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Thymoquinone Ameliorates Renal Oxidative Damage and Proliferative Response Induced by Mercuric Chloride in Rats

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Abstract: We tested the hypothesis if thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) could ameliorate renal oxidative damage and proliferative response induced by mercuric chloride ($HgCl_2$) in rats. $HgCl_2$ (3 mg/kg) was administered subcutaneously to each one of two groups of rats: (i) $HgCl_2$–thymoquinone group that received thymoquinone (10 mg/kg/day); and (ii) $HgCl_2$ group that received vehicle instead of thymoquinone. A third group of rats was reserved as control group. Rats were killed 24, 48 and 72 hr after $HgCl_2$ administration for histological and biochemical studies. Our findings show that treatment with thymoquinone offers imperative protection from $HgCl_2$-induced nephrotoxicity. The deterioration of antioxidant enzymes, increment of serum creatinine and histological damage caused by $HgCl_2$ are markedly improved by thymoquinone treatment. Apoptosis and proliferative reactions are also reduced. The maximal protection offered by thymoquinone treatment was particularly noticeable 48 and 72 hr after administration of the toxic agent at the time when histological damage, renal cell apoptosis and proliferative reactions reached their maximum. These observations may be attributed partially to the antioxidant effect of thymoquinone and suggest that it may be a clinically valuable agent in the prevention of acute renal failure caused by inorganic mercury intoxication.

Mercuric chloride ($HgCl_2$) is a potent nephrotoxicant that has been widely employed in animal models of acute renal failure. Functional alterations, haemodynamic changes and histological damage are well characterized in this experimental model [1,2]. Inorganic mercury causes renal tubular necrosis mainly in the S3 segment of the proximal tubules at least 8 hr after the peak of an apoptotic event [3]. Recent research has focused on the critical role of reactive oxygen species (ROS) in the pathogenesis of mercury-induced renal damage [4]. In $HgCl_2$-induced nephrotoxicity, the reduction in renal content of antioxidant armory such as glutathione (GSH), as well as the decrease of thiol groups, has been advocated as evidence of the role of oxidative stress in this pathology [4]. Furthermore, morphological and biochemical studies have shown that $HgCl_2$ toxicity is associated with a substantial loss of inner mitochondrial adenosine triphosphate-generating surfaces and important deactivation of the free radical scavenging systems, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) [5]. Considering that oxidative stress may play a crucial role in $HgCl_2$-induced nephrotoxicity, compounds with antioxidant potential are expected to have a protective role against it. Studies have shown that $HgCl_2$-induced injury can be ameliorated by SOD or the antioxidants *N*-acetylcysteine and melatonin [6,7]. Likewise, some plant extracts with antioxidant properties have been also shown to exert a similar effect [8].

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Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone; fig. 1) is the main active constituent of the volatile oil extracted from the black seed (*Nigella sativa* L.). Several studies point to the effect of black seed and thymoquinone to inhibit tissue inflammation and oxidative stress [9,10]. It has been reported to have strong antioxidant potentials through scavenging ability of different free radicals, scavenging power being as effective as SOD against superoxide anions [11]. In one study [12], thymoquinone protected the kidney against ifosfamide-induced damage by preventing renal GSH depletion and lipid peroxide accumulation. In addition, orally administered thymoquinone prevented cisplatin-induced nephrotoxicity in mice and rats as evidenced by significant reductions in serum urea and by a significant improvement in kidney weight and creatinine clearance [13]. Furthermore,

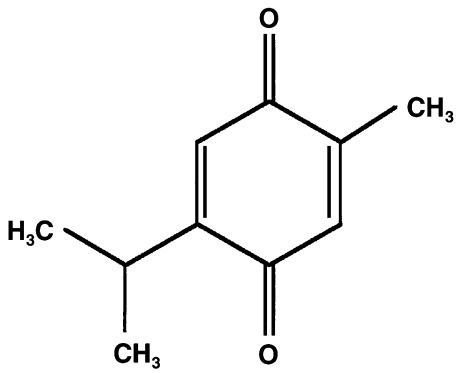

Fig. 1. Chemical structure of thymoquinone (2-isopropyl-5-methy1-1,4-benzoquinone).

pre-treatment of mice with thymoquinone 1 hr before carbon tetrachloride injection ameliorated the hepatotoxicity of carbon tetrachloride as evidenced by the significant reduction of the elevated levels of serum enzymes and significant increase of the hepatic GSH content [14]. In other studies, oral administration of thymoquinone in drinking water before a single injection of doxorubicin ameliorated drug-induced cardiotoxicity in rats [15]. Thymoquinone also prevented the nephropathy induced by doxorubicin in rats [16], and it exerted protective effects on doxorubicin damage mainly due to its superoxide-scavenging and antilipid peroxidation effects [15,16].

However, in spite of the substantial body of literature on the antioxidant power of thymoquinone in various models of oxidative stress, there is no any available information about the prevention by thymoquinone of the pathogenetic oxidative process of mercury-induced nephrotoxicity. On this basis, the purpose of this study was to investigate the potential of thymoquinone to ameliorate the oxidative nephrotoxicity induced by $HgCl_2$ *in vivo*. We reasoned that the broad spectrum antioxidant effects of thymoquinone might allow this agent to prevent the underlying oxidative processes that play an important role in the pathogenesis of $HgCl_2$-induced nephrotoxicity.

Materials and Methods

Chemicals. Thymoquinone, $HgCl_2$ and the rest of chemicals and reagents used in this study were of the highest analytical grade from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Animals. Adult male Sprague-Dawley rats (200–250 g) were obtained from the animal house of Mansoura Faculty of Medicine and fed on a standard rat chow and water *ad libitum*. Animal care and experiments were performed in accordance with the guidelines established by the National Institutes of Health Guide to the Care and Use of Laboratory Animals, National Institutes of Health publication no. 86–23.

Experimental protocol. A single dose of $HgCl_2$ (3 mg/kg body weight dissolved in 0.5 ml of 0.9% NaCl solution) was administered subcutaneously to each one of two groups of rats: (i) $HgCl_2$–thymoquinone group, consisting of 24 rats that received thymoquinone (10 mg/kg body weight/day in drinking water) starting 5 days before $HgCl_2$ administration and continued throughout the duration of the experiment (72 hr); and (ii) $HgCl_2$ group, consisting of 24 rats that received vehicle (0.9% NaCl solution) instead of thymoquinone by gastric gavage. A third group of rats reserved as control group consisting of 18 rats received 0.5 ml of 0.9% NaCl solution subcutaneously instead of $HgCl_2$. Under ether anaesthesia, six to eight rats from each group were killed 24, 48 and 72 hr after $HgCl_2$ administration. Blood was withdrawn for determination of serum creatinine (SCr). The kidneys were harvested, perfused with ice-cold saline and prepared for subsequent histological and biochemical studies.

