Thymoquinone Induces Nrf2 Mediated Adaptive Homeostasis: Implication for Mercuric Chloride-Induced Nephrotoxicity

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ABSTRACT: Background: the primary function of the kidney is to eliminate metabolic waste products and xenobiotics from the circulation. During this process, the kidney may become vulnerable to toxicity. Objective: it was aimed to investigate the impact of thymoquinone (TQ) in mercuric chloride (HgCl₂)-induced nephrotoxicity through estimation of various proteins involved in natural defense mechanisms. Material and methods: HgCl₂ (0.4 mg/kg) was administered to all groups (n = 5) except for the normal control. Three treatment groups received TQ (5, 10, and 15 mg/kg) 60 min before HgCl₂ administration. The protective effect of TQ was evaluated from renal and liver function biomarkers, urine examination, glomerulus filtration rate (GFR), histopathological features, oxidative stress biomarkers, Hsp-70, apoptosis biomarkers, and gene expression. Results: TQ significantly attenuated hazardous effects of HgCl₂ on renal and hepatic tissues. Urine albumin and glucose were considerably low in the treated groups in comparison with the HgCl₂ group. TQ treatment also enhanced % GFR in rats. TQ-enhanced superoxide dismutase, catalase, and glutathione levels by enhancing the expression level of nuclear factor erythroid 2-related factor 2 (Nrf2). TQ increased Hsp-70 and Bcl-2 levels and reduced caspase-3 activity. TQ also protected cells against HgCl₂-induced cell death and decreased % DNA fragmentation. TQ increased the expression of protective proteins metallothionein I and II and reduced the expression of kidney injury molecule-1 (Kim-1). Conclusion: TQ showed protective effects against HgCl₂-induced nephrotoxicity through modifications of various constitutive and inducible protein and enzyme levels in renal tissues.

INTRODUCTION

Mercury is known to be a potent nephrotoxic agent that is being widely used in animals for acute renal failure. Hemodynamic changes, renal functional alterations, and histological damages are well described in such experimental in vivo models.¹ The accumulation and toxicity of mercury is because of its binding potential to endogenous molecules containing thiol groups. Increased oxidative stress owing to endogenous thiol depletion is a major hallmark in mercury poisoning. By binding with thiol-containing glutathione (GSH) and other proteins, mercury acts as a catalyst in Fenton-type reactions.² Mercury is considered as a potent pro-oxidant that affects enzymatic and non-enzymatic antioxidants, including catalase (CAT), GSH, superoxide dismutase (SOD), and thioredoxin systems. The mercury–thiol interaction also contributes to the modulation of apoptosis through NF-κB and mitochondrial pathways.³ The kidney is considered as a major target for mercury intoxication. Additionally, renal proximal tubules are the most susceptible parts to toxicants as they are involved in the filtration of substances, their transportation, and relatively high energy demand for these functions.

Thymoquinone (2-isopropyl-5-methylbenzo-1,4-quinone) (TQ), monoterpen, has been regarded as a key component of volatile oil obtained from Nigella sativa. Thousands of in vitro and in vivo studies have revealed the promising role of TQ in various diseases majorly owing to its anti-oxidative potential. TQ may preserve numerous antioxidant enzymes, including CAT, SOD, glutathione peroxidase (GPX), and glutathione-S-transferase. Additionally, it also acts as a free radical scavenger.⁴ It is clearly evident from the bundle of recent scientific reports that TQ has a potential nephroprotective activity against numerous nephrotoxic xenobiotics including dioxin, cadmium, arsenic, manganese, carbon...
Results

Effect on Renal Function Tests. HgCl₂ had deleterious effects on renal functions. Urea level in the HgCl₂ group was significantly higher in comparison with a normal control. Urea level in the treatment group (5 and 10 mg/kg) have reduced considerably in the HgCl₂ group. Depending upon GFR, the HgCl₂ group categorized under stage 4 (Table 3). Moreover, TQ at a dose of 10, 15 mg/kg categorized under stage 1. Furthermore, TQ treatment also showed a statistically significant difference (p < 0.05) as compared to normal and HgCl₂ group respectively.

Effect on Urine Examination. The HgCl₂ group has shown a higher pH value as compared to the normal control. After 24 h, urine volume was significantly decreased as compared to normal control. Glucose and albumin levels were also increased owing to tubular damage, resulting in increased specific gravity. TQ treatment proved useful for the amelioration of these alterations (Table 2).

effect on kidney index. All three treatment groups showed a significant difference in the rat kidney index as compared to the HgCl₂ group (p < 0.05) (Figure 2).

