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Pyrroloquinoline quinone protects against doxorubicin-induced cardiotoxicity in mice by modulating Nrf2 and NF-kB activities

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ABSTRACT: Pyrroloquinoline quinone (PQQ) is a potent antioxidant cofactor that is widely distributed in the animal and plant tissues and is considered to be an indispensable nutrient in the mammals. In the present investigation, we examined PQQ effects on doxorubicin (DOX)-induced cardiotoxicity in mice. The animals received a single 15-mg/kg intraperitoneal dose of DOX injection at day 1. A daily oral dose of 10 mg/kg PQQ were administered to the mice in the study group. Nuclear factor kappa beta (NF-kB) and nuclear factor erythroid 2related factor 2 (Nrf2) activities were measured in the nuclear fractions of cardiac tissue homogenates. Cardiac histopathology and function, as well as serum markers of cardiac injury and tissue markers of inflammation and oxidative stress were examined. PQQ improved Nrf2 activity and at the same time suppressed NF-kB activity. PQQ reduced cardiac levels of tumor necrosis factor α (TNF-α) and malondialdehyde (MDA), and elevated tissue activities of superoxide dismutase (SOD). PQQ decreased serum levels of creatinine kinase MB (CK-MB) and cardiac troponin I (cTnI), and improved histopathological indices of cardiac injury as well as cardiac function. Altogether, these findings emphasize that PQQ is protective against DOX-induced cardiotoxicity in mice by modulating NF-kB and Nrf2 pathways.

KEYWORDS: DOX cardiotoxicity, PQQ, nuclear factor kappa beta, anti-oxidant, inflammation

INTRODUCTION

Doxorubicin (DOX) is a potent anthracycline compound, and a common anti-cancer drug for the treatment of several types of malignancies, including lymphoma, breast cancer, leukemia, lung cancer, bladder carcinoma, and sarcoma. Toxic adverse effects of anthracyclines particularly nephrotoxicity and cardiotoxicity are considered as the main limiting factors regarding the prescription of this chemotherapeutic agent [1]. Cardiotoxicity typically occurs within the first year of DOX administration. The DOX-induced cardiotoxicity, which is lethal and associated with a worse prognosis, generally appears as subacute dilated cardiomyopathy which can, in long-term, lead to congestive heart failure [2]. The incidence of cardiotoxicity after administration of DOX is dose-dependent occurring in 5–10% of patients under treatment with standard dosages of doxorubicin (400 mg/m^2) to 20% in patients after a cumulative dose of 700 mg/m^2 [3]. The higher rates of cardiotoxicity are generally detectable in elderly patients or patients with pre-existing cardiovascular disorders [4].

Several potential mechanisms have been identified for DOX cardiotoxicity including DNA and mitochondrial damage, oxidative stress, dysregulation of autophagy, necrosis, apoptosis, and iron overload (ferroptosis) [1]. The accumulation of DOX in the heart occurs through binding to cardiolipin in the inner membrane of mitochondria hindering the plasma clearance of this agent which may explain why the cardiomyocytes are so susceptible to DOX. It is evident that a multitude of signaling pathways and proteins are modulated and affected by DOX and the cardiotoxicity is not attributable to one single parameter [5]. Of note, around 50% of the cardiomyocyte volume is occupied by the mitochondria which are vital for energy generation. Mitochondrial dysfunction, mitochondrial iron accumulation, and oxidative stress derived from mitochondria have been characterized as the possible causative mechanisms. As a possible explanation for underlying mechanisms involved in DOX-induced cardiotoxicity, in recent years the role of mitochondrial iron overload and ferroptosis has been noticed the most [6].