Determination of lipid peroxidation. Malondialdehyde (MDA) in renal tissue was analysed by the method of Ohkawa *et al.* [17], in which MDA, an end-product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a coloured complex. Sections of kidney obtained after cold perfusion as outlined earlier were placed in a total volume of cold 3 ml 100 mM KCl plus 0.003 M ethylenediaminetetraacetic acid (EDTA) and homogenized. Homogenates were centrifuged at 600 ×g for 15 min. Four hundred microlitres of supernatant were added to 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% TBA and 0.6 ml water. This solution was heated to 95° for 1 hr. After addition of 1.0 ml water and 5.0 ml of *n*-butanol–pyridine mixture (15:1, v/v), all the tubes were shifted to an ice bath and centrifuged at 2500 ×g for 10 min. The amount of MDA formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm against a reagent blank. Results are expressed as nmol MDA per mg homogenate protein.

Estimation of renal GSH content. Renal GSH content was assayed by the method of Jollow *et al.* [18]. Renal tissue slices obtained after cold perfusion were suspended in 100 mM KCl plus 0.003 M EDTA and homogenized as described for the determination of MDA above. The homogenates were centrifuged at 600 ×g for 10 min. One ml of supernatant was precipitated with 1.0 ml of sulphosalicylic acid (4% w/v). The samples were kept at 4° for 1 hr and then centrifuged at 1200 ×g for 15 min. at 4°. The supernatant was used for GSH determination. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 5′,5-dithiobis-(2-nitrobenzoate) (40 mg/10 ml of phosphate buffer 0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm. GSH content was expressed as nmol of GSH per mg homogenate protein.

Estimation of renal glutathione peroxidase activity. Renal GPx activity was estimated by the method of Mohandas *et al.* [19]. The reaction mixture consisted of 50 mM potassium phosphate buffer: (in mM) 1 EDTA, 1 NaN_3, 0.2 NADPH, 1 GSH, and 0.25 H_2O_2, as well as 1 U/ml GSSG reductase, pH 7.0, in a total volume of 1 ml. Enzyme source (0.1 ml) was added to 0.8 ml of the above mixture and allowed to incubate for 5 min. at room temperature, before initiation of the reaction by the addition of 0.1 ml peroxide solution. Disappearance of NADPH at 340 nm was recorded at 25°. Enzyme activity was calculated as nmol of NADP reduced per min. per mg homogenate protein using a molar extinction coefficient of 6.22×10^3 M/cm.

Estimation of renal catalase activity. Catalase (CAT) activity was estimated by the method of Aebi [20]. Slices of renal tissue were transferred to tubes containing 50 mM potassium phosphate, pH 7.0, and homogenized. The homogenates were centrifuged at 600 ×g for 10 min. Twenty-five microlitres of the supernatant were added to 725 µl of a mixture containing 7.7 mM H_2O_2 in 10 mM phosphate buffer, pH 7.0. Changes in absorbance were recorded at 240 nm. The rate constant of a first-order reaction (k) was used: k 5 $(1/\Delta t) \times \ln(A1/A2)$, where Δt is a measured interval (30 sec.) and A1 and A2 are the initial and final absorbance measurement times, respectively. Enzyme activity was expressed as k per mg homogenate protein.

Estimation of serum creatinine. Serum creatinine was estimated by the alkaline picrate method [21]. Protein-free filtrate was prepared. To a 1.0 ml of plasma/serum, 1.0 ml of sulfuric acid (0.6 N), 1.0 ml of sodium tungstate (5% w/v) and 1.0 ml of distilled water were added and mixed thoroughly. The mixture was then centrifuged at 800 ×g for 5 min. The supernatant was added to a mixture containing 1.0 ml of picric acid (1.05% w/v) and 1.0 ml of sodium hydroxide (0.75 N) and incubated at 37° for 20 min. The absorbance was recorded at 520 nm. SCr levels were expressed as µmol/l.

Histopathological studies.

Conventional histopathology. Sections of the renal tissue were snap frozen in dry ice and acetone as well as fixed in 15% formalin and embedded in Paraplast Plus (Monoject, Sherwood Medical Scientific Division, St. Louis, MO, USA). Sections of 4 µm thickness were

stained with routine dyes (haematoxylin and eosin) for conventional histopathological evaluation.

Superoxide staining. Superoxide production in renal cells was studied by staining of superoxide-positive cells (SPC) in frozen renal sections as described previously by Briggs *et al*. [22]. Slides were incubated at 37° for 60 min. in the following staining solution: 50 ml 0.05 M Tris–HCl buffer, 1 ml 3,3′-diaminobenzidine (DAB) stock solution (5 g DAB/132 ml Tris buffer, 0.05 M, pH 7.6), 250 µl of 8% NiCl, 32.5 µl of 10% NaN_3 and 50 µl of 0.5 M $MnCl_2$. Afterwards, sections were fixed with 10% formalin for 10 min. and counterstained with 1% methyl green.

Identification of apoptosis. As described by Gavrieli *et al*. [23], the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) method was employed for renal sections using an Apoptag *in situ* apoptosis detection kit (Oncor, Gaithersburg, VA, USA). Briefly, frozen sections were fixed in 10% neutral-buffered formalin and post-fixed in ethanol:acetic acid for 5 min. at –20°. After TUNEL staining, sections were counterstained with 2% methyl green. Embryonic rat forelimb bud tissue was used as a positive control. Negative controls were renal and forelimb bud tissues incubated with distilled water instead of the TdT enzyme in the reaction buffer. Slides were mounted with SlowFade (Molecular Probes, Eugene, OR, USA) to delay fluorescence quenching.

