury, oxidative stress, Nrf2/ARE signaling pathway

Introduction

Diquat (DQ) is a bipyridyl non-selective herbicide characterized by its broad spectrum weed control, rapid action, and low residue [1]. Since the ban on paraquat sales in China, the widespread agricultural use of DQ has led to an increasing incidence of acute poisoning cases [2]. As a highly toxic compound, DQ induces multi-organ damage, inducing hepatic, renal, digestive, and myocardial injuries [3-5], posing a serious clinical challenge. Critically, no specific antidote is available for DQ poisoning, and successful management depends on early recognition and prompt intervention to reduce absorption, enhance elimination, and provide organ support. Extensive research indicates that the primary toxicological mechanism of DQ poisoning stems from intracellular overproduction of reactive oxygen species (ROS)/reactive nitrogen species (RNS) through redox cycling [2, 6]. This process induces oxidative stress and subsequent cellular dysfunction. Therefore, developing therapeutic strategies that directly target oxidative stress holds promise for the discovery of novel antidotes against DQ toxicity.

Oxidative stress arises when excessive ROS/RNS generation overwhelms the intrinsic antioxidant defense system, leading to irreversible cellular damage [7]. The transcription factor

https://doi.org/10.62347/SKBR3572

nuclear factor erythroid 2-related factor 2 (Nrf2) serves as a master regulator of antioxidant defense mechanisms [8]. Under physiologic conditions, Nrf2 is sequestered in the cytoplasm by its repressor Kelch-like ECH-associated protein 1 (Keap1) and undergoes ubiquitin-mediated degradation. Upon oxidative challenge, conformational alterations in Keap1 trigger Nrf2 release, allowing its nuclear translocation, where it binds to antioxidant response elements (ARE) to drive the transcription of cytoprotective genes, such as heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) [9]. Given its pivotal antioxidant role, pharmacologic activation of the Nrf2/ARE pathway has emerged as a promising strategy to mitigate DQ-induced toxicity. Supporting evidence shows that hydroxytyrosol ameliorates oxidative stress in DQ-treated mice by Nrf2/ARE activation [10]. Similarly, ellagic acid attenuates DQ-induced ROS generation in mice by regulating the Nrf2/HO-1/NQO1 axis [11]. Furthermore, resveratrol mitigates DQ-driven intestinal oxidative stress in weaned piglets through Nrf2/ARE pathway activation [12]. Collectively, these findings demonstrate that enhancing Nrf2-mediated antioxidant responses represents a promising strategy for developing novel antidotes against DQ toxicity.

Klotho, a well-established aging-suppressor protein, confers multi-organ protection through its potent antioxidant and anti-inflammatory properties [13, 14]. A compelling and innovative aspect of Klotho's mechanism is its role as a physiologic agonist of the Nrf2 pathway. Evidence highlights that Klotho enhances cellular antioxidant defenses by activating Nrf2 in diverse disease contexts. For example, Klotho overexpression ameliorates renal aging by suppressing Nrf2/HO-1-mediated oxidative stress [15]. Similarly, recombinant Klotho protein protects against kidney injury by inhibiting Nrf2-mediated ferroptosis [16]. In diabetic nephropathy, upregulated Klotho expression alleviates high glucose-induced oxidative damage by Nrf2 activation [17]. Most notably, and of direct relevance to the present study, exogenous Klotho supplementation has been shown to protect against myocardial infarction by attenuating cardiomyocyte oxidative stress through Nrf2/ARE activation [18]. Despite these findings, whether Klotho confers protection against DQ-induced myocardial injury remains unknown. Therefore, investigating the potential of Klotho to activate the cardiac Nrf2/ARE pathway and alleviate DQ toxicity addresses a significant knowledge gap and offers a novel therapeutic perspective.

This study aimed to evaluate the cardioprotective effect of Klotho against DQ-induced acute myocardial injury and to elucidate whether its mechanism of action involves activation of the Nrf2/ARE signaling pathway. We hypothesized that Klotho mitigated DQ-induced oxidative damage in the heart by enhancing Nrf2-driven antioxidant gene expression. Clarifying this mechanism is of paramount clinical significance, as it may not only advance the treatment of DQ poisoning by identifying a promising novel therapeutic candidate but also substantiate the broader therapeutic potential of Klotho for oxidative stress-related cardiac conditions.

Materials and methods

Cell culture and treatment

H9c2 cardiomyocytes were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DEME; 11965175, Gibco, USA) containing 10% fetal bovine serum (A3382101, Gibco, USA) and 1% penicillin-streptomycin (15140148, Gibco, USA) at 37°C in a humidified atmosphere containing 5% CO_2. To induce oxidative injury, cells were treated with 50 μM DQ (45422, Sigma-Aldrich, USA) for 24 h. For Klotho intervention, cells were treated with 1 μg/mL recombinant Klotho protein (1819-KL, R&D Systems, USA) [19, 20].

Cell Counting Kit-8 (CCK-8) assay

H9c2 cells were seeded in 96-well plates at a density of 5×10^3/well and incubated at 37°C for 24 h. Cell viability was assessed using the CCK-8 (C0040; Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, 10 μL of CCK-8 reagent was added into each well and incubated for 2 h at 37°C. Absorbance was then measured at 450 nm using a microplate reader (Model 680, Bio-Rad, USA).

Cell apoptosis assay

H9c2 cells were suspended in binding buffer and stained with Annexin V-FITC and propidium iodide (5 μL each; E-CK-A211, Elabscience, Wuhan, China) for 15 min in the dark. Apoptotic

rates were determined by flow cytometry (BD Biosciences).

Reactive oxygen species (ROS) assay

A ROS assay kit (CA1410, Solarbio, Beijing, China) was used to assess ROS levels in cells and tissues. H9c2 cells were incubated with 10 μM 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 20 min, followed by washing with phosphate-buffered saline (PBS) to remove excess probe. Myocardial tissue sections (4 μm) were incubated with 10 μM dihydroethidium (DHE) for 30-60 min at 37°C and counterstained with 4',6-Diamidino-2-Phenylindole (DAPI) for 5 min. Fluorescence signals were examined using an Olympus fluorescence microscope.