Pyrroloquinoline quinone (PQQ) is an anionic water-soluble redox compound, first characterized as a redox cofactor, with anti-inflammatory, anti-oxidative, antiosteoporotic, neuroprotective, and hepatoprotective activities [7, 8]. Previous studies have indicated that PQQ possesses free radical scavenging, antioxidative, growth-stimulating, neuroprotective, antiproliferative, and hypolipidemic properties. Human milk is reported as a major source of PQQ which can be considered as a dietary therapy for the prevention of oxidative stress, mitochondrial dysfunction, and toxic lipids-induced inflammation [9]. In recent years, the protective effects of PQQ against cyclophosphamide-induced

nephrotoxicity and hepatotoxicity have been reported by several studies [9–11]. The protective effects of PQQ against cyclophosphamide-induced nephrotoxicity and hepatotoxicity have been attributed to the activation of Nrf2 pathway and the up-regulation of anti-oxidant proteins [9, 10]. It has also been reported that PQQ can attenuate DOX-induced autophagy-dependent apoptosis in the vascular endothelial cells through lysosomal mitochondrial axis [12]. In addition, several studies have demonstrated the protective effects of PQQ against neurotoxicity [13].

The primary objective of this study is to investigate the potential protective effects of PQQ against DOX-induced cardiotoxicity. Recognizing the pivotal involvement of inflammation and oxidative stress in the pathogenesis of DOX-induced cardiac injury, our hypothesis posits that PQQ may serve as a protective agent in mitigating the adverse effects associated with DOX administration. To rigorously test this hypothesis, we conducted an experimental study utilizing a murine model of DOX-induced cardiotoxicity. Our focus was on evaluating various indices of cardiac injury, both in the systemic circulation through serum biomarkers and within the cardiac tissues themselves. By elucidating the potential cardioprotective properties of PQQ, this research aims to contribute valuable insights into novel therapeutic strategies for preventing or ameliorating the cardiotoxic effects associated with DOX, thereby offering potential benefits for individuals undergoing chemotherapy.

MATERIALS AND METHODS

Materials

PQQ and doxorubicin were obtained from the Shaanxi Iknow Biotechnology (China) and United Pharm (South Korea), respectively.

Experiment design

Twenty-four male C57BL/6 mice of 8–10 weeks old, weight 20–22 g, were included in the study. The experiment was performed with adherence to the principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and the experiment process was approved by Chinese PLA General Hospital (Ethical Code: 20220412). The mice were allocated to three groups of eight per each. The first group was the healthy controls. The remaining were injected a single intraperitoneal dose of DOX (15 mg/kg). The treatment group received daily oral doses of PQQ at a dosage of 10 mg/kg [14]. The experiment duration was 7 days. Ultimately, the anesthesia was done by ketamine (50 mg/kg) and midazolam (1 mg/kg). Blood samples were obtained directly from the heart and the hearts were excised for histopathologic evaluation.

Markers of oxidative stress and inflammation

To test for the markers of oxidative stress and inflammation, the cardiac tissue samples were homogenized in the phosphate buffered saline (PBS) solution (0.1 M, PH 7.4) and centrifuged at 5000g for 15 min at 4 °C. The supernatant was separated for the measurement of the superoxide dismutase (SOD) and activity and malondialdehyde (MDA) concentration. The quantitations were conducted using the SOD and MDA colorimetric kits (Solarbio, China). Tumor necrosis factor alpha (TNF-α) was measured by using a commercial ELISA kit (Fine Test, China).

NF-kB and Nrf2 activity assays

To measure NF-kB and Nrf2 activities in the cardiac tissue homogenates, the NF-kB (p65) Transcription Factor Assay Kit and Nrf2 Transcription Factor Assay Kit (Cayman Chemical, Missouri, USA) were implemented, respectively. Firstly, the nuclear fractions were separated from the tissue homogenates by a nuclear extraction kit (Abcam, Cambridge, UK), and protein contents were measured by Lowry-Folin-Ciocalteu method. Protein measurements were carried out in order to add equal amounts of protein (i.e. 20 µg) into each well of the kit. The microplate wells were coated with the correspondent dsDNAs capable of binding specifically to the phosphorylated p65 NF-kB and Nrf2 proteins. The absorbance was read at 450 nm, and the degree of light absorbance was regarded as the estimate of the NF-kB and Nrf2 activities.

Serum markers of cardiac injury

Creatine kinase isoform MB (CK-MB) and cardiac troponin I (cTnI) were measured as the serum markers of cardiac injury. CK-MB was measured a colorimetric assay kit (Pars Azmoon, Iran), and (cTnI) was assayed with an enzyme-linked immunosorbent assay (ELISA) kit (Fine Test).

