POTENTIAL PROTECTIVE ROLE OF QUERCETIN AGAINST CHRONIC DOXOMBICIN-INDUCED CARDIOTOXICITY IN RATS

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ABSTRACT:

Doxorubicin (DOX) is an anthracycline antibiotic that is used as a potent anticancer agent. However, the clinical use of DOX is limited by its acute and chronic cardiotoxicity. It was found that generation of reactive oxygen species (ROS) represents the main mechanism that underlines its cardiotoxicity. Moreover, other studies showed a possible contribution of the proinflammatory cytokine, tumor necrosis factor-α (TNF-α) in DOX-induced cardiotoxicity. In this setting, our study was conducted in a trial to protect the heart against DOX-induced deleterious effects by using the natural antioxidant falvonoid, quercetin (QRN). Male albino rats were intraperitoneally injected with DOX (2.5 mg/kg/48 h for six consecutive doses for two weeks) to induce cardiotoxicity. Concurrent administration of QRN (10 mg/kg/day, orally) was started at the same day of DOX administration and continued for further 4 weeks after stopping of DOX (6 weeks total period).

The results revealed that chronic DOX administration induced an elevation in serum levels of the inflammatory mediator, TNF-α, creatine kinase (CK-MB) and lactate dehydrogenase (LDH). Also, a decline in myocardial antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) was reported. In addition, DOX treatment resulted in an increase in myocardial levels of malondialdehyde (MDA) and nitric oxide (NO), as markers for oxidative stress. The histopathological examination of cardiac tissues treated with DOX showed leukocyte infiltration, intermuscular hemorrhage and focal necrosis. On the other hand, administration of QRN for 6 weeks resulted in a decline in serum TNF-α, CK-MB and LDH levels and in myocardial MDA and NO levels compared to DOX group. Moreover, QRN resulted in a significant elevation of myocardial antioxidant enzymes. The histopathological examination revealed that treatment with QRN ameliorated the effects of DOX on cardiac tissues with minimal signs of leukocyte infiltration and necrosis. Our results suggest that QRN confers a cardioprotective effect against DOX-induced cardiotoxicity via both its antioxidant and ant-inflammatory activities.

KEYWORDS:

Cardiotoxicty Doxorubicin Quercetin
Oxidative stress Inflammation.

INTRODUCTION:

Doxorubicin (DOX) is used as an efficacious antineoplastic agent for many haemopoietic and solid cancers. Despite being used for more than 30 years; DOX continues to be considered as a first line antineoplastic drug (Mordente et al., 2001). Unfortunately, its clinical usefulness is limited by the development of a cumulative dose-dependent cardiotoxicity (Jain, 2000; Singal et al., 1998). Acute effects can occur immediately after treatment and are characterized by transient arrhythmias, reversible hypotension and pericarditis (Jain, 2000) while, chronic cardiotoxicity can manifest years to decades after treatment. It is irreve-

rsible and dose dependent cardiot-oxicity that is characterized by progressive left ventricular dysfunction and may lead to congestive heart failure (Corna et al., 2004). Several reports have shown that generation of free radicals and increased oxidative stress are the main pathways involved in DOX cardiotoxicity (Tokarska et al., 2006). On the other hand, a strong association between oxidative stress and cardiac inflammatory response including cytokine release after DOX treatment was studied (Bien et al., 2007). One of the proinflammatory cytokines involved, which mediate cardiac damage, is tumor necrosis factor-α (TNF-α). Previous studies have shown that DOX may lead to an increase in cardiac TNF-α expression (Riad et al., 2009).