Identification of proliferating cells. As described previously [7], the avidin-biotin-peroxidase technique was used to study proliferating cell nuclear antigen (PCNA). Frozen sections (4 µm) were post-fixed in 100 and 95% ethanol, incubated for 10 min. in 3% H_2O_2, rinsed with phosphate-buffered saline (PBS), and incubated with avidin (2 µg/ml) for 20 min., then after being washed with PBS, incubated with 0.001% biotin solution for 20 min. After that, sections were incubated for 90 min. with monoclonal anti-PCNA antibody (Zymed Laboratories, San Francisco, CA, USA) diluted 1:20 in Tris-buffered saline (TBS; pH 7.8). Subsequently, the sections were incubated with 2.5% low-fat milk solution in TBS for 15 min. and then treated with biotinylated $F(ab')_2$ rat anti-mouse immunoglobulin G (IgG) antibody (Organon Teknica, Cappel, Durham, NC, USA) at a dilution of 1:20 in TBS for 1 hr. Finally, sections were incubated with peroxidase-conjugated extravidin for 30 min. after being washed in TBS. The reaction product was exposed by using 0.005% H_2O_2 containing 0.02% DAB in 50 mmol/l Tris (pH 7.6).

Evaluation of histopathological findings. At least six sections of each biopsy were used for evaluation of histopathological findings. The extension of tubular injury was evaluated in terms of the percent area of the sections showing a given level of histological damage. The severity of the tubular lesions was classified as described by Nava *et al*. [7], no injury = intact tubules with normal tubular cells and preserved brush border; tubular cell damage = loss of the brush border, cytoplasmic extrusion into the lumen, and intraluminal cellular debris; focal tubular necrosis = patchy necrosis of tubular epithelial cells with intraluminal debris and preservation of the tubular basement membrane; and complete tubular necrosis = disruption of the tubular basement membrane and necrosis of the tubule. Examination of the TUNEL-positive cells, PCNA-positive cells, and severity of toxic damage was done by using a microscope with epifluorescence device and with a grid fitted an ocular piece. The number of TUNEL and PCNA-positive cells per cubic millimetre in tubulointerstitial areas was counted as described by Soto *et al*. [24].

Statistical analysis. All statistical calculations were performed with SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± S.D. Statistical significance between means was done by one-way ANOVA, followed by Dunnett's *post hoc* tests. P-values of 0.05 or less were considered significant.

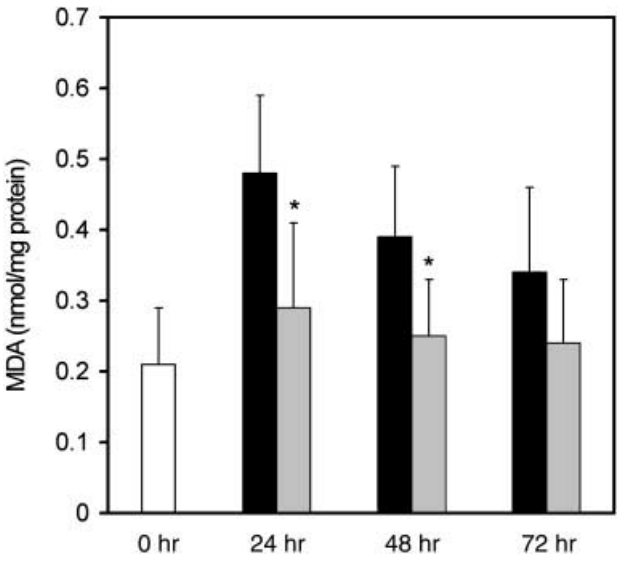

Fig. 2. Renal content of malondialdehyde (MDA) in rats receiving $HgCl_2$ alone (black bars) or with thymoquinone (greay bars). Open bar represents control group. *P < 0.05; $HgCl_2$–thymoquinone group versus the corresponding $HgCl_2$ group (mean ± S.D., ANOVA).

Results

Effect of thymoquinone on $HgCl_2$ modulation of renal lipid peroxidation.

Figure 2 shows serial changes of renal MDA levels over 72 hr after $HgCl_2$ administration. In the control rats, renal MDA level was 0.21 ± 0.08 nmol/mg protein. Injection of $HgCl_2$ significantly raised the renal MDA content over 72 hr after injection being more than twice its normal level on day 1 (0.48 ± 0.11 nmol/mg protein, P < 0.001). In comparison with their corresponding vehicle-treated rats, rats treated with thymoquinone had lower MDA contents over the 72 hr after exposure to the toxic agent (table 1 and fig. 2).

Effect of thymoquinone on $HgCl_2$ modulation of renal GSH content.

Figure 3 shows serial changes of renal GSH contents over 72 hr after $HgCl_2$ administration. In the control rats, renal GSH content was 10.0 ± 1.9 nmol/mg protein. In the first day after $HgCl_2$ injection, renal GSH decreased by ~4-fold (2.63 ± 0.92 nmol/mg protein, P < 0.001), then showed gradual spontaneous up-trend over the next 2 days. In contrast, rats that received thymoquinone treatment had significantly higher GSH contents over the 72 hr after exposure to the toxic agent (table 1 and fig. 3).

Effect of thymoquinone on $HgCl_2$ modulation of renal GPx activity.

Figure 4 shows serial changes of renal GPx activity over 72 hr after $HgCl_2$ administration. Control rats had GPx activity equal to 107.8 ± 16.7 nmol NADPH oxidized/min./mg protein. After $HgCl_2$ administration, GPx activity was reduced significantly in $HgCl_2$ group being 51.3% of its normal

Table 1.

Serum creatinine levels (SCr) and markers of oxidative stress: malondialdehide (MDA), reduced glutathione (GSH), glutathione peroxidase (GPx) and catalase (CAT) activity at various times in renal homogenates of rats receiving 3 mg/kg $HgCl_2$ alone or with 10 mg/day of thymoquinone (TQ) orally.

	24 hr		48 hr		72 hr	
	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 7)	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 8)	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 7)
MDA (nmol/mg protein)	0.48 ± 0.11	0.29 ± 0.12[1]	0.39 ± 0.1	0.25 ± 0.08[1]	0.34 ± 0.12	0.24 ± 0.09
GSH (nmol/mg protein)	2.63 ± 0.92	5.52 ± 1.42[2]	4.11 ± 1.55	7.63 ± 2.15[2]	5.83 ± 1.82	8.32 ± 2.11[1]
GPx (nmol NADPH-oxidized/min./mg protein)	55.3 ± 12.5	88.7 ± 14.2[2]	66.3 ± 13.9	93.7 ± 16.2[2]	78.2 ± 14.4	98.5 ± 17.2[1]
CAT (k/mg protein)	0.2 ± 0.04	0.29 ± 0.06[2]	0.25 ± 0.05	0.32 ± 0.06[1]	0.29 ± 0.06	0.33 ± 0.07
SCr (μmol/l)	163.6 ± 33.7	76.8 ± 19.6[3]	233.7 ± 46.5	96.5 ± 31.4[3]	183.2 ± 42.5	95.8 ± 33.4[2]

The values in each cell represent mean ± S.D.
Significant differences between TQ-treated groups and corresponding vehicle-treated groups: [1]P < 0.05; [2]P < 0.01; [3]P < 0.001 (ANOVA).

activity at day 1 (55.3 ± 12.5 nmol NADPH oxidized/min./mg protein, P < 0.001) with partial recovery at day 3 (78.2 ± 14.4 nmol NADPH oxidized/min./mg protein). In rats treated with thymoquinone, there was a significant recovery of reduced levels of this antioxidant enzyme over the 72 hr after $HgCl_2$ administration (table 1 and fig. 4).