Heart histopathology

Specimens obtained from heart tissue were fixed in 10% neutral buffered formalin (NBF), processed in paraffin cubes, and 5-µm thickness sections were obtained. After staining with the Hematoxylin and Eosin (H&E), the slides were examined using a light microscope (Kern, Germany) according to the published protocols [15].

Heart function

To assess the heart function, firstly, the coronary (Qe) and aortic (Qa) flow rates (ml/min), and aortic pressure (PAO, mmHg), as well as the peak systolic pressure (PSP) and the heart rate (HR) were measured by using the Viggo-Spectramed devise (Viggo-spectramed limited, Windlesham, England) Then, the cardiac output and stroke volume were calculated through the following equation: Cardiac output (CO) (ml/min) =

(Qe + Qa); Stroke volume (SV) (ml/min) = (CO/HR) [16]. The cardiac function tests were conducted on the 7th day of the study, immediately preceding tissue samplings.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from heart tissues using TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA), and cDNA was synthesized using a reverse transcription kit (InterLabServices, Moscow, Russia) as per the provided instructions. Real-time PCR was conducted on Mic thermocycler (BioMolecular Systems, Upper Coomera, Australia) using SYBR Premix Ex Taq II, with GAPDH serving as the internal control. The relative expression of each target gene was calculated by the $2^{-\Delta\Delta CT}$ method [17]. The primers employed are shown in Table S1.

Statistical analyses

All values are presented as mean ± standard deviation (SD). Obtained data were analyzed by using the SPSS, version 24. To examine the statistical significance between the groups, the Kruskal-Wallis (KW) test followed by the Dunn-Bonferroni post hoc test was used; p values < 0.05 were considered significant.

RESULTS

PQQ alleviated oxidative stress in the cardiac tissues

To evaluate the oxidative stress status within cardiac tissues, we examined two key indicators of oxidative stress, namely MDA and SOD, in the homogenized heart tissue samples. In sham mice, MDA levels were recorded at 55.2 ± 7.4 nmol/g. This value notably rose to 135.4 ± 13.6 nmol/g in mice subjected to DOX-induced cardiotoxicity, but significantly decreased to 93.8 ± 9.2 nmol/g in those treated with PQQ following DOX administration ($p < 0.05$). Conversely, SOD activities exhibited a decline in the hearts of sham mice, decreasing from 109.4 ± 5.7 U/g to 59.2 ± 8.3 U/g in the DOX group. Notably, treatment with PQQ led to a significant increase in SOD levels, reaching 80.4 ± 6.9 U/g compared to the control DOX group ($p < 0.05$) (Table 1).

Nrf2 activities within the nuclear fractions of cardiac tissues were measured at 2.10 ± 0.15 and 1.05 ± 0.18 in sham and DOX mice, respectively. However, following treatment with 10 mg/kg PQQ, there was a significant increase to 1.42 ± 0.22 compared to the levels observed in DOX mice (Table 1). Additionally, we assessed the gene expressions of Nrf2 target genes, namely NAD(P)H quinone oxidoreductase 1 (*NQO1*), heme oxygenase 1 (*HMOX1*), and glutamate-cysteine ligase (*GCL*) in cardiac tissues. Expressions of *NQO1*, *HMOX1*, and *GCL* were found to be decreased in DOX mice, and notably, PQQ treatment significantly elevated their expressions (Fig. 1).

PQQ attenuated inflammation in the heart tissues

We examined two parameters to assess the inflammatory status within cardiac tissues. Initially, we measured TNF-α levels, a pivotal inflammatory mediator. In normal control mice, TNF-α levels registered at 188 ± 10.3 pg/ml. However, in control DOX mice, these levels escalated to 361 ± 11.2 pg/ml, and PQQ treatment significantly attenuated TNF-α levels to 274 ± 8.7 pg/ml in DOX mice ($p < 0.05$). Additionally, the light absorbance of p65 NF-kB at 450 nm was 0.53 ± 0.25 in normal mice, contrasting with 1.82 ± 0.36 in DOX mice. Notably, this value significantly decreased to 1.16 ± 0.28 after treatment with PQQ ($p < 0.05$) (Table 1). Furthermore, our analyses revealed a notable increase in the gene expression of key inflammatory cytokines, including TNF-α, interleukin 1 (IL-1), and IL-6, in the cardiac tissues of DOX mice. Remarkably, PQQ treatment significantly mitigated the expression of these aforementioned genes (Fig. 1).