Quercetin (QRN) is one of the most abundant natural flavonoids, present in a large number of fruits and vegetables (Hertog et al., 1996). There has been an upsurge of interest to explore the cardioprotective potential of such natural product (Yogeeta et al., 2006). Previous in vitro and in vivo studies have focused on the antioxidant potential of QRN (Ahn et al., 2008; Annapurna et al., 2009). Animal evidence suggests the antioxidant effects of quercetin that could afford protection of the brain, heart, and other tissues against ischemia-reperfusion injury, toxic compounds, and other factors that can induce oxidative stress. The antioxidant properties of QRN might be due to its ability to chelate transition metal ions, such as Fe^{2+} and Cu^{2+} and scavenge free radicals (Iqbal et al., 2008). Since that the increased free radical and cytokine production may be major mechanisms of DOX-induced myocardial damage therefore, the therapeutic intervention that could impair inflammatory responses or diminish free radical production has been of great interest.

This encouraged us to conduct the present study in order to evaluate the protective effect of QRN in rats exposed to chronic DOX-induced cardiotoxicity. Also, the possible mechanisms underlying this effect were investigated.

MATERIALS AND METHODS:

Doxorubicin hydrochloride was purchased from Pharmacia Italia, S.P.A., Italy, Quercetin powder was purchased from Sigma-Aldrish (St. Louis, MO, USA). Other chemicals were purchased from Al-Gomhorria and El-Motaheda, Cairo, Egypt. Quercetin was suspended in saline solution 0.9% NaCl.

Animals and experimental design

Adult male albino rats weighing 190-220 g were used and obtained from the animal house, Faculty of Agriculture, Minia Unive-rsity. The animals were kept in stand-ard conditions (24±1°C and 12h light/dark cycle). They were supplied with standard laboratory chow and water ad libitum, and left to acclima-tize for two weeks before experiments. Rats were randomly divided into 4 groups:

1- Control group (8 rats): Normal healthy rats were orally given 0.5 ml saline solution (0.9% NaCl) for 6 weeks.

2- QRN-treated group (8 rats): Normal healthy rats were orally treated with QRN (10 mg/kg/day) for 6 weeks (Haleagrahara et al., 2009).

3- DOX- treated group (15 rats): Rats were injected with DOX in a dose of 2.5mg/kg, i.p in six equal injections every 48h intervals for two consecutive weeks to achieve accumulative dose of 15 mg/kg (Herman and Ferrans, 1998; Ibrahim et al., 2009).

4- DOX + QRN- treated group (15 rats): Rats were concurrently treated with QRN (10 mg/kg/day, orally) and DOX (2.5 mg/kg/48 h, i.p) injection. Administration of QRN was started at the same day of DOX administration and continued for further 4 weeks after stopping of DOX (6 weeks total period).

Serum and tissue sampling

Twenty four hours following the last doses of the treated drugs, rats were sacrificed by decapitation. A blood sample of each animal was collected into a dry centrifuge tube. Serum and heart tissues were collected and stored at -80°C till the time of analysis. Serum was separated by centrifugation at 8000 x g for 10 minutes and used for determination of creatine kinase (CK-MB), lactate dehydrogenase (LDH) and TNF-α serum levels. Hearts were rapidly exposed and excised, washed in cold phosphate buffered solution, blotted dry on a filter paper and weighed. Heart weight index was calculated according to the formula: (heart weight/body weight) ×100 (Sampaio et al., 2002). Immediately, the hearts were flash-frozen in liquid nitrogen and kept frozen at - 80°C until the time of analysis.

Determination of serum TNF-α, CK-MB and LDH levels.

Serum TNF-α level was assessed in this study using enzyme-linked immunosorbent assay (ELISA) using a microplate reader (Spectra 111, Austria) as previously described (Beutler & Cerami, 1987). CK-MB was determined according to previously described method (Morin, 1977) using diagnostic kit (Stanbio laboratory, Texas, U.S.A). The increase in absorbance at 340nm is measured spectrophotometrically to calculate CK-MB level as U/L.

LDH activity was determined according to previously described method (Rotenberg et al., 1988) using diagnostic kit provided from Biogamma (Rome-Italy). The increase in absorbance is measured spectrophotometrically at 340 nm at 1 min intervals for 3 min. Serum total LDH activity was calculated as U/L.