Effect of thymoquinone on $HgCl_2$ modulation of renal catalase activity.

Figure 5 shows serial changes of renal CAT activity over 72 hr after $HgCl_2$ administration. Control rats had CAT activity equal to 0.34 ± 0.05 k/mg protein. In the first 2 days after $HgCl_2$ administration, CAT activity was reduced significantly in $HgCl_2$ group (0.2 ± 0.04 k/mg protein at day 1, P < 0.001, and 0.25 ± 0.05 k/mg protein at day 2, P < 0.01). Compared to their corresponding vehicle-treated rats, rats treated with thymoquinone had significantly higher CAT activity during days 1 and 2. Day 3 studies showed that CAT activities were comparable in all groups (table 1 and fig. 5).

Effect of thymoquinone on $HgCl_2$-induced elevation of serum creatinine.

Figure 6 shows serial changes of SCr levels over 72 hr after $HgCl_2$ administration. Control rats had SCr levels equal to 39.5 ± 13.3 μmol/l. There was significant elevation of SCr levels over the 72 hr after $HgCl_2$ administration being 7.3 times its normal level at day 2 (233.7 ± 46.5 μmol/l, P < 0.001). Compared to their corresponding vehicle-treated rats, rats that were treated with thymoquinone had significantly lower levels of SCr over the 72 hr after the nephrotoxic

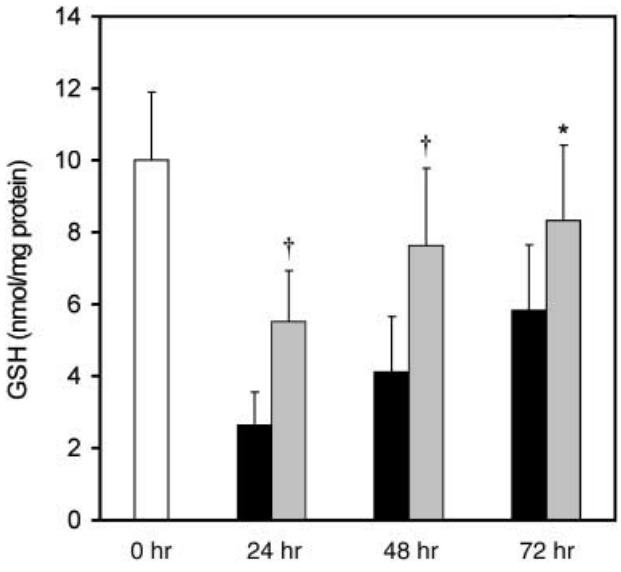

Fig. 3. Renal content of glutathione (GSH) in rats receiving $HgCl_2$ alone (black bars) or with thymoquinone (gray bars). Open bar represents control group. *P < 0.05; †P < 0.01; $HgCl_2$–thymoquinone group versus the corresponding $HgCl_2$ group (mean ± S.D., ANOVA).

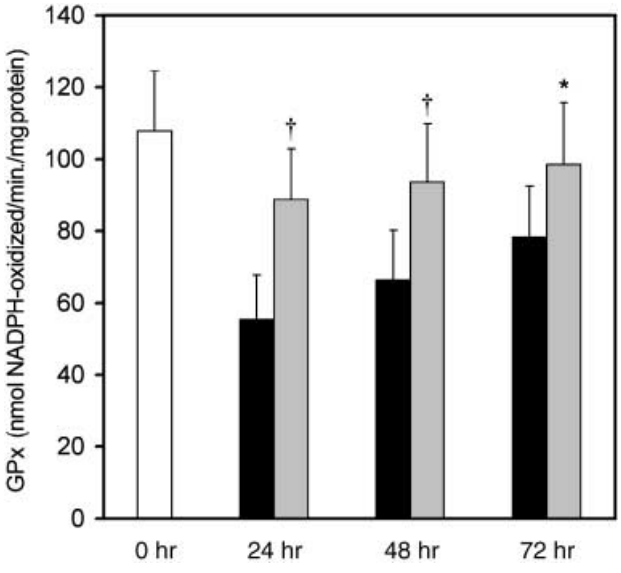

Fig. 4. Renal glutathione peroxidase (GPx) activity in rats receiving $HgCl_2$ alone (black bars) or with thymoquinone (gray bars). Open bar represents control group. *P < 0.05; †P < 0.01; $HgCl_2$–thymoquinone group versus the corresponding $HgCl_2$ group (mean ± S.D., ANOVA).

Table 2.

Histopathological observations in proximal tubular cells at various times in renal sections of rats receiving 3 mg/kg $HgCl_2$ alone or with 10 mg/day of thymoquinone (TQ) orally.

	24 hr		48 hr		72 hr	
	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 7)	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 8)	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 7)
Percentage of no injury	15.2 ± 9.8	33.4 ± 13.8[1]	9.1 ± 5.3	29.9 ± 14.7[2]	26.5 ± 15.2	56.2 ± 21.0[1]
Percentage of tubular cell damage	41.4 ± 15.3	23.4 ± 11.2[1]	52.4 ± 15.2	25.5 ± 13.5[2]	34.2 ± 10.7	15.6 ± 9.5[2]
Percentage of focal necrosis	33.5 ± 13.9	19.8 ± 9.4	59.6 ± 20.7	22.8 ± 11.6[2]	41.7 ± 15.4	19.3 ± 10.7[1]
Percentage of complete tubular necrosis	39.6 ± 13.2	18.8 ± 10.5[2]	67.3 ± 22.4	23.2 ± 12.1[3]	45.2 ± 17.5	17.0 ± 11.2[2]

The values in each cell represent mean ± S.D.
Significant differences between TQ-treated groups and corresponding vehicle treated groups: [1]P < 0.05; [2]P < 0.01; [3]P < 0.001 (ANOVA).

insult. The maximal protection offered by thymoquinone treatment was particularly noticeable at day 2 when SCr reached its peak (96.5 ± 31.4 µmol/l compared to 233.7 ± 46.5 µmol/l in vehicle-treated rats, P < 0.001; table 1 and fig. 6).