Effect on Glomerulus Filtration Rate. Treatment with HgCl₂ decreased the glomerulus filtration rate (GFR) considerably in the HgCl₂ group. Depending upon GFR, the HgCl₂ group categorized under stage 4 of kidney disease according to the classification provided by National Kidney Foundation as the GFR was less than 60%. While the TQ 5 mg/kg group falls in stage 1. Moreover, TQ at a dose of 10, 15 mg/kg categorized under stage 2 (Table 3).

Effect on Liver Function Tests. Similar to renal functions, HgCl₂ also showed deleterious effects on liver functions resulting in the increased hepatic enzymes found in the serum. All treatment groups reduced the alkaline phosphatase level (ALP) level significantly (p < 0.05) in comparison to the HgCl₂ group (Figure 3A). Similarly, TQ treatment also reduced the alanine aminotransferase (ALT) level at three

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Figure 1. Protective effect of TQ on HgCl₂ induced alteration in kidney function tests. (A) Effect on urea level, (B) effect on uric acid level, and (C) effect on creatinine level. Values are presented as mean ± standard error of the mean (SEM) (n = 6) and analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison post-test. Here "a" and “b” shows statistically significant difference (p < 0.05) as compared to normal and HgCl₂ group respectively, ns: non-significant.

Table 1. Effect of TQ on HgCl₂-Induced Alterations in Urine Volume, pH, Specific Gravity, and Albumin and Glucose Levels

| parameters          | normal control | HgCl₂ group | HgCl₂ + TQ (5 mg/kg) | HgCl₂ + TQ (5 mg/kg) | HgCl₂ + TQ (15 mg/kg) |
|---------------------|----------------|-------------|----------------------|----------------------|-----------------------|
| volume (mL)         | 12.60 ± 0.6760 | 5.220 ± 0.3397 | 10.88 ± 0.8255       | 6.920 ± 0.6851       | 6.900 ± 0.4980        |
| pH                  | 7.2 ± 0.12     | 8.1 ± 0.56  | 7.4 ± 0.31           | 7.6 ± 0.26           | 7.5 ± 0.12            |
| specific gravity    | 1.001          | 1.015       | 1.011                | 1.012                | 1.012                 |
| albumin             | +++            | ++          | +                    | +                    | ++                    |
| glucose             | +++            | ++          | +                    | +                    | ++                    |

+: mild changes, ++: moderate changes, +++: severe changes.

Table 2. Effect of TQ on HgCl₂-Induced Alterations in GFR and Classification of Disease Stage

| groups              | GFR (mL/min) | % age GFR | disease stage |
|---------------------|--------------|-----------|---------------|
| normal control      | 1.750 ± 0.23 | 100%      | 3             |
| HgCl₂ group         | 0.966 ± 0.07 | 55.2%     | 3             |
| HgCl₂ + TQ (5 mg/kg)| 1.635 ± 0.36 | 93.4%     | 1             |
| HgCl₂ + TQ (10 mg/kg)| 1.359 ± 0.13 | 77.6%     | 2             |
| HgCl₂ + TQ (15 mg/kg)| 1.195 ± 0.27 | 68.2%     | 2             |

Effect on Kidney Index. All three treatment groups showed a significant difference in the rat kidney index as compared to the HgCl₂ group (p < 0.05).

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tetrachloride, and chemotherapy. Potential mechanisms behind nephron-protective action of TQ include antioxidant effects, amelioration of inflammation, and anti-apoptosis effects.1-10

Currently, there are some good chelators (e.g., 2,3-dimercaprol) for acute mercury intoxication but they have no effect on low-level constant mercury exposure, as they cannot remove metals from intracellular sites. These chelators cannot subside hazardous effects of mercury exposure. Additionally, they have various associated side effects, including the redistribution of the metal, renal or liver dysfunction, and loss of essential metals along with heavy metals. The purpose of this study was to investigate the role of TQ against inorganic HgCl₂-induced nephrotoxicity through the estimation of various determinants of oxidative stress. It was aimed to explore the defensive mechanisms enhanced by TQ against HgCl₂ intoxication.

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glomerulus shrinkage, dilatation of bowman presented with severe pathological conditions including HgCl$_2$ group (Figure 5B). Similar to CAT, the same trend has showed a signi fi cant change in the CAT level (p < 0.05) with respect to normal and HgCl$_2$ groups, respectively.