Enzyme-linked immunosorbent assay (ELISA)

Levels of oxidative stress markers - including malondialdehyde (MDA) contents, glutathione peroxidase (GSH-PX), glutathione sulfotransferase (GSH-ST), and total superoxide dismutase (T-SOD), were examined using commercial ELISA kits (BC1195, BC0355, BC0170, Solarbio, Beijing, China) following the manufacturer's instructions.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA extraction was conducted using Trizol (15596026, Invitrogen, USA). The cDNAs were synthesized using the Prime Script RT reagent Kit (RR037Q, Takara, Dalian, China). Quantitative PCR was then performed with SYBR Green qPCR Master Mix (A46110, Takara, Dalian, China) on an ABI 7500 Real-time PCR System (CA, USA). Relative mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences used were as below: Nrf2 (forward: 5'-TGC TGC CAT TAG TCA GTC GC-3', reverse: 5'-TGC CGG AGT CAG AGT CAT TG-3'), HO-1 (forward: 5'-ACA TGG CCT TCT GGT ATG GG-3', reverse: 5'-CAC CTC GTG GAG ACG CTT TA-3'), NQO1 (forward: 5'-CCA CGC AGA GAG GAC ATC AT-3', reverse: 5'-TCA GAT TCG ACC ACC TCC CA-3').

Western blot analysis

Protein isolation was conducted using radioimmunoprecipitation assay (RIPA) solution (89900, Invitrogen, USA). Equal amounts of protein were subjected to electrophoresis in 12% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membranes (IPVH00010, Millipore, USA). After blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, membranes were incubated with primary antibodies overnight at 4°C. The membranes were then washed with TBST and incubated with corresponding secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (RPN2236, Amersham, UK), and band intensities were quantified using ImageJ software. Primary antibodies were as follows: Klotho (1:1000, ab181373, Abcam), Nrf2 (1:1000, ab313825, Abcam), HO-1 (1:2000, ab189491, Abcam), NQO1 (1:1000, ab97385, Abcam), and GAPDH (1:2500, ab9485, Abcam).

Animal experiments

Male Sprague-Dawley rats (5-6 weeks, 150-200 g) were provided by the Guangzhou Twelfth People's Hospital and housed under standard conditions. Animal experiments were approved by the Animal Ethics Committee of Guangzhou Twelfth People's Hospital. Rats were randomly divided into three groups (n=8 per group): control group, DQ group, and DQ+Klotho group. Rats in DQ group were intragastrically administered with 115.5 mg/kg DQ (50% of the median lethal dose) [21], while control rats were administered with equal volume of normal saline. The expression of oxidative stress markers and Nrf2/ARE signaling associated molecules was detected at 12, 24, 48, 72, 120 h after DQ exposure. Rats in DQ+Klotho group received intraperitoneal injection of recombinant Klotho protein (0.01 mg/kg/day) for 5 consecutive days [22, 23], whereas rats in the control and DQ groups were administered an equal volume of normal saline. At the end of the experiment, rats were euthanized by intraperitoneally injecting 150 mg/kg pentobarbital sodium, and myocardial tissues were harvested for further examination.

Hematoxylin and eosin (H&E) staining

Myocardial tissue samples were fixed in 10% neutral buffered formalin, embedded in paraf-

fin, and sectioned at a thickness of 4 μm. Following deparaffinization and rehydration, sections were stained with H&E (G1120, Solarbio, Beijing, China) and then imaged using an Olympus microscope.

Immunohistochemistry

After deparaffinization and rehydration, tissue sections subjected to antigen retrieval using microwave heating in sodium citrate buffer. After cooling to room temperature, endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 10 min. Sections were blocked with 5% bovine serum albumin (BSA) for 30 min and then incubated with primary antibody at 4°C overnight. After washing with phosphate-buffered saline (PBS), sections were incubated with secondary antibody for 1 h at room temperature. Immunoreactivity was visualized using diaminobenzidine chromogen (DA1010, Solarbio, Beijing, China), with microscopic examination using an Olympus microscope. ImageJ software was used for quantitative analysis.

Statistical analysis

Statistical analyses were conducted using SPSS version 22.0 software. All experiments were performed in triplicate, and data were presented as mean ± standard deviation (SD). Differences between two groups were performed using Student's t-test, while multi-group comparisons were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc test. A $P<0.05$ was considered significant.

Results

DQ stimulation promoted myocardial oxidative stress injury

To evaluate DQ-induced myocardial oxidative stress, rats were intragastrically administered DQ, and oxidative stress markers were assessed at 12, 24, 48, 72, and 120 hours post-treatment. Myocardial ROS fluorescence signal was markedly enhanced in DQ-treated rats compared with controls, rising as early as 12 h and peaking at 48 h (**Figure 1A**, **1B**). Furthermore, DQ exposure significantly increased MDA level (**Figure 1C**) and GSH-ST activity (**Figure 1D**), while reducing GSH-PX (**Figure 1E**) and SOD activities (**Figure 1F**) in myocardial tissues. These oxidative stress markers reached their maximal alterations at 24-48 h post-treatment. Collectively, these findings suggest that DQ administration induced significant oxidative stress in myocardial tissues.

DQ stimulation activated the Nrf2/ARE signaling pathway

To investigate the role of the Nrf2/ARE pathway in response to DQ-induced oxidative stress, we examined the expression of Nrf2 and its downstream effectors HO-1 and NQO1 in myocardial tissues at 12, 24, 48, 72, and 120 hours after DQ treatment. DQ administration significantly increased the mRNA expression of Nrf2, HO-1, and NQO1 in myocardial tissues compared to controls (**Figure 2A-C**), with transcriptional activation observed at 24 h and peaking at 48 h post-treatment. Consistent with these results, western blot analysis revealed a corresponding increase in Nrf2, HO-1, and NQO1 protein levels, also peaked at 48 hours post-treatment (**Figure 2D-G**). Immunohistochemical staining further confirmed these results (**Figure 2H-K**). Together, these findings demonstrate that DQ exposure activates the Nrf2/ARE signaling pathway during myocardial oxidative stress injury.

Klotho alleviated DQ-induced oxidative stress in cardiomyocytes

H9c2 cardiomyocytes were exposed to different concentrations of DQ for 24 h or to 50 μM DQ for varying durations. DQ exposure significantly reduced H9c2 cell viability in both dose- and time-dependent manners (**Figure 3A**, **3B**). Based on these findings, 50 μM DQ for 24 hour - causing approximately a 50% reduction in viability - was selected as the optimal condition for subsequent experiments.