Estimation of myocardial antioxidant enzyme activities and lipid peroxidation level

Myocardial superoxide dismutase (SOD) activity was determined using a comercially available kit (Biodiagnostic, Egypt) based on a previously described colorimetric method (Nishikimi et al., 1972).

The activity of catalase (CAT) enzyme in myocardial tissue homogenates was determined by a colorimetric method (Aebi et al., 1984) using a commercially available kit (Biodiagnostic, Egypt).

Lipid peroxidation was determined in heart tissue homogenates in the form of thiobarbituric acid reactive species (TBARS; sometimes referred to as malondialdehyde, MDA). Lipid peroxidation products as MDA react with thiobarbituric acid to form a pink colored adduct. The color intensity is measured spectrophotometrically at 532 nm. Concentration of TBARS was calculated for each sample after reference to the standard curve (Buege and Aust. 1978). Total protein concentration was determined by a colorimetric method (Lowry et al., 1951) using commercially available kit, following the instructions of the manufacturer (Biodiagnostic, Egypt).

Assessment of myocardial total nitrate/nitrite concentration

Nitrate and nitrite are assayed colorimetrically as indicators of NO in

the tissue. The total of nitrate/nitrite in the samples was assayed as nitrite after reduction of nitrate into nitrite using the cadmium reduction method. Then the total nitrite is then measured by employment the Griess reaction (Tsikas, 2007).

Histopathological examination of the cardiac tissues

Heart samples were taken, fixed with 10% (v/v) formaldehyde, embedded in paraffin wax and stained with hematoxylin and eosin (H&E) for assessment of histopathological changes.

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM) and were analyzed for statistically significant differences using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post analysis test to compare all groups.

RESULTS:

1. Effect on the heart weight and heart weight index

The effects of DOX on the heart weight and heart weight index and their alterations by QRN treatment are shown in Table 1. DOX treated group showed significant increase in the heart weight and the heart weight index when compared to control group. Administration of QRN for 6 weeks resulted in significant protection against DOX-induced elevation of the heart weight and heart weight index when compared to DOX-treated group.

2. Effect on serum TNF-α, CK-MB and LDH levels

DOX produced a significant increase in serum TNF-α level, while concurrent treatment with QRN for 6 weeks inhibited this change as shown in Fig. 1.

Administration of DOX caused a significant increase in serum levels of both LDH and CK-MB as compared with control group. The combined administration of QRN and DOX resulted in a significant decline in serum LDH and CK-MB levels compared with their respective DOX-treated rats as shown in Fig. 2 (A and B).

3. Effect on myocardial antioxidant enzyme activity (myocardial levels of SOD and CAT)

Rats treated with DOX exhibited a marked decline in myocardial antioxidant activity. This effect was evident by the significant decrease in myocardial SOD and CAT activities as compared with control group. Concurrent administration of QRN and DOX resulted in a significant increase in the activities of SOD and CAT compared with their respective DOX-treated rats as shown in Fig. 3 (A and B).

4. Effect on myocardial lipid peroxidation (MDA) and total nitrite levels

Chronic DOX administration induced a significant increase in myocardial MDA and nitric oxide contents as compared with control group. Compared to DOX treated group, administration of QRN resulted in a significant decline in myocardial MDA and nitric oxide levels as shown in Fig. 4 (A and B).

5- Histopathological changes

Histopathological analysis of the heart muscle showed leukocyte infiltration, intramuscular hemorrhage and focal necrosis in the cardiac tissues of DOX-treated group (Fig. 5 C and D). However, administration of QRN in combination with DOX significantly attenuated the extent and severity of the histological features of the damaging effect of DOX in the cardiac tissues (Fig. 5 E).

Table 1: Effect of DOX administration (2.5 mg/kg/48h x 6 doses, i.p) on body weight, heart weight and heart weight index and their modulations by the concurrent treatment with QRN (10 mg/kg/day, orally for 6 weeks).