Histopathological observations.

Table 2 shows serial histopathological observations in proximal tubular cells of rats after administration of $HgCl_2$ alone or with thymoquinone 10 mg/day. The protective effect of thymoquinone in $HgCl_2$-induced renal injury was also apparent from histopathological examination. In rats that received $HgCl_2$ alone, a mixture of tubular cell damage, focal tubular necrosis and complete tubular necrosis was evident from the first day after the nephrotoxic insult. At the peak of the nephrotoxic insult (at day 2), 67.3 ± 22.4% of the tubules showed complete necrosis with disruption of the tubular basement membrane. Stroma also showed congestion, oedema and focal neutrophilic and lymphocytic infiltration. On day 3, there were 45.2 ± 17.5% of the tubules still showing complete necrosis with basement membrane disruption. In contrast, the severity of the tubular lesions was significantly reduced in rats pre-treated with thymoquinone. The extension of complete tubular necrosis observed in day 2 was reduced nearly three times (23.2 ± 12.1%, P < 0.001). On day 3, significant tubular improvement was seen running towards normal morphology and 56.2 ± 21.0 of the tubules were within normal limits (P < 0.05). Representative micrographs are seen in fig. 7A and B.

Table 3 shows numbers of superoxide-positive cells, TUNEL-positive cells representing renal tubular cell apoptosis, and a number of PCNA-positive cells in renal sections at various times after $HgCl_2$ administration of $HgCl_2$ alone or with thymoquinone 10 mg/day. Examination for superoxide production in stained renal sections showed a very little number of SPCs in the control rats (0.35 ± 0.03 SPC/100 tubulointerstitial cells). Significant increase in SPC was observed from the first day after $HgCl_2$ administration

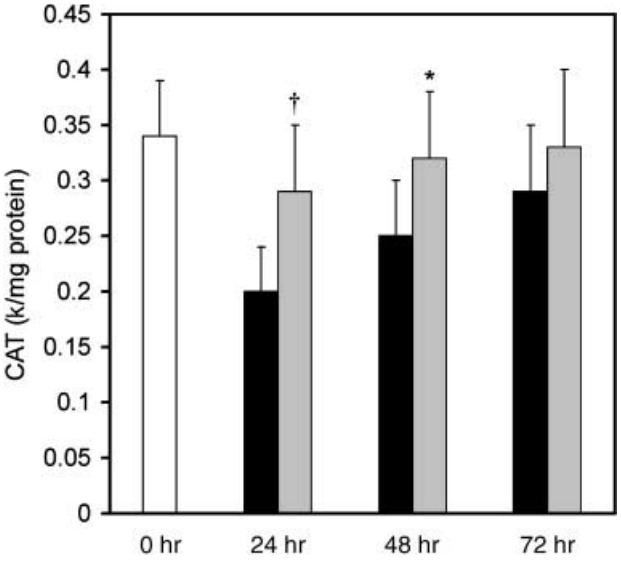

Fig. 5. Renal catalase (CAT) activity in rats receiving $HgCl_2$ alone (black bars) or with thymoquinone (gray bars). Open bar represents control group. *P < 0.05; †P < 0.01; $HgCl_2$–thymoquinone group versus the corresponding $HgCl_2$ group (mean ± S.D., ANOVA).

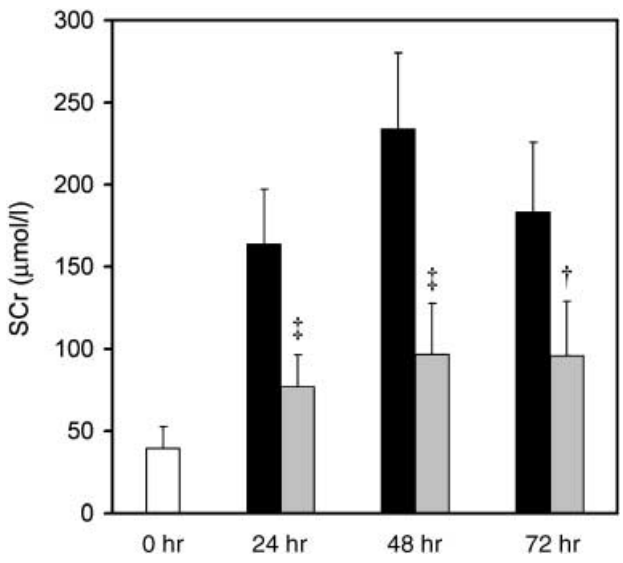

Fig. 6. Serum creatinine (SCr) in rats receiving $HgCl_2$ alone (black bars) or with thymoquinone (gray bars). Open bar represents control rats. †P < 0.01; ‡P < 0.001; $HgCl_2$–thymoquinone group versus the corresponding $HgCl_2$ group (mean ± S.D., ANOVA).