To evaluate the protective effects of Klotho, DQ-stimulated H9c2 cardiomyocytes were treated with recombinant Klotho protein. Western blot analysis confirmed a marked increase in Klotho protein expression following recombinant Klotho administration (**Figure 3C**). Klotho treatment effectively preserved cell viability (**Figure 3D**) and reduced apoptosis (**Figure 3E**, **3F**) of DQ-stimulated H9c2 cells. Furthermore, DQ exposure significantly increased ROS levels (**Figure 3G**, **3H**), MDA content (**Figure 3I**), and GSH-ST activity (**Figure 3J**), whiledecreasing GSH-PX (**Figure 3K**) and SOD

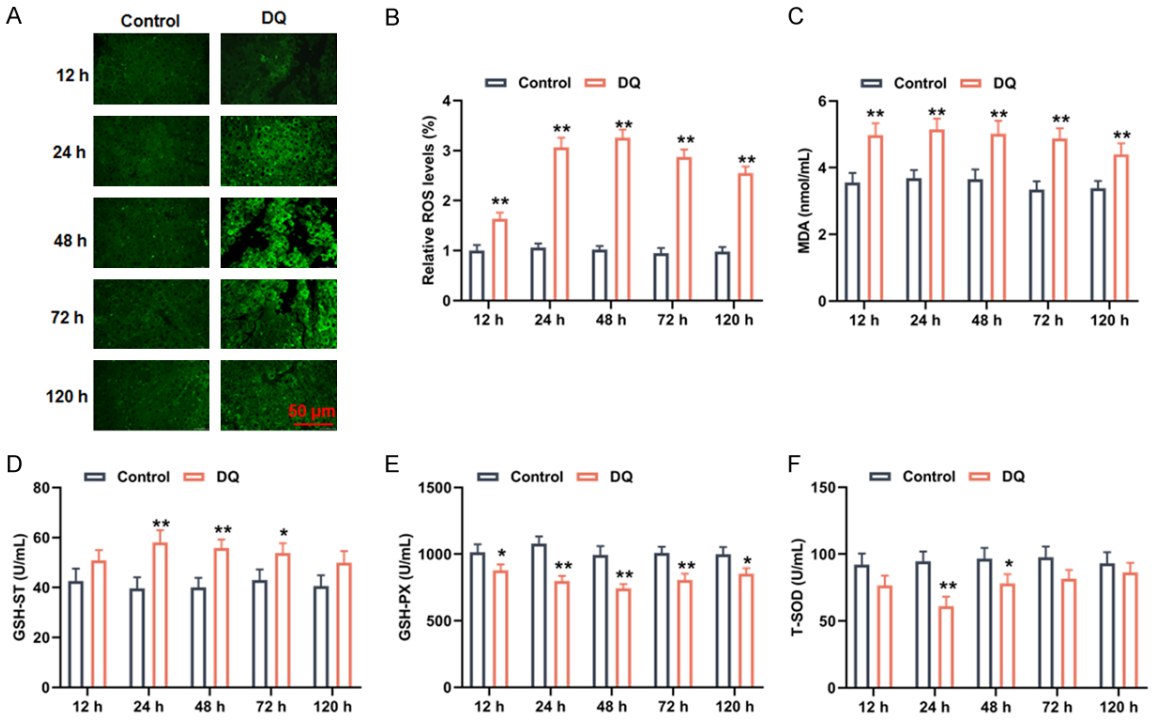

Figure 1. Diquat (DQ) exposure promoted myocardial oxidative stress injury. (A, B) Reactive oxygen species (ROS) fluorescence signal, (C) Malondialdehyde (MDA), (D) glutathione sulfotransferase (GSH-ST), (E) glutathione peroxidase (GSH-PX), and (F) total superoxide dismutase (T-SOD) activities in myocardial tissues were assessed at 12, 24, 48, 72, and 120 hours after DQ treatment using enzyme-linked immunosorbent assay (ELISA) kits. Data were presented as mean ± standard deviation (SD). **P*<0.05, ***P*<0.01, compared to control group.

activities (**Figure 3L**) in H9c2 cells. Notably, Klotho treatment reversed these DQ-induced oxidative changes. Consistent with its antioxidant effects, Klotho significantly upregulated the expression of Nrf2 and its downstream effectors HO-1 and NQO1 (**Figure 3M**, **3N**). Collectively, these findings demonstrate that Klotho alleviated DQ-induced oxidative stress in cardiomyocytes through activation of the Nrf2/ARE signaling pathway.

Nrf2 inhibition abrogated the protective effects of Klotho on DQ-induced oxidative stress in cardiomyocytes

To investigate the role of Nrf2/ARE signaling in Klotho-mediated protection, H9c2 cardiomyocytes were pretreated with the Nrf2 inhibitor ML385. Klotho markedly upregulated Nrf2, HO-1, and NQO1 protein levels (**Figure 4A**, **4B**), whereas ML385 effectively suppressed their expression. Moreover, Klotho treatment significantly alleviated DQ-induced viability inhibition (**Figure 4C**) and apoptosis (**Figure 4D**, **4E**). However, ML385 reversed these protective effects. Furthermore, Klotho mitigated DQ-induced oxidative stress, as evidenced by decreased ROS fluorescence intensity (**Figure 4F**, **4G**), MDA content (**Figure 4H**), and GSH-ST activity (**Figure 4I**), along with restoration of GSH-PX (**Figure 4J**) and SOD activities (**Figure 4K**). However, ML385 abrogated these regulatory effects of Klotho. Collectively, these findings indicate that Klotho alleviated DQ-triggered cardiomyocyte injury through Nrf2/ARE pathway activation.