PQQ decreased serum markers of cardiac injury

We noticed increased serum levels of both CK-MB and cTnI in the cardiac tissues of DOX mice compared with normal control mice ($p < 0.05$) (Table 1). According to our findings, CK-MB levels decreased from 21.33 ± 2.74 U/l to 14.29 ± 2.14 U/l in PQQ receiving mice. Similarly, cTnI levels were reduced from 7.35 ± 1.22 ng/ml to 4.22 ± 0.95 ng/ml in DOX mice treated with PQQ ($p < 0.05$).

PQQ improved cardiac histoarchitecture and cardiac function

We observed profound tissue deterioration in the cardiac sections of DOX mice as evidenced by myofibrillar loss, inflammatory cell infiltration, cell vacuolization, and necrosis (Fig. 2). It was found that treatment with 10 mg/kg PQQ significantly preserved cardiac architecture and protected the hearts against the detrimental toxic effects of DOX.

According to our findings, both cardiac output (CO) and stroke volume (SV) were decreased from 71.3 ± 6.1 ml/min and 2.18 ± 0.31 ml/beat in normal control mice to 43.5 ± 4.9 ml/min and 1.69 ± 0.24 ml/beat in DOX group, respectively ($p < 0.05$). Treatment with 10 mg/kg PQQ significantly increased CO as well as SV values to 55.8 ± 5.4 ml/min and 1.95 ± 0.27 ml/beat, respectively, compared with the DOX group ($p < 0.05$) (Table 1).

DISCUSSION

PQQ is an aromatic water-soluble quinone which serves as a cofactor for dehydrogenase enzymes. Moreover, PQQ possesses free radical scavenging properties,

Table 1 List of measured parameters in the cardiac tissues of mice.

	Sham (N = 8)	DOX (N = 8)	DOX + PQQ (N = 8)
MDA (nmol/g tissue)	55.2 ± 7.4	135.4 ± 13.6[a]	93.8 ± 9.2[a,b]
SOD (U/g tissue)	109.4 ± 5.7	59.2 ± 8.3[a]	80.4 ± 6.9[a,b]
TNF-α (pg/ml)	188 ± 10.3	361 ± 11.2[a]	274 ± 8.7[a,b]
NF-kB p65 (A_{450})	0.53 ± 0.25	1.82 ± 0.36[a]	1.16 ± 0.28[a,b]
Nrf2 (A_{450})	2.10 ± 0.15	1.05 ± 0.18[a]	1.42 ± 0.22[a,b]
CK-MB (U/l)	6.45 ± 1.89	21.33 ± 2.74[a]	14.29 ± 2.14[a,b]
cTnI (ng/ml)	2.25 ± 0.75	7.35 ± 1.22[a]	4.22 ± 0.95[a,b]
Cardiac output (ml/min)	71.3 ± 6.1	43.5 ± 4.9[a]	55.8 ± 5.4[a,b]
Stroke volume (ml/beat)	2.18 ± 0.31	1.69 ± 0.24[a]	1.95 ± 0.27[a,b]

Sham, healthy control mice; DOX, DOX-induced cardiotoxic control mice; DOX + PQQ, DOX-induced cardiotoxic mice treated with 10 mg/kg PQQ. Values are expressed as mean ± SD. CK-MB, creatine kinase MB; cTnI, cardiac troponin I; DOX, doxorubicin, GPX, glutathione peroxidase; MDA, malondialdehyde; NF-kB, nuclear factor kappa beta; Nrf2, nuclear factor erythroid 2 (NFE2)-related factor 2; SOD, superoxide dismutase, TNF-α, tumor necrosis factor α.
[a] $p < 0.05$ vs. Control; [b] $p < 0.05$ vs. DOX.