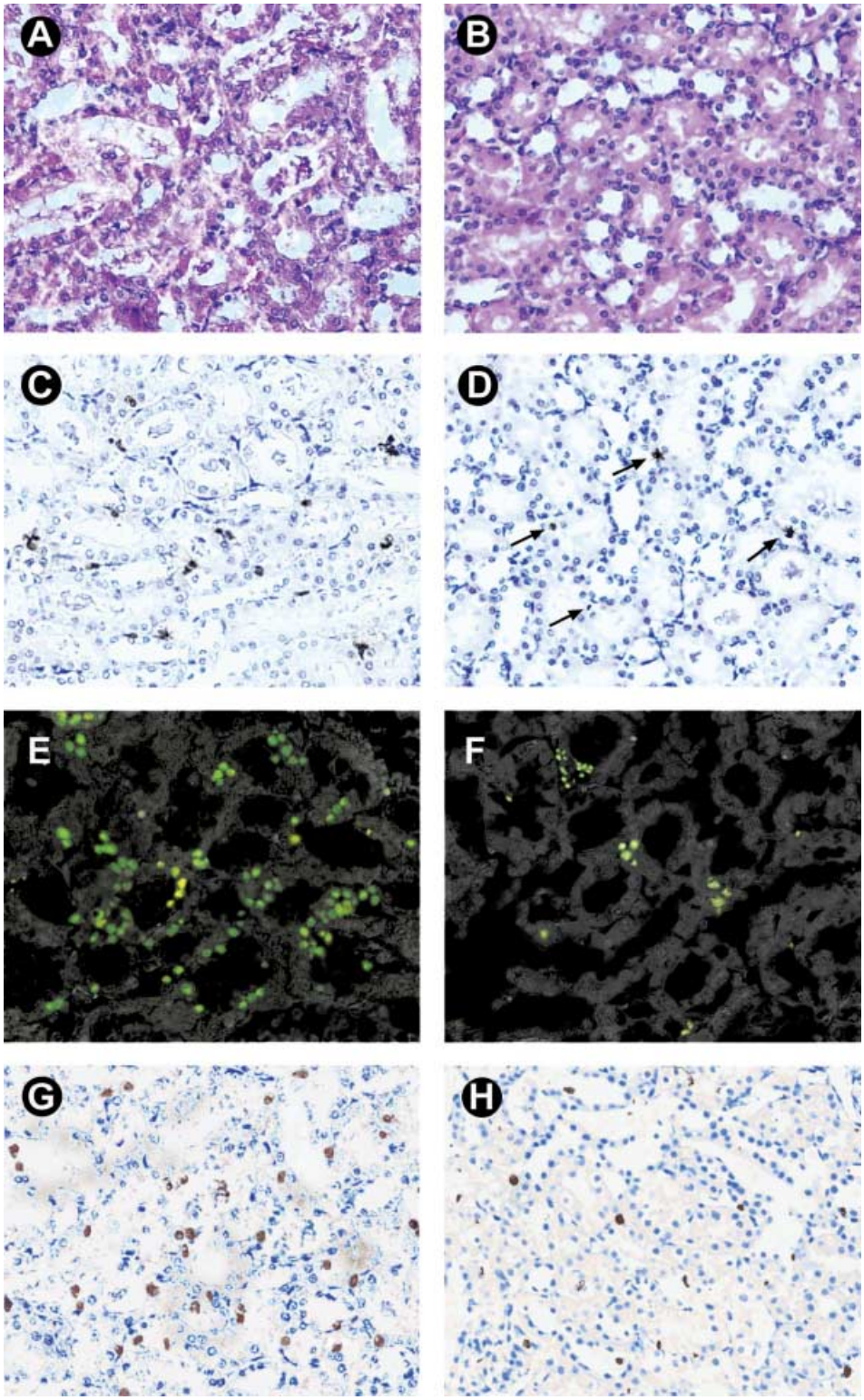

Fig. 7. Micrographs of renal biopsies obtained 48 h from rats treated with $HgCl_2$ alone (left-sided images), and $HgCl_2$ plus thymoquinone (right-sided images). (A, B) Haematoxylin and eosin staining showing significant preservation of renal tubular histology in rats treated with thymoquinone. (C, D) Superoxide staining of superoxide-positive cells showing significant reduction in the SPC count in rats treated with thymoquinone. (E, F) Fluorescent apoptotic cells (TUNEL-positive cells) stained with Apoptag in situ apoptosis detection. Rats that were treated wtih thymoquinone had significantly lower counts of apoptotic cells. (G, H) Proliferating cells (PCNA-positive cells, immunoperoxidase staining). Significant reduction in the PCNA-positive cells was seen in rats treated with thymoquinone. All micrographs are presented at ×400 magnification.

Table 3.

Number of superoxide positive cells (SPC), TUNEL-positive cells representing renal tubular cell apoptosis, and number of proliferating cell nuclear antigen (PCNA)-positive cells at various times in renal sections of rats receiving 3 mg/kg $HgCl_2$ alone or with 10 mg/day of thymoquinone (TQ) orally.

	24 hr		48 hr		72 hr	
	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 7)	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 8)	$HgCl_2$ (n = 6)	$HgCl_2$–TQ (n = 7)
SPC/100 tubulointerstitial cells	8.6 ± 2.8	4.2 ± 1.4[2]	11.4 ± 3.2	5.3 ± 2.5[2]	9.8 ± 3.1	4.6 ± 2.1[2]
TUNEL-positive cells/mm^2	55.7 ± 25.2	25.9 ± 14.3[1]	166.8 ± 39.3	68.7 ± 22.4[3]	223.5 ± 47.9	106 ± 42.4[3]
PCNA-positive cells/mm^2	37.2 ± 15.4	19.7 ± 8.6[1]	133.4 ± 36.5	62.5 ± 23.6[3]	258.6 ± 53	95.7 ± 37[3]

The values in each cell represent mean ± S.D.
Significant differences between TQ-treated groups and corresponding vehicle treated groups: [1]P < 0.05; [2]P < 0.01; [3]P < 0.001 (ANOVA).

and reaching a peak at day 2 (11.4 ± 3.2 SPC/100 tubulointerstitial cells, P < 0.001). Significant reduction in the SPC count in the $HgCl_2$–thymoquinone group was observed during days 1, 2 and 3. At day 2, the reduction in SPC count by thymoquinone treatment was 53.5% (5.3 ± 2.5 SPC/100 tubulointerstitial cells, P < 0.001; table 3, figs 7C,D and 8).

To detect concomitant apoptosis, *in situ* TUNEL staining was employed for the renal sections. In the $HgCl_2$ group, the number of TUNEL-positive cells representing apoptosis showed progressive increase from 55.7 ± 25.2 TUNEL-positive cells/mm^2 at day 1 to 223.5 ± 47.9 TUNEL-positive cells/mm^2 at day 3. In contrast, rats that were treated with thymoquinone had significantly lower counts of apoptotic cells over 72 hr after the nephrotoxic insult. The maximal reduction of apoptotic cell count in the renal interstitium offered by thymoquinone treatment was seen at days 2 and 3 where 58.8% (P < 0.001) and 52.6% (P < 0.001) reductions in the TUNEL-positive cells were seen at days 2 and 3, respectively (table 3, figs 7E,F and 9).