Klotho alleviated DQ-induced acute myocardial injury in rats through Nrf2/ARE activation

The protective role of Klotho was further validated in a rat model of DQ-induced acute myocardial injury. Rats were intragastrically administered with DQ to induce acute myocardial injury and treated with recombinant Klotho protein for 5 days. Immunohistochemistry results showed markedly decreased Klotho protein expression in myocardial tissues of the DQ group compared to controls, while recombinant Klotho administration effectively restored Klo-

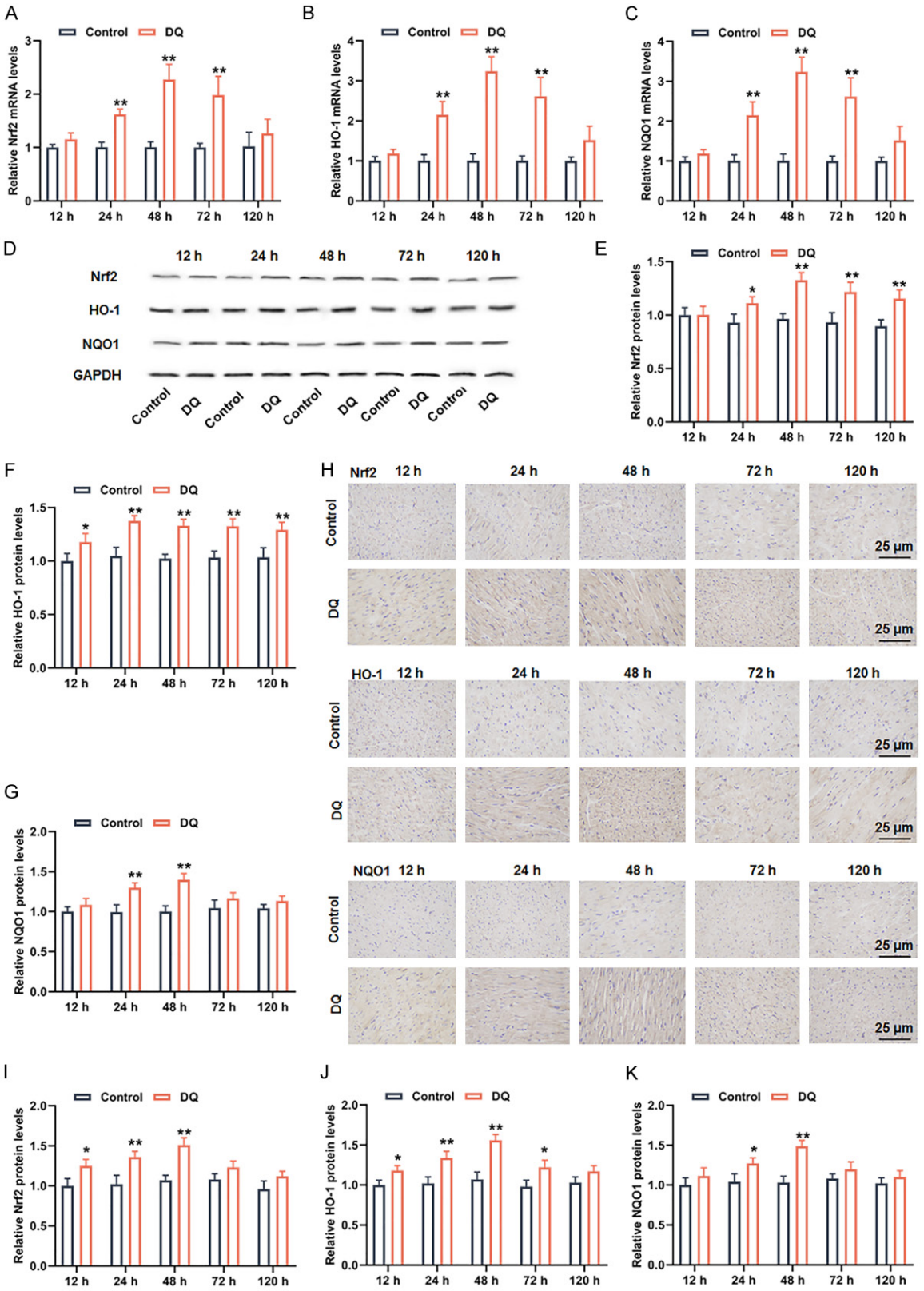

Figure 2. DQ exposure activated nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response elements (ARE) pathway. The mRNA and protein levels of Nrf2, heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase 1 (NQO1) at 12, 24, 48, 72, and 120 hours after DQ treatment were examined using (A-C) reverse transcription quantitative polymerase chain reaction (RT-qPCR), (D-G) Western blot, and (H-K) immunohistochemistry analysis. Data were presented as mean ± SD. **P*<0.05, ***P*<0.01, compared to control group.