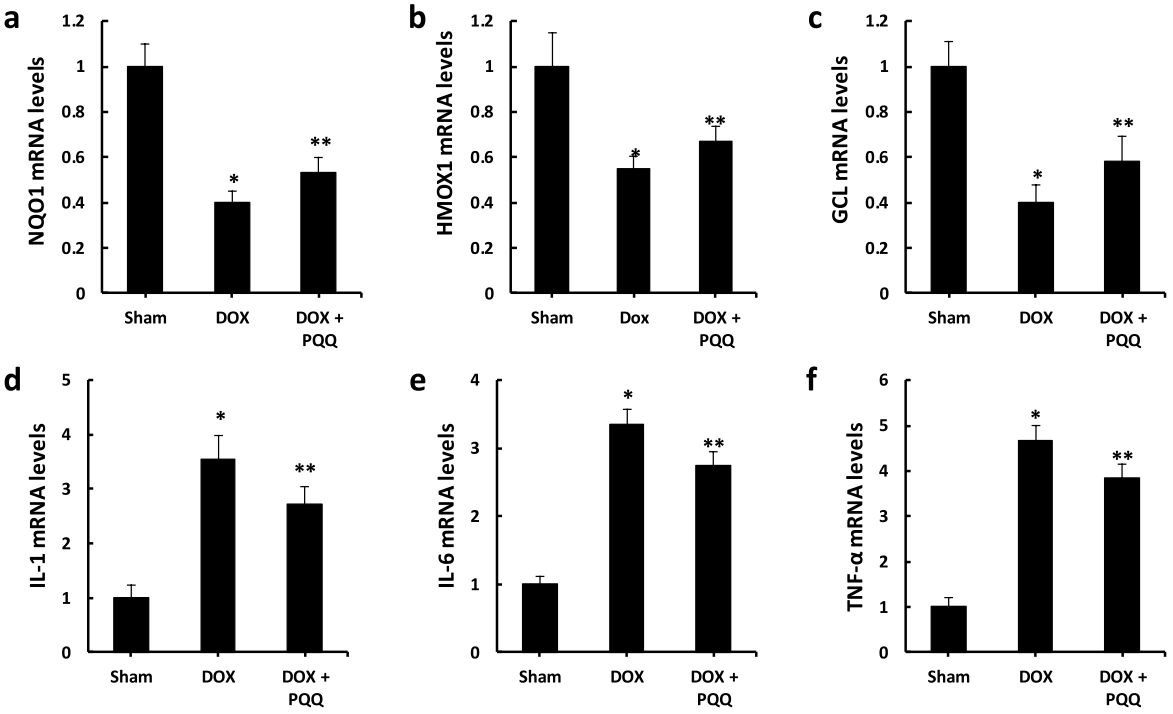

Fig. 1 PQQ effects on the expression of antioxidant genes (a to c) and inflammatory genes (d to f) in heart tissue. DOX suppresses the expression of antioxidant genes *NQO1*, *HMOX1*, and *GCL*, whereas PQQ augments their expression. Conversely, DOX upregulates the expression of inflammatory genes *IL-1*, *IL-6*, and *TNF-α*, while PQQ significantly decreases their expression. (a) *NQO1*, NAD(P)H quinone oxidoreductase 1; (b) *HMOX1*, heme oxygenase 1; (c) *GCL*, glutamate-cysteine ligase; (d) *IL-1*, interleukin 1; (e) *IL-6*, interleukin 6; (f) *TNF-α*, tumor necrosis factor alpha. * $p < 0.05$ vs. Control; ** $p < 0.05$ vs. DOX.

and serves as a catalyst for amine oxidases [18]. Apart from its protective effects against oxidative stress mediated cell damage, PQQ is able to relieve oxidative stress induced changes in cell morphology and mobility [19]. Protective effects of PQQ against DOX-induced toxicity was first proved by Jiang et al[12] who conducted an *in vitro* cell culture study and reported that PQQ attenuated autophagy-induced apoptosis in cultured cardiomyocytes. PQQ is chemically categorized as a vitamin B compound, therefore PQQ is a clinically safe and tolerable agent and a recent clinical trial reported that the daily dose of 20 mg/kg PQQ for 12 weeks improved brain function and cognition in adults above 45 years' age, without any side effects [20].