Groups	Body weight (g)	Heart weight (g)	Heart weight index (g/g %)
Control	226 ± 0. 9	0.69 ± 0.026	0.305 ± 0.028
QRN	229 ± 1. 17	0.69 ± 0.019	0.301 ± 0.034
DOX	245 ± 2.01	0.93 ± 0.039 [a]	0.379 ± 0.027 [a]
DOX +QRN	229 ± 1.08	0.68 ± 0.014 [b]	0.296 ± 0.031 [b]

Data represent the mean ± S.E.M. of observations from 10 rats.
[a] Significantly different from control group at $P < 0.05$.
[b] Significantly different from DOX group at $P < 0.05$.
QRN: Quercetin**;** DOX: Doxorubicin.

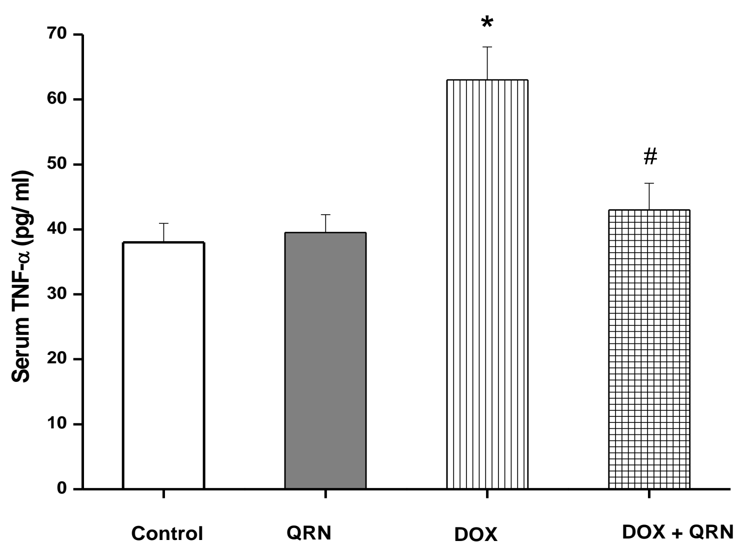

Fig. (1): Effect of DOX (2.5 mg/kg/48 h x 6 doses) and QRN (10 mg/kg/day, orally for 6 weeks) on the serum TNF-α level. Data are expressed as mean ± SEM, * is significantly different from the control at $P < 0.01$. # is significantly different from DOX group at $P < 0.01$.