Table 3. List of Primers Used in qRT-PCR

| parameter                              | primer sequence (5′→3′)                              | product size (bp) | accession no. |
|----------------------------------------|-----------------------------------------------------|-------------------|---------------|
| kidney injury molecule-1 (KIM-1)       | forward GGGCCGTATTTTCAAGAAGGA reverse TGTTTGAACCATCCAGGAA | 105               | AF_035963.1    |
| metallothionein-I (MT-I)               | forward ACCTCCTGCAAAGAAAGCCTG reverse GGAGGTGTACGGCAAGACTC | 181              | NM_138826.4    |
| metallothionein-II (MT-II)             | forward ACCTCCTGCAAAGAAAGCCTT reverse ACCTCCTGCAAAGAAAGCCT | 92               | H_32024.1      |
| nuclear factor erythroid 2-related factor 2 (Nrf2) | forward AAGTTGCCGCTCAAACACTGT reverse CCTCTTTTAATGTCGCCCAGG | 81               | NM_031789.2    |
| glycer aldehyde 3-phosphate dehydrogenase (GAPDH) | forward GGAGTCCCCTATCCAAAACTCA reverse GCCCATAAACCCCACACAC | 173              | XM_017592435.1 |
Additionally, TQ also boosted the second line of defense antioxidant, primarily GSH. Khalife and Lupidi explored that TQ reacted chemically with GSH, NADH, and NADPH at physiological conditions in the liver which resulted in reduced end products, glutathionylated-dihydrothymoquinone and dihydrothymoquinone, respectively. These reduced forms of TQ showed better scavenging activity than TQ alone and their antioxidant potential was equivalent to trolox. In addition to GSH, TQ also interacted with serum albumin and induced conformational modifications resulting in its enhanced antioxidant activity in a thiol-dependent manner. In the current study, TQ reduced the MDA level in treatment groups. It revealed that TQ provided protection against lipid peroxidation.

It is evident from literature studies that heavy metal exposure is responsible for the induction of the defense mechanism known as a stress response. The stress response is being accompanied by the production of stress proteins including Hsps or MTs. These are highly conserved proteins produced to maintain homeostasis and protect the essential proteins impaired by metals. Hsps are produced due to extreme and prolonged stress exposure. Hsps have been recognized as cyto-protective agents respond to various cellular insults through multiple ways including protein folding, assembling, and translocation of different organelles across membranes, repairing of peptides, and finally degradation of irreparable peptides. Although there are various families of Hsps depending upon their molecular weight but Hsp-70, a molecular weight of 70 kDa, has been recognized as a sensitive biomarker for HgCl2 intoxication and Hsp-70 level begun to increase even from the administration of a single dose of HgCl2 (0.1 mg/kg). There was increased production of Hsp-70 after exposure to HgCl2 in the current study similar to the study conducted by Han et al. Whenever a cell undergoes a prolonged stress, two opposing responses may happen. There may be either enhanced production of stress proteins in order to resist stress or apoptosis may occur in order to prevent inflammation. In the current study, there was the increased level of Hsp-70 in TQ-treated groups in comparison to the HgCl2 group.

Bcl-2, an anti-apoptotic protein, is found at the mitochondrial membrane, where it is involved in the stabilization of the transmembrane potential and decreases permeability across the membrane and finally inhibits the release of cytochrome C. Jiang et al. found that Hsp-70 increased the expression of Bcl-2 and provided the protection against oxidative stress-induced apoptosis. Similarly, the Bcl-2 level was considerably high in TQ-treated groups in comparison to the HgCl2 group. TQ triggered anti-apoptotic signaling by enhancing the expression of Bcl-2 against hepatic ischemic injury. Hsp-70 inhibited apoptosis by directly associating with apoptotic peptidase activating factor-1 (Apaf-1). Hsp-70 may obstruct Apaf-1 oligomerization and sustain its conformation, which is incompatible for pro-caspase-9 recruitment and activation. Activated caspase-9 is responsible for the activation of the caspase cascade. Therefore, we have observed the decreased activity of caspase-3 in treated groups compared to the HgCl2 group.

**Figure 3.** Protective effect of TQ on HgCl2 induced alteration in liver function tests. (A) Effect on ALP, (B) effect on ALT level, and (C) effect on the total bilirubin level. Values are presented as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison post-test. Here, “a” and “b” shows statistically significant difference (p < 0.05) as compared to normal and HgCl2 groups, respectively.