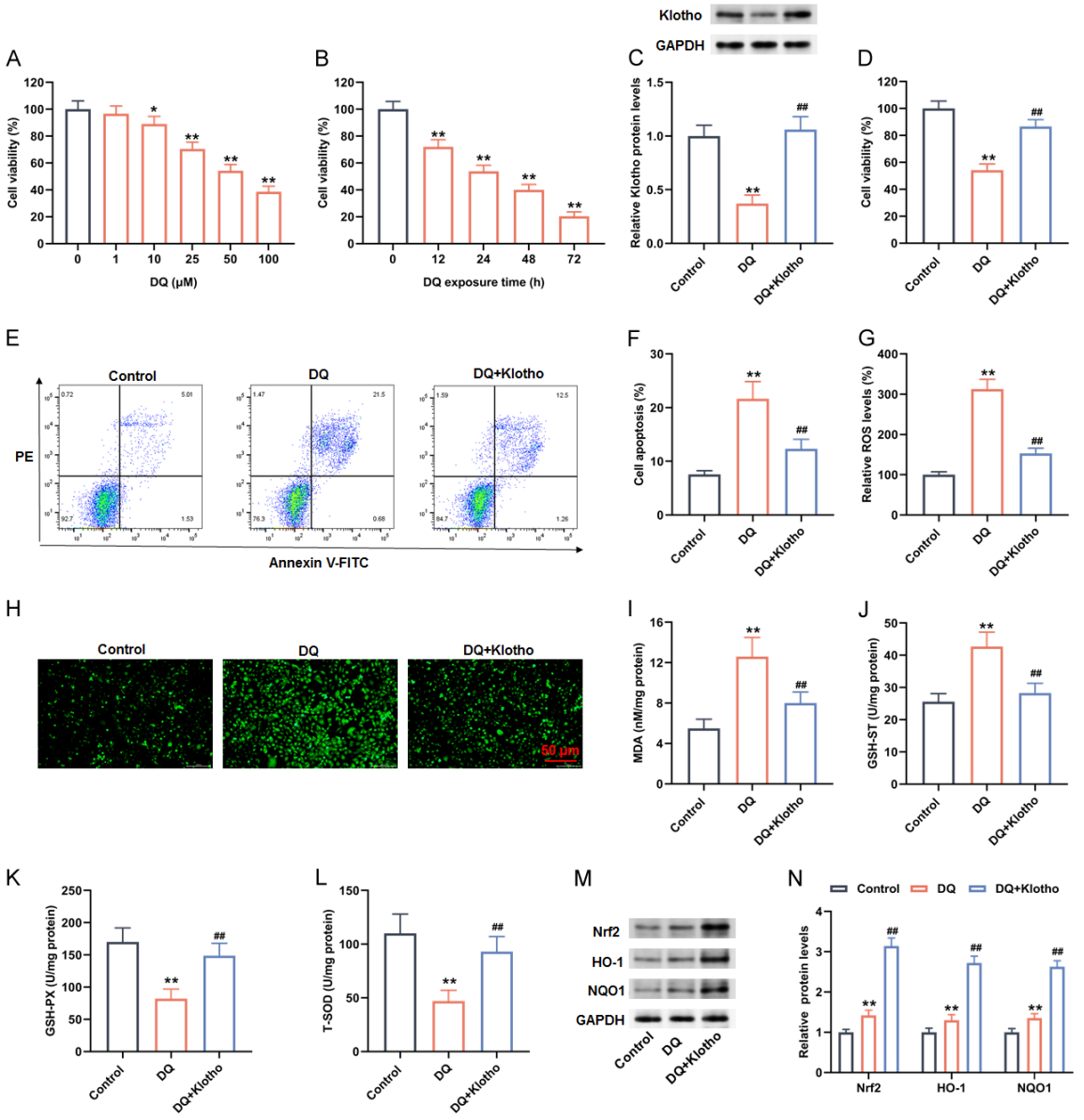

Figure 3. Klotho alleviated DQ-induced cardiomyocyte oxidative stress. (A, B) Cell viability was examined using Cell Counting Kit-8 (CCK-8) assay. (C) Klotho protein levels were examined using western blot analysis. (D) DQ-stimulated H9c2 cardiomyocytes were treated with recombinant Klotho protein, followed by viability assessment using CCK-8 assay. (E, F) Apoptosis was examined using flow cytometry. (G, H) ROS fluorescence signal, (I) MDA level, (J) GSH-ST, (K) GSH-PX, and (L) SOD activities in H9c2 cells were assessed using ELISA kits. (M, N) Nrf2, HO-1, and NQO1 protein levels were examined using western blot analysis. Data were presented as mean ± SD. **P*<0.05, ***P*<0.01, compared to control group. ##*P*<0.01, compared to DQ group.

tho expression (**Figure 5A**, **5B**). H&E staining (**Figure 5C**) demonstrated well-organized cardiomyocytes with clear interstitial spaces and no inflammatory infiltration in control tissues. In contrast, the DQ group exhibited myocardial fiber disarray, necrosis, edema, and inflammatory cell infiltration. Notably, Klotho treatment markedly alleviated these histopathologic changes. Biochemically, DQ administration increased ROS fluorescence intensity (**Figure 5D**, **5E**), MDA content (**Figure 5F**), and GSH-ST activity (**Figure 5G**), while reducing GSH-PX (**Figure 5H**) and SOD activities (**Figure 5I**). Klotho treatment normalized their expression to control levels. Furthermore, immunohistochemistry further indicated that Klotho treat-

