PROTECTIVE EFFECT OF THYMOQUINONE AGAINST
DOXORUBICIN–INDUCED CARDIOTOXICITY IN RATS: A POSSIBLE
MECHANISM OF PROTECTION

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Administration of thymoquinone (10 mg kg⁻¹ day⁻¹, p.o.) with drinking water starting 5 days before a single injection of doxorubicin (15 mg kg⁻¹ i.p.) and continuing during the experimental period ameliorated the doxorubicin-induced cardiotoxicity in rats. This protection was evidenced from the significant reduction in serum enzymes: lactate dehydrogenase elevated level, 24 h and creatine phosphokinase elevated levels, 24 h and 48 h after doxorubicin administration. The cardiotoxicity of doxorubicin has been suggested to result from the generation of superoxide free-radical. The protective action of thymoquinone was examined against superoxide anion radical either generated photochemically, biochemically or derived from calcium ionophore A23187 stimulated polymorphonuclear leukocytes. The results indicate that thymoquinone is a potent superoxide radical scavenger, scavenging power being as effective as superoxide dismutase against superoxide. In addition thymoquinone has an inhibitory effect on lipid peroxidation induced by Fe³⁺/ascorbate using rat heart homogenate. The superoxide scavenging and anti-lipid peroxidation may explain, in part, the protective effect of thymoquinone against doxorubicin-induced cardiotoxicity.

KEY WORDS: thymoquinone, lactate dehydrogenase, creatine phosphokinase, cardiotoxicity, doxorubicin.

INTRODUCTION

Doxorubicin (adriamycin) is a quinone-containing antitumour antibiotic that is used to treat several types of cancer [1]. However, its clinical use has been restricted by dose-limiting cardiotoxicity which lead to cardiomyopathy and heart failure [1–3]. At present, oxygen radical-induced injury of membrane lipids is considered to be the most important factor responsible for the development of doxorubicin-induced cardiotoxicity [4–7]. The mechanism involves one-electron reduction of doxorubicin for generation of a semiquinone radical. Doxorubicin semiquinone radical reduces oxygen to produce superoxide and to regenerate doxorubicin. The net result of this process is that doxorubicin catalyzes the reduction of oxygen by NADPH, to form a superoxide radical, which is subsequently reduced to hydrogen peroxide (H₂O₂) by the antioxidant enzyme, superoxide dismutase. In the presence of Fe²⁺, the H₂O₂ is further reduced to the extremely reactive hydroxyl radical (‘OH), which can react with polyunsaturated fatty acids to yield lipid hydroperoxide. This initiates a lipid radical chain reaction, which can cause oxidative damage to cell membranes [5].

Thymoquinone (TQ), the main constituent of the volatile oil from Nigella sativa seeds has been reported to inhibit eicosanoid generation in leucocytes and non-enzymatic peroxidation in ox brain phospholipid liposome [8]. Previous studies have shown that pretreatment with TQ protected organs against oxidative damage induced by a variety of free radical generating agents, including carbon tetrachloride [9, 10], cis-platin [11] and doxorubicin [12]. In this study, we investigated the effect of TQ on doxorubicin induced cardiotoxicity in rats, the ability of TQ to scavenge superoxide radical and the effect of TQ on the non-enzymatic lipid peroxidation of rat heart homogenate induced in vitro by Fe³⁺/ascorbate.

MATERIALS AND METHODS

Chemicals
Thymoquinone, superoxide dismutase (SOD),
Table I

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Treatment</th>
<th>Serum enzyme activities Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LDH</td>
</tr>
<tr>
<td>24 h</td>
<td>Control</td>
<td>223 ± 22.9</td>
</tr>
<tr>
<td></td>
<td>TQ</td>
<td>216.6 ± 21.2</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>420 ± 19.9***</td>
</tr>
<tr>
<td></td>
<td>TQ + Dox</td>
<td>292 ± 33.9†</td>
</tr>
<tr>
<td>48 h</td>
<td>TQ</td>
<td>220 ± 29.4</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>340 ± 52.9</td>
</tr>
<tr>
<td></td>
<td>TQ + Dox</td>
<td>229 ± 23</td>
</tr>
</tbody>
</table>

***Significantly different from control group.
†Significantly different from doxorubicin.
§P < 0.05, ¶P < 0.01, §§P < 0.001.

The remaining chemicals were of the highest analytical grade. The drugs were dissolved in normal saline just before use.