**Figure 4.** Histopathological examination of renal tissues (A−E). (A) Normal control: normal histological appearance of renal tissues. (B) HgCl2 group: glomerulus shrinkage (arrow), dilatation of Bowman’s capsule (arrow head) cystic dilatation in proximal tubules (star), tubular hydropic degeneration (triangle), eosinophilic cytoplasm with nuclear chromatin fragmentations reflecting cell apoptosis (square), and necrotic tubular epithelia (circle). (C) HgCl2 + TQ (5 mg/kg), (D) HgCl2 + TQ (10 mg/kg), and (E) HgCl2 + TQ (15 mg/kg).
reflecting the reduced extent of apoptosis. Caspase-3 is being considered as the most important reliable component of the caspase cascade to determine the extent of apoptosis. DNA fragmentation is being frequently detected in cells as a result of reactive oxygen species (ROS)-induced genotoxicity, but it is prevented with increased Hsp-70 expression. Interestingly, Hsps work hand-in-hand along with the redox system in order to neutralize the damaging effects of oxidative stress.25 It might be a possible mechanism behind reduced DNA fragmentation in treated groups compared to the HgCl2 group in the current study. Previously, Ghani and his colleagues also reported protective effects of TQ against DNA fragmentation.32

Furthermore, heavy metal exposure also triggers another adaptive response, that is, the induction of metallothionein. After heavy metal intoxication, metallothionein genes are rapidly activated. Metallothionein is a group of low-molecular weight (6–7 kDa), cysteine-rich, and metal-binding proteins which acts as scavengers of heavy metal ions and ROS.33 We
have observed elevated metallothionein-I and II mRNA expression after HgCl2 exposure. Interestingly, a considerably higher metallothionein expression was observed when HgCl2 was given in combination with TQ as compared to HgCl2 alone. Actually, TQ also involved in the increased expression of this protective proteins. Elsherby and El-Sherbiny have reported that TQ increased Nrf2 mRNA level. Nrf2 has been recognized as a transcriptional activator that acts as a key role in a cellular response to oxidative stress and heavy metal intoxication by inducing the expression of antioxidant enzymes and metallothioneins.34

Besides of metallothioneins, HgCl2 increased mRNA expression of Kim-1 in renal tissues, which was attenuated by TQ treatment. Kim-1 is a transmembrane protein, which is not detectable in a healthy kidney. The expression of Kim-1 is strongly upregulated after toxic injury. Kim-1 has been recognized as a good biomarker of renal damage as compared to other traditional biomarkers, including blood urea nitrogen, serum creatinine, and urinary N-acetyl-b-D-glucosaminidase, as a Kim-1 expression is rapidly elevated even after exposure of lower doses of nephron-toxicants.35

**MATERIALS AND METHODS**

**Chemicals.** Chemicals, including TQ (Glentham Life Sciences, CAS: 490-91-5), HgCl2 (BDH, Prod: 10154), trizole, cDNA kit, primers, and cyber green (Thermo Fischer Scientific), were used in the current study. Elisa kits for estimation of Bcl-2 and Hsp-70 were purchased from Elabscience Biotechnology Inc.

**Ethical Approval.** The current study design has been approved by the Institutional Review Board of Government College University Faisalabad, Faisalabad with reference no. GCUF/ERC/14. Animals care and experiments were accomplished according to the protocols established by the National Institutes of Health Guide to the Care and Use of Laboratory Animals, National Institutes of Health publication no. 86−23.12

**Animals.** Twenty-five healthy rats of either sex were used in this study. Animals were purchased from physiology department, Government college university, Faisalabad and fed with standard chow diet and water ad libitum.

**Experimental Protocol.** Animals were classified into five groups. Group I taken as the normal control and normal saline (1 mL/100 gm) was administered by the oral route. Group II taken as the disease control and HgCl2 (0.4 mg/kg for 30 days orally) was administered. Group III−V served as treatment groups and received TQ at doses of 5, 10, and 15 mg/kg/day, respectively, for 30 days. HgCl2 (0.4 mg/kg) was given to the treated groups after 1 h of TQ. After 24 h of the last dose administration, rats were anesthetized with isoflurane (5%) in an induction chamber. Cardiac puncture was carried out before

**Figure 9.** Effect on % age DNA strand breakage level. Values are presented as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison post-test. Here, “a” and “b” shows statistically significant difference (p < 0.05) as compared to normal and HgCl2 groups, respectively.