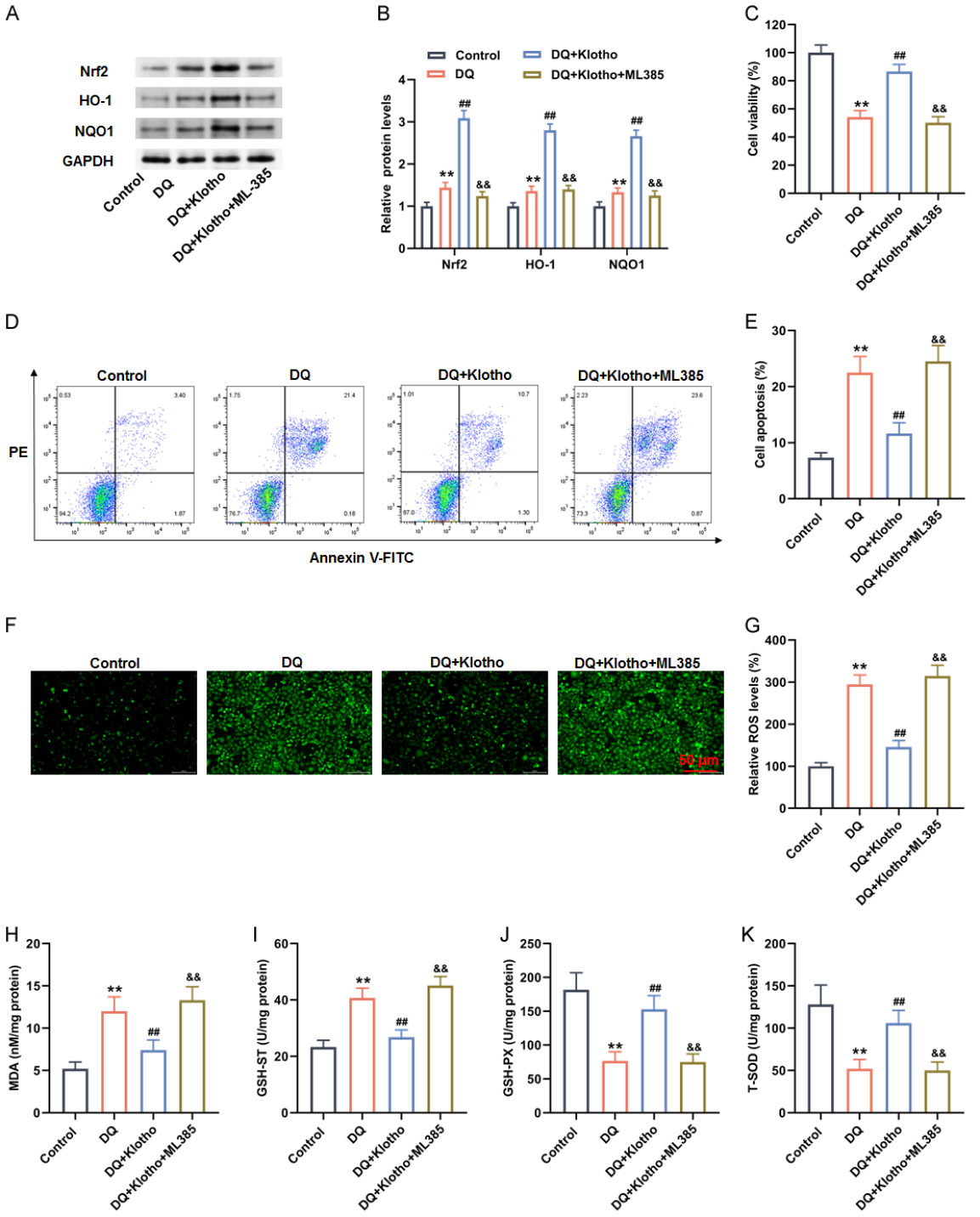

Figure 4. Nrf2 inhibition abrogated the protective effects of Klotho on DQ-induced oxidative stress. DQ-stimulated H9c2 cells received recombinant Klotho protein and Nrf2 inhibitor ML385. (A, B) Nrf2, HO-1, and NQO1 protein levels were examined using western blot analysis. (C) Cell viability was examined using CCK-8 assay. (D, E) Apoptosis was examined using flow cytometry. (F, G) ROS fluorescence signal, (H) MDA level, (I) GSH-ST, (J) GSH-PX, and (K) SOD activities in H9c2 cells were assessed using ELISA kits. Data were presented as mean ± SD. $^{**}P<0.01$, compared to control group. $^{\#\#}P<0.01$, compared to DQ group. $^{\&\&}P<0.01$, compared to DQ+Klotho group.

ment activated the Nrf2/ARE pathway, as evidenced by increased Nrf2, HO-1, and NQO1 expression (**Figure 5J**, **5K**). Collectively, these findings confirm that Klotho alleviates DQ-induced acute myocardial injury in rats through Nrf2/ARE activation.

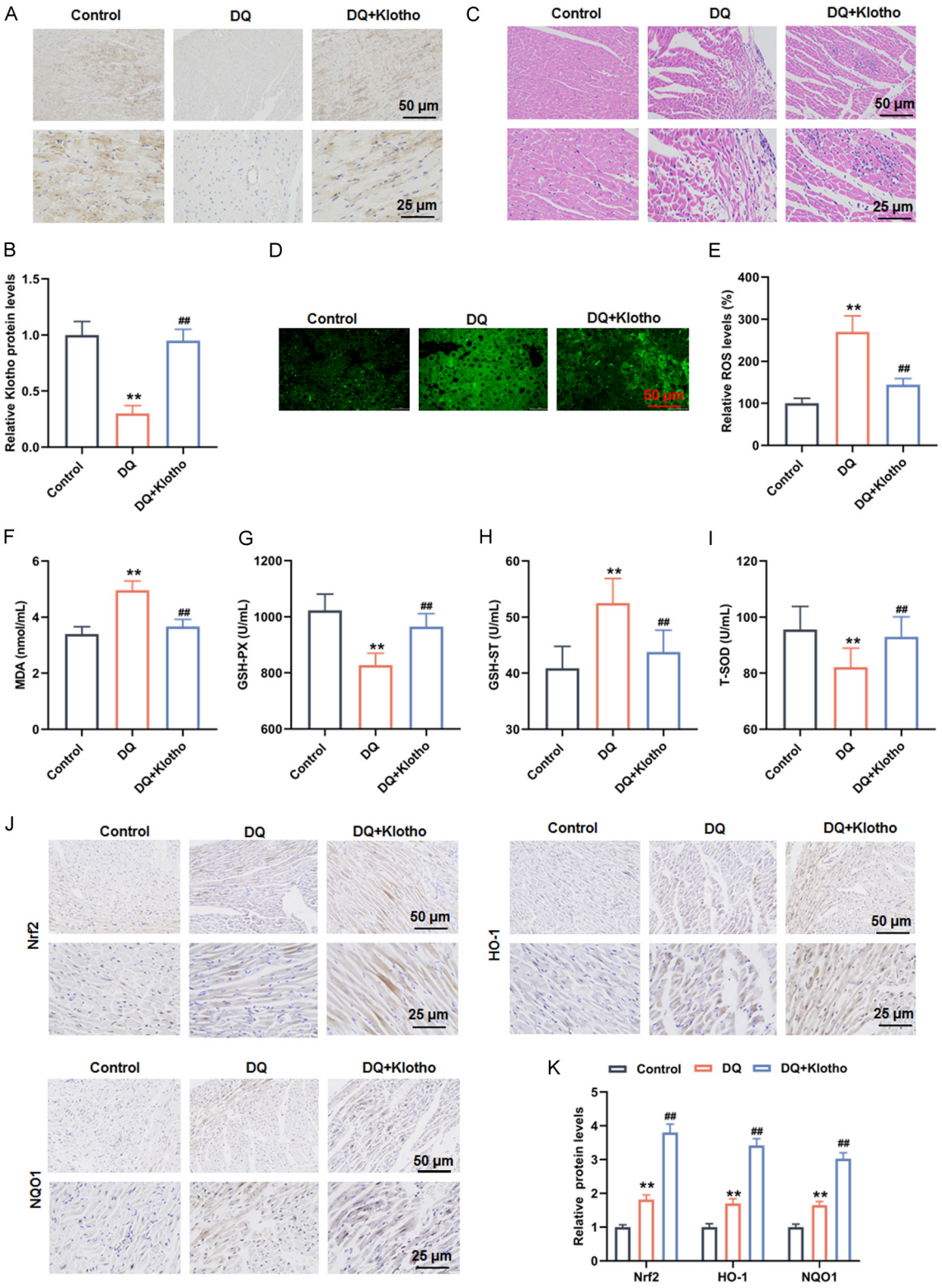

Figure 5. Klotho alleviated DQ-induced acute myocardial injury in rats through Nrf2/ARE activation. Rats were intragastrically administered with DQ to induce acute myocardial injury and treated with recombinant Klotho protein for 5 days. (A, B) Klotho protein expression in myocardial tissues was assessed using immunohistochemistry. (C) H&E staining of myocardial tissue of rats in each group (D, E) ROS fluorescence signal, (F) MDA level, (G) GSH-ST, (H) GSH-PX, and (I) SOD activities in H9c2 cells were assessed using ELISA kits. (J, K) Nrf2, HO-1, and NQO1 protein levels in myocardial tissues was assessed using immunohistochemistry. $^{**}P<0.01$, compared to control group. $^{\#\#}P<0.01$, compared to DQ group.