Concomitantly, interstitial cell proliferation was seen from the first day after the nephrotoxic insult in both the $HgCl_2$ and the $HgCl_2$–thymoquinone groups and increased with time to a maximal value 72 hr after the nephrotoxic insult. Increased numbers of mononuclear cells also appeared transiently in the renal interstitium from day 1 and thereafter. Most of these cells were macrophages and T lymphocytes surrounded preferentially the severely injured tubules. However, significant reduction in these events was seen in rats receiving thymoquinone treatment over the 72 hr after the nephrotoxic insult. As with apoptotic cells, the maximal reduction of proliferating cell count in the renal interstitium offered by thymoquinone treatment was seen at days 2 and

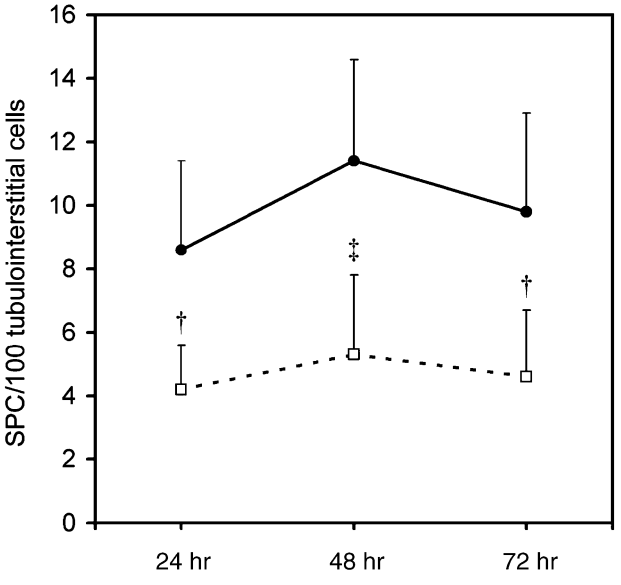

Fig. 8. Number of superoxide positive cells in renal sections of rats receiving $HgCl_2$ alone (filled circles) or with thymoquinone (open squares). †P < 0.01; ‡P < 0.001; $HgCl_2$-thymoquinone group versus the corresponding $HgCl_2$ group (mean ± S.D., ANOVA).

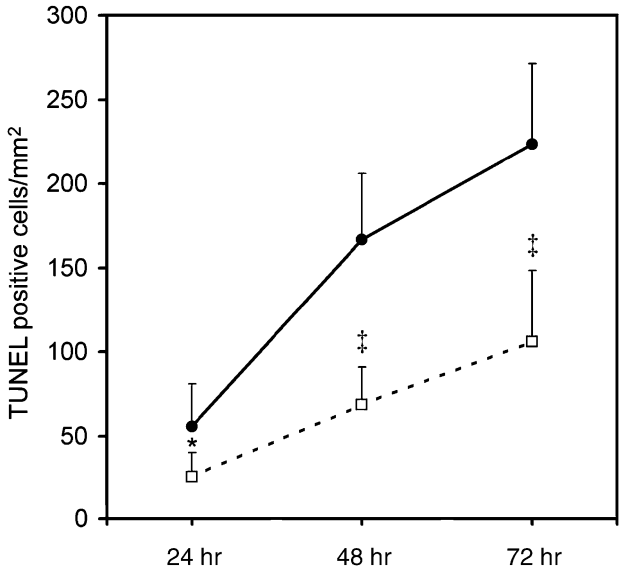

Fig. 9. Apoptosis in renal tubular cells as determined by the number of terminal deoxynucleotidyl transferse-mediated UTP-biotin nick-end labeling (TUNEL)-positive cells per square millimetre of tubulointerstitial areas in rats receiving $HgCl_2$ alone (filled circles) or with thymoquinone (open squares). *P < 0.05; ‡P < 0.001; $HgCl_2$–thymoquinone group versus the corresponding $HgCl_2$ group (mean ± S.D., ANOVA).

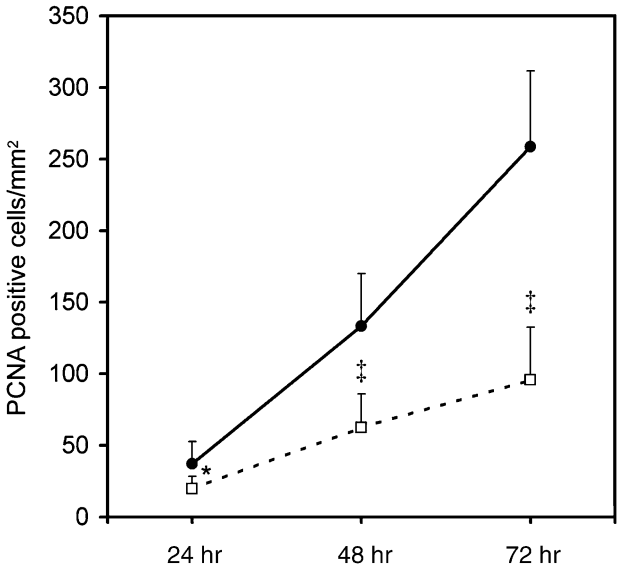

Fig. 10. Interstitial cell proliferation as determined by the number of proliferation cell nuclear antigen (PCNA)-positive cells per square millimetre of tubulointerstitial areas in rats receiving $HgCl_2$ alone (filled circles) or with thymoquinone (open squares). *$P < 0.05$; ‡$P < 0.001$; $HgCl_2$–thymoquinone group versus the corresponding $HgCl_2$ group (mean ± S.D., ANOVA).

3, where 53.2% ($P < 0.001$) and 63% ($P < 0.001$) reductions in the PCNA-positive cells were seen at days 2 and 3, respectively (table 3, figs 7G,H and 10).

Discussion

In this study, we investigated thymoquinone as a protective agent against $HgCl_2$-induced nephrotoxicity, and our results obtained herein demonstrate a clear nephroprotective action by thymoquinone in this experimental model. We chose the dose and route of administration of thymoquinone based on the following data; it has previously been shown that thymoquinone 10 mg/kg protected rats against doxorubicin-induced cardiotoxicity [15] and nephropathy [16], when given once daily in the drinking water 5 days before doxorubicin and continued throughout the duration of the experiments. In addition, thymoquinone 10 mg/kg given once daily orally for 3 consecutive days protected rats from acetic acid-induced colitis [25]. The LD_{50} of thymoquinone was 90.3 mg/kg [26].

Our findings show that a single injection of a nephrotoxic dose of $HgCl_2$ into rats caused rapid increment of the biomarkers of oxidative stress associated with marked renal cellular injury. The changes in the renal MDA, GSH content, and GPx and CAT activities were more pronounced on the first day, with subsequent return towards the control levels, while signs of histological damage, apoptotic events and proliferative reactions were seen from the first day and increased with time to a maximum 48 to 72 hr after the nephrotoxic insult.