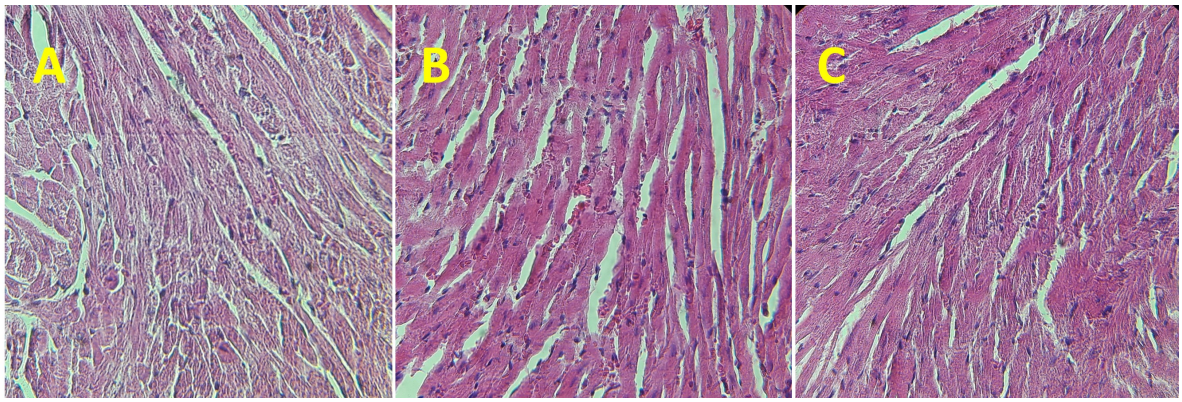

Fig. 2 PQQ effects on heart histoarchitecture (400×). Micrographs illustrate the hematoxylin and eosin stained sections of cardiac tissue. Sham mice (N = 8) (A), DOX-induced cardiotoxic control mice (N = 8) (B), and DOX-induced cardiotoxic mice treated with 10 mg/kg PQQ (N = 8) (C). (A) normal heart histology. (B) myofibrillar loss, necrotic foci, and infiltration of inflammatory cells. (C) PQQ (10 mg/kg) improves DOX-induced pathological.

Nrf2 belongs to the cap 'n' collar (CNC) family in the category of basic region leucine zipper (bZip) transcription factors. It is now known that Nrf2 is not essential for the differentiation of the blood cells, rather, it induces the expression of a group anti-oxidant enzymes including SOD and NQO1 [21]. Increased levels of reactive oxygen specieic (ROS) mediates the translocation of Nrf2 from cytosol to the nucleus; then, Nrf2 binds to a common DNA sequence termed antioxidant response element (ARE), initiating the expression of an array of antioxidant enzymes [22]. Despite being a potent chemotherapeutic agent, cardiotoxicity is the major sequela of DOX, limiting its clinical utility. DOX causes a strong oxidative stress in cardiomyocytes, leading to cardiac cell death due to apoptosis and ferroptosis [23, 24]. Here, we found that PQQ alleviated oxidative stress in cardiac tissues as evidenced by decreased levels of MDA, increased activities of SOD as well as increased nuclear presence of Nrf2. Similarly, it has previously been reported that PQQ preserves mitochondrial membrane integrity and suppresses oxidative stress in cultured cardiomyocytes [25]. Moreover, similar experimental studies examining the effects of natural antioxidant agents like curcumin, resveratrol, and quercetin have all reported decreased levels of ROS and elevated levels of antioxidant enzymes in the cardiac tissues of animals [26–28]. It has also been shown that PQQ is a potent activator of sirtuin 3 (SIRT3), another antioxidant protein [29].

PQQ has been proposed to enhance mitochondrial function and stimulate biogenesis. Improved mitochondrial function can reduce the production of ROS, which may indirectly influence Nrf2 activation [30]. PQQ has been shown to activate AMPK, a key regulator of cellular energy homeostasis. Activated AMPK can modulate Nrf2 signaling by influencing the redox status of the cell and promoting antioxidant defenses [31]. Mitogen-Activated Protein Kinases (MAPKs) are signaling proteins that play a role in cellular response to stress. PQQ has been suggested to modulate MAPK signaling, and these pathways can intersect with Nrf2 activation [32]. The phosphoinositide 3-kinase (PI3K)/Akt pathway is involved in cell survival and growth. PQQ has been shown to activate Akt, and this pathway may intersect with Nrf2 signaling to regulate cellular responses to oxidative stress [33].