Animal treatment

Adult male albino rats weighing 220–250 g were obtained from the Experimental Animal Care Centre of King Saud University, Riyadh, KSA. Animals were divided into four groups of six and allowed ad libitum access to food (Purina chow) and tap water. One group received doxorubicin (15 mg kg⁻¹, i.p) and the other group received doxorubicin (15 mg kg⁻¹, i.p) and thymoquinone (10 mg kg⁻¹ day⁻¹, p.o.) in the drinking water starting 5 days before doxorubicin and continuing throughout the duration of the experiment (48 h). Two control groups received drinking water with or without thymoquinone.

Assessment of cardiotoxicity

Blood samples were drawn from the orbital plexus, under light ether anaesthesia, into non-heparinized capillary tubes at 24 and 48 h following doxorubicin treatment. Serum was separated by centrifugation at

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**Fig. 1.** (a) Thymoquinone scavenging of superoxide radical as a function of its concentration measured by reduction of NBT with NADH and PMS. Each point represents the mean of three different experiments. (b) Same as in (a) except thymoquinone was replaced by different concentrations of superoxide dismutase.
4000 rpm for 4 min and stored at −20°C until analysis. LDH and CPK levels were assayed using commercially available reagents based on the method of Buhl and Jackson [13] and Gruber [14], respectively.

**Assays for superoxide anion radical**

Phenazine methosulphate (PMS), NADH and NBT method [15]. The reaction mixture contained in a total volume of 1 ml, 25 mM Tris–HCl, pH 8.8, 50 μM NBT, 100 μM NADH and different concentrations of TQ or SOD. The reaction was initiated by adding 5 μM PMS and the production of formazan was followed at 560 nm at 20°C for 5 min. Data were plotted as a percentage of the remaining absorbance in the presence of SOD or TQ.

Glucose–glucose oxidase and NBT method [16]. The reaction mixture contained in a total volume of 1.0 ml, 10 mM glycine–NaOH, pH 9.5, 0.2 mM glucose, 50 μM NBT and different concentrations of TQ or SOD. The reaction was initiated by the addition of 10 units of glucose oxidase and the initial rate of the reaction was measured from the rate of change of absorbance at 560 nm at room temperature. Data were plotted as a percentage of the remaining activity in the presence of TQ or SOD.

Riboflavin, methionine and NBT method [17]. The reaction mixture contained in a total volume of 2 ml, 50 mM Tris–HCl, pH 7.8, 10 mM methionine, 5 μM riboflavin, 50 μM NBT and different concentrations of TQ or SOD. Reduction of NBT after 10 min of illumination was measured at 560 nm. Data were plotted as a percentage of the remaining absorbance in the presence of SOD or TQ.

Calcium ionophore (A23187) stimulated polymorphonuclear leucocytes (PMNL) and NBT [18]. The ability to reduce NBT was assayed by incubating rat PMNL 10 × 10⁶ cells with 0.1% NBT dissolved in phosphate-buffer saline, pH 7.4) the reaction was initiated by the addition of calcium ionophore (10 μM) for 20 min at 37°C. Termination of the assay was done by adding 0.6 ml of glacial acetic acid (final assay volume 3 ml) into which the reduced NBT dye was extracted and the extract was read at 560 nm. Data were plotted as a percentage of remaining absorbance in the presence or absence of SOD or TQ.

**Assay of non-enzymatic lipid peroxidation in rat heart homogenate**[8]

The heart of a normal male rat was isolated,

Fig. 2. (a) Thymoquinone scavenging of a superoxide radical as a function of its concentration measured by NBT reduction with riboflavin and methionine. Each point represents the mean of three different experiments. (b) Same as in (a) except thymoquinone was replaced by different concentrations of superoxide dismutase.
washed with saline, weighed and homogenized in ice-cold saline and 10% homogenate was made in a Branson sonifier (250, VWR, Scientific, Danbury, CT, USA).

The reaction mixture contained varying amounts of TQ, 0.75 ml phosphate buffer (50 mM, pH 7.4), 50 μl rat heart homogenate (10%), 0.1 ml of 1 mM ferric chloride and 0.1 ml of 1 mM ascorbic acid. The tubes were incubated at 37°C for 30 min and the extent of peroxidation was measured by thiobarbituric acid (TBA) test. To the above test tube, the following were added 0.1 ml butylated hydroxytoluene (BHT) (2% w/v) to stop further lipid peroxidation, 1.0 ml TBA (1% w/v in 0.05 M NaOH) and 1.0 ml trichloroacetic acid (2.8% w/v) and placed in a waterbath at 80°C for 20 min. At the end of incubation, the tubes were cooled and centrifuged for 5 min at 3200 rpm. The chromogen was extracted with 2.0 ml n-butanol and absorbance was read at 532 nm. Tubes containing reagent blank, heart homogenate and subjected to TBA test served as control tubes. Data were plotted as a percentage of the remaining absorbance in the presence of TQ.