Findings from several studies suggest that an important mechanism involved in renal cellular injury induced by exposure to inorganic mercury involves the induction of oxidative stress. The high affinity of mercuric ions for binding to thiols naturally suggests that the ensuing depletion of intracellular thiols (especially GSH) either directly causes, or predisposes, proximal tubular cells to oxidative damage [4]. The activity of several antioxidant enzymes also appears to be markedly diminished after *in vivo* exposure of rats to nephrotoxic doses of $HgCl_2$. Gstraunthaler *et al*. have reported that administration of $HgCl_2$ to male Sprague-Dawley rats caused marked increase in the formation of MDA in renal cortical homogenates with decreased activity of SOD, CAT, GPx and glutathione disulfide reductase in the renal cortex [27]. Decreases in the activities of these protective enzymes would be expected to enhance the susceptibility of renal epithelial cells to oxidative injury. In this work, we found that the rapid increment in renal MDA content, as an indicator of oxidative damage, was significantly reduced by thymoquinone pre-treatment. The thymoquinone-treated rats had also marked preservation of GSH content and GPx and CAT activities. Moreover, 48 to 72 hr after the nephrotoxic insult, the MDA and GSH contents and the GPx and CAT activities in the thymoquinone-treated group had returned to the control levels. In some studies [4,7], inorganic mercury did not influence basal CAT activity. Other studies reported significant decrease in CAT activity following $HgCl_2$ administration into rats [8,27] and mice [28]. In our study, however, pre-treatment with thymoquinone produced an increment in CAT activity as has been reported with other protective enzymes. Indeed, thymoquinone proved to be beneficial in restoring declined SOD and CAT due to ischaemia insult [29].

Glutathione may play an important role in the prevention of mercury-induced oxidative damage as a direct scavenger or by scavenging as a collaborating factor with GPx. In this experimental model, the $HgCl_2$-induced depletion of renal GSH is probably the cause for the early reduction in GPx and consequently the CAT activity as CAT could compensate for the depletion of GPx to a certain degree by scavenging peroxide radicals [30]. The rapid improvement of these cellular defence enzymes may be partially due to prevention by thymoquinone of the rapid depletion of intracellular GSH [31] either by acting as a shield against free radical attacks, or by stimulating the early regeneration of these antioxidant enzymes as well as its ability to prevent the energy decline in kidney tissues [32]. We also observed that GSH content and GPx and CAT activities were partially recovered with no treatment by day 3, when the values in the $HgCl_2$ group were not statistically significant in terms of control. Similar results were also reported by other authors [7,33] who reported partial recovery of oxidative stress biomarkers towards the control levels 3 to 4 days after injection of a nephrotoxic dose of $HgCl_2$.

The antioxidant activities of thymoquinone have been previously reported [10,14,15]. Thymoquinone acts as a scavenger of superoxide, hydroxyl radical and singlet molecular oxygen [9,11]. Previous studies have shown that pre-treatment with thymoquinone protected organs against oxidative

damage induced by a variety of free radical generating agents, including cisplatin [13], carbon tetrachloride [14] and doxorubicin [15]. Moreover, thymoquinone supplementation has recently been shown to prevent deterioration of the biochemical and histological indices of gentamicin-induced nephrotoxicity, which is coincided with the increase in the total antioxidant status in renal cortex, including GSH concentration, GPx and CAT activities [32]. The strong antioxidant potentials of thymoquinone may be related to the redox properties of the quinone structure of thymoquinone molecule and its unrestricted crossing of morphophysiological barriers, and easy access to subcellular compartments facilitates the ROS scavenging effect [11]. Previously, Brunmark *et al*. [34] has shown that quinone reductase, first described as DT-diaphorase [35], catalyses the two electron reduction of quinones to hydroquinones, preventing their participation in redox cycling, and subsequent generation of ROS. Quinone reductase activity was found in all rat tissues including the kidney [36,37] and can catalyse the two electron reduction of thymoquinone to form dihydro thymoquinone [38]. Furthermore, thymoquinone ministration showed a significant induction in the enzyme activity of hepatic quinone reductase [39], but whether quinone reductase is induced in the kidney tissue by administration of thymoquinone or its metabolite dihydro thymoquinone is not known.

In this study, we also show that the number of SPC increased 37-fold after 48 hr of $HgCl_2$ administration. Apoptosis and concomitant tubular cell proliferation increased as well to a maximal value at day 3. Similar results were also reported by other authors [7,33]. Treatment of rats with thymoquinone reduced the number of SPC by 70% and offered imperative protection from $HgCl_2$-induced renal damage. The maximal protection offered by thymoquinone treatment was particularly noticeable 48 and 72 hr after the administration of the toxic agent at the time when histological damage, apoptotic events and proliferative reactions reached their maximum.

Apoptosis is known to be induced by mercury in renal tubular cells both *in vivo* [3] and *in vitro* [40]. Impairment of the cell membrane and mitochondrial dysfunction has been considered to be important actions for mercury-induced renal cell apoptosis [40]. The onset of subsequent renal regeneration is marked by proliferation of surviving tubular cells [41]. Numerous agents are known to trigger apoptosis in kidney cells. Among the physiological activators of particular interest are members of the tumour necrosis factor family, notably, tumour necrosis factor α (TNF-α) [42]. Under basal conditions, tubular cells are quite resistant to TNF-α-induced apoptosis, as would be expected by its constitutive expression in these cells. In contrast, sensitivity to apoptotic cell death induced by TNF-α has been reported to be increased by inhibition of nuclear factor kappa B (NF-κB) [43,44]. Dieguez-Acuña *et al*. [44] have reported that mercury ions within a low concentration specifically impairs thiol-dependent signal transduction processes that are involved in activation of NF-κB and that these effects may increase the susceptibility of kidney cells to the apoptosis-inducing effects of TNF-α and other toxicants to which kidney cells are normally resistant. Unfortunately, we have no reports on the influence of thymoquinone on the expression of NF-κB and/or TNF-α in the renal tubular cells, but alternatively, thymoquinone has been recently shown to inhibit TNF-α production in rat model of rheumatoid arthritis [45]. Additionally and more recently, El Gazzar *et al*. [46] have shown that although thymoquinone has no effect on nuclear expression of NF-κB, but rather modulates its nuclear transactivation thus blocking transcription and production of TNF-α and consequently attenuates the inflammatory response in activated mast cells.

In summary, the present work documents that treatment with thymoquinone offers imperative protection from $HgCl_2$-induced nephrotoxicity. The deterioration of antioxidant enzymes, increment of SCr and histological damage caused by $HgCl_2$ are markedly improved by thymoquinone treatment. Apoptosis and proliferative reactions are also reduced by thymoquinone. These observations may be attributed partly to the considerable antioxidant effect of thymoquinone molecule and suggest that it may be a valuable therapeutic agent against a variety of conditions where cellular damage is a consequence of oxidative stress such as acute renal failure caused by inorganic mercuric intoxication.

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