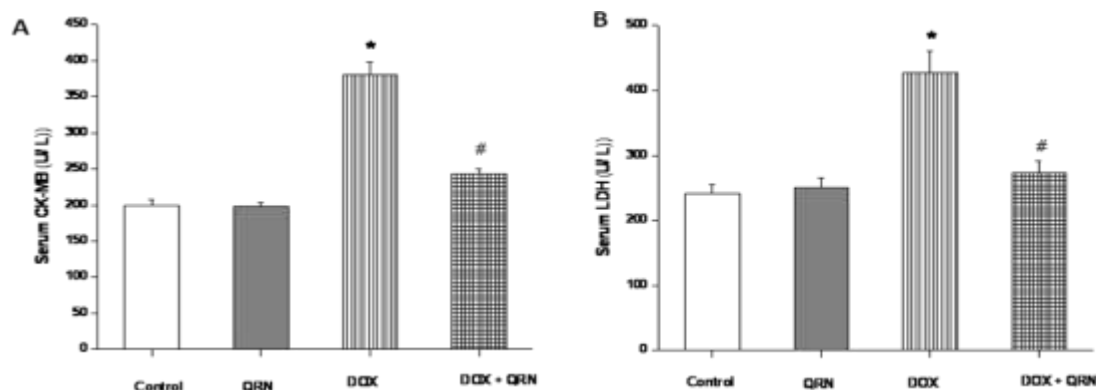

Fig. (2): **A**) Effect of DOX (2.5 mg/kg/48 h x 6 doses) and QRN (10 mg/kg/day, orally for 6 weeks) on the serum creatine kinase (CK-MB) level. Data are expressed as mean ± SEM, * is significantly different from the control at P < 0.01. # is significantly different from DOX group at P < 0.01.
B) Effect of DOX (2.5 mg/kg/48 h x 6 doses) and QRN (10 mg/kg/day, orally for 6 weeks) on serum lactate dehydrogenase (LDH) level. Data are expressed as mean ± SEM, * is significantly different from the control at P < 0.01. # is significantly different from DOX group at P < 0.01.

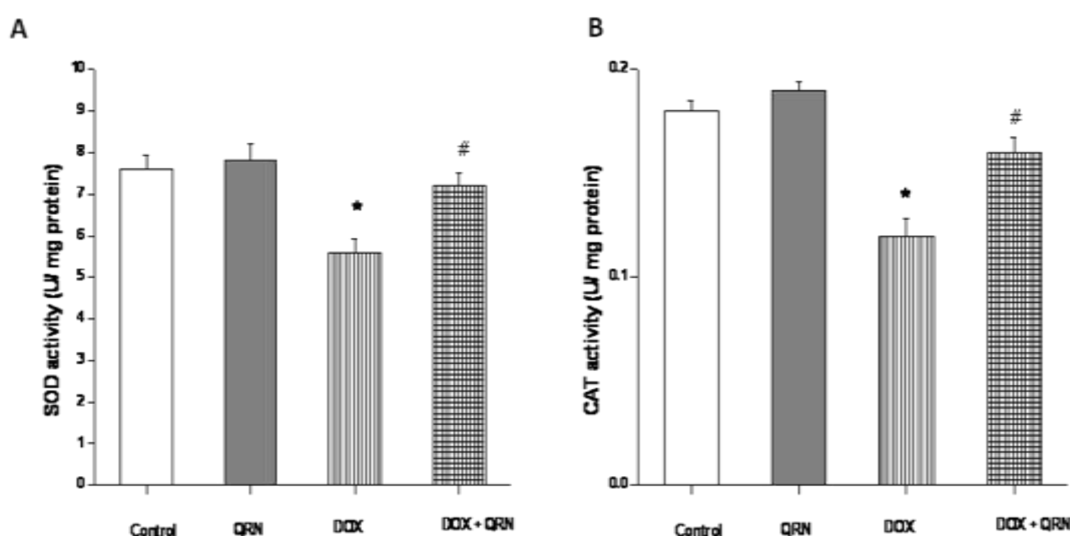

Fig. (3): **A**) Effect of DOX (2.5 mg/kg/48 h x 6 doses) and QRN (10 mg/kg/day, orally for 6 weeks) on myocardial superoxide dismutase (SOD) level. Data are expressed as mean ± SEM. * is significantly different from the control at P < 0.01. # is significantly different from DOX group at P < 0.01.
B) Effect of DOX (2.5 mg/kg/48 h x 6 doses) and QRN (10 mg/kg/day, orally for 6 weeks) on myocardial catalase (CAT) level. Data are expressed as mean ± SEM, * is significantly different from the control at P < 0.01. # is significantly different from DOX group at P < 0.01.