NF-kB is a member of a group of inducible transcription factors that regulates a large set of genes implicated in diverse processes of the inflammation as well as innate immune response [34]. The effector component of NF-kB is the p65 subunit which is normally sequestered in cytoplasm by binding to the protein called inhibitor of kappa beta (IKK). However, upon activation by various cell stressors, p65 is released and then translocated into the nucleus to bind to its correspondent response element on DNA [35]. Thereby, the expression of an array of pro-inflammatory cytokines including TNF-α, interleukin 1 beta (IL-1β) and IL-6 is induced to stimulate inflammation and the activation and aggregation of the immune cells [36]. According to our findings, PQQ is effective in suppressing inflammation in cardiac tissues as it significantly reduced TNF-α levels and NF-kB activities in the nuclear fractions of DOX mice. The pivotal role of NF-kB activation in DOX-induced cardiotoxic states has long been known [37].

Attenuation of oxidative stress and inflammation of cardiac tissues means nothing, unless it is corroborated by the demonstration of reduced cardiomyocyte damage and improved cardiac function. Therefore, we examined serum markers of cardiac damage (CK-MB and cTnI), cardiac histopathology as well as cardiac function (cardiac output and stroke volume). While 10 mg/kg PQQ decreased both CK-MB and cTnI levels

in the serum of DOX mice, it significantly improved cardiac histoarchitecture as evidenced in H&E stained cardiac sections. Moreover, PQQ improved both cardiac output and stroke volume in DOX mice, proving that PQQ not only alleviates cardiac injury, but also improves cardiac function. In line with these findings, an *in vitro* study conducted on hypoxia-induced cardiomyocytes damage have reported that curcumin-PQQ mixture prevented cardiomyocyte damage by relieving mitochondrial stress [38].

It is noteworthy to acknowledge a potential limitation in our study concerning the restoration of gene expression changes by PQQ treatment following DOX-induced alterations. While our findings suggest a trend towards restoration, it is crucial to recognize that the observed fold changes were subtle. It is conceivable that these subtle alterations may be attributed to technical variations or individual fluctuations inherent in the experimental setup. Therefore, although our data tentatively supports the claim that PQQ treatment could influence the restoration of gene expressions altered by DOX, it is imperative to approach this conclusion with caution. The statistical analysis conducted revealed significant differences among the study groups, indicating the need for prudence in asserting the extent of gene expression restoration. Future studies incorporating larger sample sizes and employing complementary methodologies could provide a more comprehensive understanding of the nuanced effects of PQQ on gene expression in the context of DOX-induced changes.

In conclusion, PQQ is protective against DOX-induced cardiotoxicity in mice via suppressing NF-kB activity and inducing Nrf2 activity, and reduced serum levels of CK-MB and cTnI, besides improved cardiac histoarchitecture and cardiac function, corroborate the protective role of PQQ.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.080.

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Appendix A. Supplementary data

Table S1 The list of primers.

Gene name	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
NQO1	GCCGAACACAAGAAGCTGGAAG	GGCAAATCCTGCTACGAGCACT
HMOX1	CACTCTGGAGATGACACCTGAG	GTGTTCCTCTGTCAGCATCACC
GCL	TCCTGCTGTGTGATGCCACCAG	GCTTCCTGGAAACTTGCCTCAG
IL-1	ACGGCTGAGTTTCAGTGAGACC	CACTCTGGTAGGTGTAAGGTGC
IL-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
GAPDH	CGGAGTCAACGGATTTGGTCGTA	AGCCTTCTCCATGGTGGTGAAGA

NQO1, NAD(P)H quinone oxidoreductase 1; HMOX1, heme oxygenase 1; Glutamate-cysteine ligase; IL-1, interleukin 1; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.