Discussion

The present study demonstrates that Klotho protein protects against DQ-induced myocardial injury by activating the Nrf2/ARE signaling pathway and suppressing oxidative stress. DQ exposure triggers significant oxidative damage in myocardial tissues, as evidenced by increased ROS and MDA levels, and elevated GSH-ST activity, along with reduced SOD and GSH-PX activities. These changes were associated with Nrf2/ARE pathway activation, suggesting a compensatory antioxidant response. DQ poisoning is known to cause multi-organ injury, with oxidative stress playing a central role in its toxicity [24-26]. Consistent with prior reports, our data confirm that DQ generates excessive ROS via redox cycling, overwhelming the endogenous antioxidant defenses and leading to cellular dysfunction. The observed upregulation of Nrf2, HO-1, and NQO1 in DQ-treated myocardial tissues indicates an adaptive antioxidant defense that is, however, insufficient to fully prevent tissue injury, thereby highlighting the need for therapeutic interventions to enhance antioxidant capacity.

Klotho has emerged as a promising therapeutic candidate for oxidative stress-related disorders [15-17]. Notably, it exhibits potent cardioprotective effects in myocardial (ischemia/reperfusion) I/R by attenuating ROS accumulation and pro-inflammatory cytokine release [27]. Moreover, Klotho supplementation mitigates oxidative damage in myocardial infarction by activating the Nrf2/ARE pathway [18] and decreasing ROS/RNS generation while maintaining metabolic homeostasis in cardiomyocytes [19]. Our results extend these findings by revealing that Klotho attenuates DQ-mediated myocardial damage through Nrf2-mediated antioxidant responses. This conclusion aligns with prior evidence that Klotho enhances Nrf2 nuclear translocation and upregulates its downstream antioxidant targets in both renal and myocardial injury models [15, 18]. Importantly, our rescue experiments using the Nrf2 inhibitor ML385 confirmed that inhibition of Nrf2 abolishes Klotho's cardioprotective effects, thereby establishing the essential role of Nrf2/ARE signaling in mediating this protection.

Of particular relevance, a recent study revealed that paraquat exposure greatly downregulates cardiac Klotho expression in rats [28], which is consistent with our observation that DQ reduces Klotho protein levels in myocardial tissues. Moreover, cardiac-specific Klotho overexpression alleviates paraquat-induced myocardial injury by inhibiting oxidative stress and inflammation through Nrf2/ARE pathway activation [28]. These independent results corroborate our data and collectively establish Klotho's essential protective mechanism against DQ/paraquat cardiotoxicity.

Our findings hold considerable clinical relevance, particularly in the context of DQ poisoning for which no specific antidote is currently available, leaving management largely dependent on supportive care and early decontamination [2]. Our data suggest that recombinant Klotho protein may serve as a therapeutic agent to mitigate DQ-induced cardiac damage by activation of the Nrf2/ARE pathway. This aligns with emerging evidence that Nrf2 activators such as hydroxytyrosol, ellagic acid, and resveratrol effectively reduce DQ toxicity [10-12]. However, further studies are needed to optimize Klotho dosing, evaluate its long-term safety, and explore clinically feasible delivery routes. Moreover, developing indirect therapeutic approaches targeting Klotho/Nrf2 axis represents a promising therapeutic direction.

Several limitations should be acknowledged. First, although our findings establish that Klotho exerts cardioprotection through Nrf2 activation, the potential involvement of other cytoprotective mechanisms, such as autophagy modulation or anti-inflammatory pathways, was not assessed. Future investigations should explore these interactions to provide a more comprehensive understanding of Klotho's multifaceted actions. Second, our findings were derived from rodent models (rats *in vivo* and rat H9c2 cardiomyocytes *in vitro*), which may not fully replicate human cardiac physiology, antioxidant response pathways, or drug metabolism. Therefore, further preclinical validation and translational studies are warranted. Finally, this study did not explore dose-response relationships or optimal therapeutic time windows. Future studies should systematically evaluate alternative delivery strategies, multiple dosing levels, and varied treatment durations to identify the most effective and translatable protocol.

Conclusion

This study elucidated the critical role of Nrf2/ARE signaling in mediating DQ-triggered myocardial injury. Klotho conferred robust cardioprotection by enhancing Nrf2-mediated antioxidant defenses, offering a promising therapeutic strategy for DQ poisoning. These findings advance our understanding of DQ cardiotoxicity mechanisms, and support the development of Klotho-based interventions for oxidative stress-related conditions.

Acknowledgements

This study was supported by Science and Technology Program of Guangzhou (2025-A03J3533).

Disclosure of conflict of interest

None.

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References

[1] Magalhães N, Carvalho F and Dinis-Oliveira RJ. Human and experimental toxicology of diquat poisoning: toxicokinetics, mechanisms of toxicity, clinical features, and treatment. Hum Exp Toxicol 2018; 37: 1131-1160.

[2] Ren Y, Guo F and Wang L. Imaging findings and toxicological mechanisms of nervous system injury caused by diquat. Mol Neurobiol 2024; 61: 9272-9283.

[3] He C, Cai G, Jia Y, Jiang R, Wei X and Tao N. Effect of Diquat on gut health: molecular mechanisms, toxic effects, and protective strategies. Front Pharmacol 2025; 16: 1562182.

[4] Huang Y, Zhang R, Meng M, Chen D and Deng Y. High-dose diquat poisoning: a case report. J Int Med Res 2021; 49: 3000605211026117.

[5] Aloise DM, Memon A and Zaldivar A. Diquat herbicide organophosphate poisoning and multi-organ failure: a case report. Cureus 2022; 14: e27241.

[6] Xing J, Chu Z, Han D, Jiang X, Zang X, Liu Y, Gao S and Sun L. Lethal diquat poisoning manifesting as central pontine myelinolysis and acute kidney injury: a case report and literature review. J Int Med Res 2020; 48: 300060520943824.