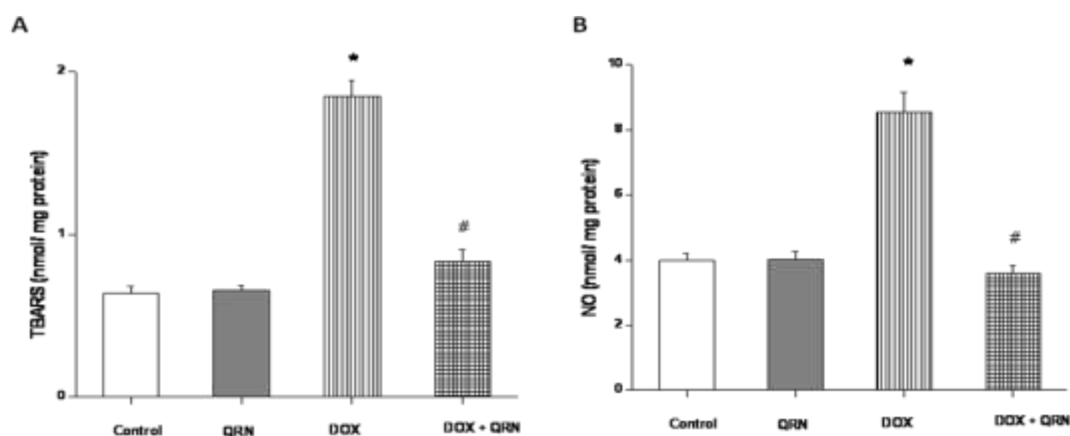

Fig. (4): **A**) Effect of DOX (2.5 mg/kg/48 h x 6 doses) and QRN (10 mg/kg/day, orally for 6 weeks) on myocardial thiobarbituric acid reactive species (TBARS) level. Data are expressed as mean ± SEM, * significantly different from the control at $P < 0.01$. # is significantly different from DOX group at $P < 0.01$. **B**) Effect of DOX (2.5 mg/kg/48 h x 6 doses) and QRN (10 mg/kg/day, orally for 6 weeks) on myocardial nitric oxide (NO) level. Data are expressed as mean ± SEM, * significantly different from the control at P< 0.0 1. # is significantly different from DOX group at $P < 0.01$.