**Statistical analysis**

Data are expressed as (means ± SEM). Statistical comparison between different groups were done by using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. Significance was accepted at $P < 0.05$.

**RESULTS**

**Effect of thymoquinone on the elevated serum LDH and CPK activities induced by doxorubicin administration**

Serum LDH and CPK were significantly ($P < 0.001$) elevated 24 h after a single injection of doxorubicin reaching 420 ± 19.9 and 1123 ± 16.5 U l$^{-1}$, respectively, as compared with the control group. Pretreatment with TQ decreased significantly the enzyme activities by 30.5 and 62.3%, respectively (Table I). After 48 h, the serum CPK level was still significantly elevated over the control values ($P < 0.001$). TQ reduced significantly CPK activity (Table I).

**Scavenging of superoxide anion radical by thymoquinone**

Superoxide anions were generated in enzymatic

![Fig. 3.](image-url) (a) Thymoquinone scavenging of superoxide radical as a function of its concentration measured by NBT reduction with calcium ionophore stimulated polymorphnuclear leukocytes. Each point represents the mean of three different experiments. (b) Same as in (a) except thymoquinone was replaced by different concentrations of superoxide dismutase.
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Fig. 4. (a) Thymoquinone scavenging of superoxide radical as a function of its concentration measured by NBT reduction using glucose–glucose oxidase. Each point represents the mean of three different experiments. (b) Same as in (a) except thymoquinone was replaced by different concentrations of superoxide dismutase.

and non-enzymatic systems. In all systems used in this study, SOD inhibited the reduction of NBT in a concentration-dependent manner. Similar inhibition curves were obtained when SOD was replaced by TQ (Figs. 1–4). The IC50 values for TQ ranged from 8 to 20 μM in non-cellular system and 60 μM in the cellular system.

Effect of thymoquinone on lipid peroxidation induced by Fe3+/ascorbate in vitro

The antioxidant nature of TQ as an inhibitor of in-vitro non-enzymatic lipid peroxidation was examined, using rat heart homogenate. TQ inhibited lipid peroxidation in a concentration-dependent manner (Fig. 5). The IC50 for TQ was 5 μM.

DISCUSSION

In the present study, a single dose of doxorubicin (15 mg kg⁻¹ i.p.) induced cardiotoxicity manifested biochemically by a significant increase in serum LDH after 24 h and serum CPK after 24 and 48 h. These results are consistent with the previous studies reported by other investigators [2, 4, 7] that doxorubicin induced cardiotoxicity in normal rats. The results of the present study clearly demonstrate that TQ, when given in the drinking water protects rats from doxorubicin-induced cardiotoxicity as evidenced from the significant reduction in serum enzymes, LDH and CPK (Table I). These results confirm our previously published data on the protective action of TQ on doxorubicin-induced cardiotoxicity in mice [12].

The biochemical mechanisms involved in the development of doxorubicin cardiotoxicity have been well studied and documented. It is now believed that the formation of superoxide radical from doxorubicin recycling is a crucial factor in the pathogenesis of doxorubicin cardiotoxicity [4–7]. An important finding in this study is that TQ shows a superoxide radical scavenging activity, like superoxide dismutase, using four different systems for the generation of superoxide (Figs. 1–4) The IC50 values for TQ in these assays were in the micromolar range which indicates that TQ is a good scavenger of superoxide radical. The effect of TQ on lipid peroxidation, a free radical mediated process, can provide some information to its antioxidant capability. The studies on the rat heart homogenate showed that TQ had
an inhibitory effect, the magnitude of which was concentration-dependent (Fig. 5). These results are consistent with alleviation of doxorubicin mediated cardiotoxicity by other antioxidants, such as coenzyme Q₁₀ [19], N-acetylcysteine [20], alpha tocopherol [21], vitamin A [22], probucol [23] and butylated hydroxyanisol [24]. It has been shown that TQ pretreatment protected other organs against oxidative damage induced by a variety of free radical generating agents, including carbon tetrachloride [9, 10] and cisplatin [11]. On the basis of these findings, we suggested that TQ might play an important role as an endogenous antioxidant and could also be applicable as a cytoprotective agent against tissue damage mediated by chemotherapeutic agents. The results of this study, when combined with the knowledge that TQ has low toxicity [25], support a potential role of TQ as an antioxidant drug in doxorubicin cardiotoxicity.

REFERENCES


Fig. 5. Effect of thymoquinone on non-enzymatic lipid peroxidation induced by Fe³⁺/ascorbate in vitro. The assay conditions are described under Materials and Methods. Results are expressed as a percentage of control. Each point represents a mean of triplicate experiments.