[7] Jomova K, Raptova R, Alomar SY, Alwasel SH, Nepovimova E, Kuca K and Valko M. Reactive oxygen species, toxicity, oxidative stress, and antioxidants: chronic diseases and aging. Arch Toxicol 2023; 97: 2499-2574.

[8] Yu C and Xiao JH. The Keap1-Nrf2 system: a mediator between oxidative stress and aging. Oxid Med Cell Longev 2021; 2021: 6635460.

[9] Kaur K, Narang RK and Singh S. Role of Nrf2 in oxidative stress, neuroinflammation and autophagy in Alzheimer's disease: regulation of Nrf2 by different signaling pathways. Curr Mol Med 2025; 25: 372-387.

[10] Han H, Zhong R, Zhang S, Wang M, Wen X, Yi B, Zhao Y, Chen L and Zhang H. Hydroxytyrosol attenuates diquat-induced oxidative stress by activating Nrf2 pathway and modulating colonic microbiota in mice. J Nutr Biochem 2023; 113: 109256.

[11] Zhang X, Wang S, Wu Y, Liu X, Wang J and Han D. Ellagic acid alleviates diquat-induced jejunum oxidative stress in C57BL/6 mice through activating Nrf2 mediated signaling pathway. Nutrients 2022; 14: 1103.

[12] Xun W, Fu Q, Shi L, Cao T, Jiang H and Ma Z. Resveratrol protects intestinal integrity, alleviates intestinal inflammation and oxidative stress by modulating AhR/Nrf2 pathways in weaned piglets challenged with diquat. Int Immunopharmacol 2021; 99: 107989.

[13] Hosseini L, Babaie S, Shahabi P, Fekri K, Shafiee-Kandjani AR, Mafikandi V, Maghsoumi-Norouzabad L and Abolhasanpour N. Klotho: molecular mechanisms and emerging therapeutics in central nervous system diseases. Mol Biol Rep 2024; 51: 913.

[14] Prud'homme GJ, Kurt M and Wang Q. Pathobiology of the klotho antiaging protein and therapeutic considerations. Front Aging 2022; 3: 931331.

[15] Xu Y, You J, Yao J, Hou B, Wang W and Hao Z. Klotho alleviates oxidative stress and mitochondrial dysfunction through the Nrf2/HO-1 pathway, thereby reducing renal senescence induced by calcium oxalate crystals. Urolithiasis 2025; 53: 61.

[16] Zhou P, Zhao C, Chen Y, Liu X, Wu C and Hu Z. Klotho activation of Nrf2 inhibits the ferroptosis signaling pathway to ameliorate sepsis-associated acute kidney injury. Transl Androl Urol 2023; 12: 1871-1884.

[17] Xing L, Guo H, Meng S, Zhu B, Fang J, Huang J, Chen J, Wang Y, Wang L, Yao X and Wang H. Klotho ameliorates diabetic nephropathy by activating Nrf2 signaling pathway in podocytes. Biochem Biophys Res Commun 2021; 534: 450-456.

[18] Xu Z, Zheng S, Feng X, Cai C, Ye X and Liu P. Klotho gene improves oxidative stress injury

after myocardial infarction. Exp Ther Med 2021; 21: 52.
[19] Olejnik A, Banaszkiewicz M, Krzywonos-Zawadzka A and Bil-Lula I. The Klotho protein supports redox balance and metabolic functions of cardiomyocytes during ischemia/reperfusion injury. Cardiol J 2022; 29: 836-849.
[20] Olejnik A, Krzywonos-Zawadzka A, Banaszkiewicz M and Bil-Lula I. Klotho protein contributes to cardioprotection during ischaemia/reperfusion injury. J Cell Mol Med 2020; 24: 6448-6458.
[21] Cui S, Zhang X, Wang C, Sun C, Shi L, Kan B, Li Q and Jian X. Study on the therapeutic effect of glucocorticoids on acute kidney injury in rats exposed to diquat. Biomed Pharmacother 2023; 166: 115310.
[22] Vázquez-Sánchez S, Blasco A, Fernández-Corredoira P, Cantolla P, Mercado-García E, Rodríguez-Sánchez E, González-Lafuente L, Poveda J, González-Moreno D, Matutano A, Peribañez S, García-Consuegra I, Volpe M, Fernández-Velasco M, Ruilope LM and Ruiz-Hurtado G. Recombinant Klotho administration after myocardial infarction reduces ischaemic injury and arrhythmias by blocking intracellular calcium mishandling and CaMKII activation. J Pathol 2025; 265: 342-356.
[23] Liu X, Niu Y, Zhang X, Zhang Y, Yu Y, Huang J, Li J and Yu C. Recombinant α-Klotho protein alleviated acute cardiorenal injury in a mouse model of lipopolysaccharide-induced septic cardiorenal syndrome type 5. Anal Cell Pathol (Amst) 2019; 2019: 5853426.
[24] Wu F, Yang X, Wang F, Liu Y, Han S, Liu S, Zhang Z and Chen B. Dietary curcumin supplementation alleviates diquat-induced oxidative stress in the liver of broilers. Poult Sci 2023; 102: 103132.
[25] Peng WS, Gao M, Yao XF, Tong YY, Zhang HH and He X. Magnolol supplementation alleviates diquat-induced oxidative stress via PI3K-Akt in broiler chickens. Anim Sci J 2023; 94: e13891.
[26] Xu Q, Liu M, Chao X, Zhang C, Yang H, Chen J, Zhao C and Zhou B. Acidifiers attenuate diquat-induced oxidative stress and inflammatory responses by regulating NF-κB/MAPK/COX-2 pathways in IPEC-J2 cells. Antioxidants (Basel) 2022; 11: 2002.
[27] Myung J, Beom JH, Kim JH, Woo JS, Park I, Chung SP, Chung YE and You JS. Recombinant Klotho protein ameliorates myocardial ischemia/reperfusion injury by attenuating sterile inflammation. Biomedicines 2022; 10: 894.
[28] Feng XQ, Deng AP, Wu YQ, Cai CZ, Ye XQ, Liu PF, Huang XJ, Li ZJ and Xu ZF. Cardiac-specific overexpression of Klotho attenuates paraquat-induced myocardial injury by enhancing the Nrf2/ARE signaling pathway. J Cardiovasc Pharmacol 2025; [Epub ahead of print].