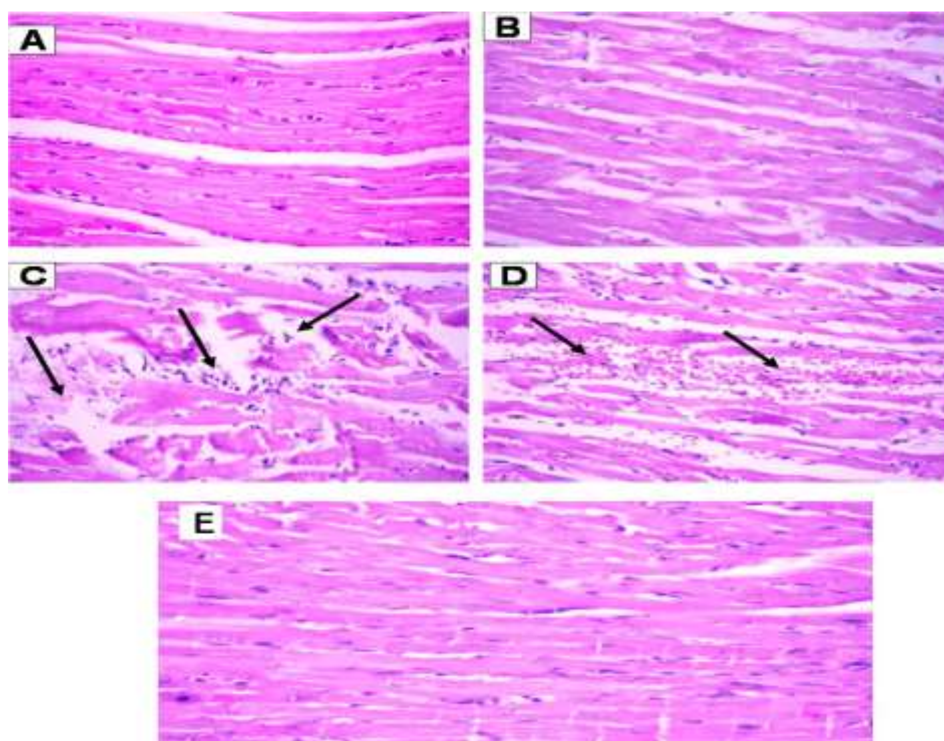

Fig. (5): Microscopical examination of heart sections of control and DOX-treated groups. (**A**) control and (**B**) QRN-treated group showed normal cardiac muscle fibers without necrosis and absence of any damage; (**C**) DOX-treated group revealed focal necrosis of cardiomyocytes associated with leukocyte infiltration (+++); (**D**) DOX-treated group showed intramuscular hemorrhage and revealed focal myocarditis (++); (**E**) the combined administration of QRN and DOX, resulted in amelioration of histopathological changes of cardiac muscle fibers with minimal necrosis, edema and inflammatory cell infiltration (+). Original magnification H &E x: 200.

DISCUSSION:

DOX is a member of anthracycline antibiotics which is extensively used as an effective and broad spectrum anti-cancer agent. Chronic DOX administration induces irreversible and life-threatening cardiac dysfunctions that may lead to heart failure or even death. Cardiotoxicity is believed to occur by DOX-induced mitochondrial dysfunction and subsequent oxidant production (Perego et al., 2001). The majority of experimental evidence has shown that DOX-induced cardiotoxicity occurs through formation of reactive oxygen species (ROS) (Corna et al., 2004). These ROS cause membrane lipid peroxidation of cardiomyocytes leading to cardiac damage (Vora et al., 1996).

Pharmacological activation of endogenous myocardial antioxidants has been identified as a promising therapeutic approach in diseases associated with increased oxidative stress. DOX-induced oxidative stress in cardiac tissues, as manifested by the alterations observed in cardiac antioxidant defense systems, is both enzymatic and non-enzymatic. In this sense, the anthracycline drug reduced significantly the cardiac GSH content, besides it notably lowered the cardiac enzymatic activities of SOD and CAT (Powell & Chevion, 1991). In this regard, our current work revealed declined activities of SOD and CAT in the cardiac DOX-treated group. This is likely due to superoxide generation at the site of damage, which diminishes SOD and CAT activities and lead to myocardium damage (Sharma et al., 2001). In a line of this concept, the cardioprotection due to the direct effect of antioxidant in DOX cardiotoxicity has been previously reported (Morishima et al., 1998). QRN administration ameliorated the deterioration in the activities of these enzymes in DOX-treated rats. Thus, QRN scavenges superoxide radicals and reduces myocardial damage due to free radicals production in cardiac tissue. Indeed, an increasing number of evidence indicates the cardioprotective properties of QRN due to its antioxidant activity (Ahn et al., 2008; Annapurna et al., 2009). The cardioprotective effects of QRN were observed in models of pharmacologically-induced hypertension (Duarte et al., 2001). QRN is an excellent metal chelator. It chelates transition metals such as iron which can initiate the formation of oxygen free radicals (Afanas'ev et al., 1989). Both, scavenging of free radicals and chelating effects, are involved in its cardio-protective effects (Cheng and Breen, 2000).

Increased levels of lipid peroxidation products such as heart TBARS indicate oxidative stress in DOX-treated rats. It has been demonstrated that as a result of lipid peroxidation, inflammatory cells accumulate in cardiac myocytes (Saad et al., 2001). Superoxide radicals play an important role in the formation of other ROS such as peroxynitryl (ONOO-), hydroxyle (OH•), and singlet oxygen, which induce oxidative damage to lipids, proteins, and DNA (Pietta, 2000). $ONOO^-$ is an important contributor in DOX-induced cardiotoxicity (Shuai et al., 2007). $ONOO^-$ is considered as a major initiator of lipid peroxidation (Denicola and Radi, 2005). This suggestion is based on that $ONOO^-$ causes nitration of the protein tyrosine (Mihm et al., 2001). The first targets of DOX-mediated free radical damage are cellular membranes, which are rich in lipids prone to peroxidation. This radical damage results in production of many relatively stable and highly toxic aldehydes, such as MDA. Interestingly,

the increased levels of lipid peroxidation products might be due to decreased antioxidant system and this was normalized by QRN.

Additionally, the present data demonstrate that DOX administration induced a significant elevation of serum cardiac isoenzymes levels (LDH and CK-MB) which are considered important markers of early and late cardiac injury during DOX therapy (Fadillioglu and Erdogan, 2003). The increased LDH and CK-MB in DOX-treated rats observed in this study could be due to DOX-induced cardiac necrosis. The leakage of these enzymes and the increase of their serum levels may be accounted for free radicals generated by DOX which may attack the cardiac membrane causing damage of several macromolecular cellular components (DeAtley et al., 1999). Noteworthy, pretreatment with QRN normalized the elevated serum level of these cardiac isoenzymes in DOX-treated rats. QRN possess free radical scavenging and antioxidant activity that may explain their ability to protect the myocardium from DOX-induced damage by preventing leakage of cardiac LDH and CK-MB isoenzymes.

On the other hand, previous studies have postulated the possible role of the proinflammatory cytokine TNF-α in the pathogenesis of DOX cardiotoxicity. TNF-α is a potentially important stimulus for increased oxidative stress in the myocardium and has the ability to stimulate free radical production (Meier et al., 1989; Torre-Amione et al., 1996). TNF-α induces depression of cardiac functions and is involved in the progression of congestive heart failure, inducing cardiac dysfunction, apoptosis, ventricular remodelling and dilatation (Edmunds et al., 1999; Ferrari et al., 1995). ROS have been demonstrated to trigger the production and the release of TNF-α via up-regulation of nuclear factor-kB (NF-KB) (Nian et al., 2004). Thus, these reports indicated the patho-physiological relevance of TNF-α in DOX-induced cardiotoxicity. In consistent with this concept, the present study showed an elevation of serum TNF-α level. Importantly, QRN administration effectively inhibited the elevated TNF-α in DOX-treated rats nearly to the normal level. In harmony with this finding, it has been demonstrated that QRN is able to inhibit NF-KB via its antioxidant and free radical scavenging activities (Rogler et al., 1998).

Furthermore, the cardioprotective effects of QRN were confirmed by the histopathological examination of cardiac tissues that revealed a well-preserved normal mor-phology of cardiac muscle with minimal necrosis and inflammatory cell infiltration compared to DOX-treated rat.

Besides the histopathological changes induced by DOX, our results showed an increase in the heart weight index (serves as an index of cardiomegaly). This observation is consistent with previous studies and indicates the cardiac remodeling and hypertrophy effects induced by DOX (Sacco et al., 2001; Soga et al., 2006). DOX-induced cardiac hypertrophy may be related to increased ROS production. This may be based on the previous study that showed the association between the release of ROS and proinflammatory cytokines, TNF-α, during DOX administration. TNF-α-induced cardiac hypertrophy may be mediated via the release of free radicals (Yokoyama et al., 1997). This effect was previously described in neonatal cardiac myocytes (Nakamura et al., 1998). In contrast,

our results revealed that treatment with QRN ameliorated the increase in the heart weight index induced by DOX. This effect may be attributed to the fact that TNF-α -induced cardiac hypertrophy is mediated via free radicals (Nakamura et al., 1998). Consequently, QRN could inhibit the hypertrophic actions by its antioxidant and free radical scavenging activities that were documented in many experimental studies (Ahn et al., 2008; Annapurna et al., 2009).

CONCLUSION:

In conclusion, our results suggest that QRN exhibited a cardio-protective effect against cardio-toxicity-induced by chronic DOX administration in rats and this is attributed to its antioxidant and anti-inflammatory properties. Thus, QRN can be supplemented as cardio-protectant against cardiactoxic effects of DOX. Nevertheless, additional human studies using QRN are required for further elucidation its protective role against agents that induce cardiotoxic effects.

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