**Figure 10.** Protective effect of TQ on HgCl2 induced modifications in mRNA expression of different proteins. (A) Effect on metallothionein-I mRNA expression, (B) effect on metallothionein-II mRNA expression, (C) effect on Kim-1 mRNA expression, and (D) effect on Nrf2 mRNA expression. Values are presented as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Bonferroni’s multiple comparison post-test. Here, “a” and “b” shows statistically significant difference (p < 0.05) as compared to normal and HgCl2 groups, respectively.
euthanizing the animals and blood was collected; set aside for 30 min and then centrifuged in a temperature-controlled centrifuge machine (D3024R, DLAB) at 4500 rpm for 15 min at 4 °C for serum collection. Rats were euthanized by cervical dislocation. Serum samples were stored in portions at −20 °C for various biochemical parameters. The right kidney and a part of liver were dissected and fixed immediately in 10% formalin solution for histological studies.

**Estimation of Kidney Function Tests.** Urea, creatinine, and uric acid (Bio Vision) were determined from serum samples via an assay method by a chemical analyzer (Microlab-300, EliTech Group) according to the manufacturer’s protocols.

**Urine Examination.** Urine output and urine contents were obtained during a 24 h period from 09:00 AM of one day to 09:00 AM next morning. Food was withheld for few hours in order to collect urine free of food contamination. Urine samples were collected on dry ice, centrifuged, and stored at −10 °C till assayed. Urine volume, pH, specific gravity, and albumin and glucose levels were determined from 24 h of The urine sample.

**Alteration in the Kidney Index.** Right kidney weight was used for the calculation of the kidney index in accordance with the following formula

\[
\text{kidney index} = \frac{\text{right kidney weight}}{\text{body weight}} \times 100
\]

**Estimation of GFR.** GFR was estimated by using renal creatinine clearance as an indicator according to the following formula

\[
\text{GFR} = \frac{U_c \times V}{P_c}
\]

\(U_c\): urinary creatinine level (mg/mL), \(V\): volume of urine (mL/min), and \(P_c\): plasma creatinine level (mg/mL).

**Estimation of Liver Function Tests.** Serum total bilirubin, ALT, and ALP (Bio Vision) were estimated by the assay kit method by using a chemical analyzer.

**Histopathological Examinations.** Small sections of renal tissues were washed with the help of normal saline and fixed in 10% formalin solution, embedded in liquid paraffin; and was cut; and was finally stained by eosin and hematoxylin. They were observed under a light microscope at 40X.

**Oxidative Stress Biomarkers. Preparation of Tissue Homogenate.** The left kidney was weighed and homogenized in 10 volume of ice-cold phosphate-buffered saline (2.68 mM KCl, 136.75 mM NaCl, 1.76 mM KH2PO4, and 10.14 mM Na2HPO4, pH 7.4) in a bullet blender homogenizer (BBYSE-CE, Next Advance, Inc.) for 10 min. Homogenates was centrifuged at 3500 rpm at 4 °C for 5 min. The fractions of the supernatant were collected for further biochemical analysis.

**Estimation of CAT Activity.** For the assessment of CAT activity, the mixture comprises 50 μL of the tissue homogenate, potassium phosphate buffer (50 mM, 1.95 mL, pH 7.4), and H2O2 (30 mM, 1 mL). Absorbance was observed at a wavelength of 240 nm by using a spectrophotometer (CE-7400S, CECIL instruments).13

\[
\text{CAT} = \frac{\delta \text{OD}}{E \times V \times \text{mg of protein}}
\]

\(\delta\): variation in absorbance/min, \(V\): volume of sample, and \(E\): extinction co-efficient of H2O2 (0.071 mmol/cm)

**Estimation of SOD Activity.** The reacting mixture comprises 100 μL of tissue homogenate, 2.8 mL of potassium phosphate buffer (pH 7.4), and 10 μL of pyrogallol solution. Absorbance was recorded at 325 nm.13

\[
Y = 0.0095x + 0.1939
\]

**Estimation of the MDA Level.** The MDA concentration reflects the extent of lipid peroxidation. For this estimation, tissue homogenate (1 mL) was added in thiobarbituric acid (3 mL); the solution was shaken and placed on ice for 15 min. After cooling, the solution was centrifuged at 3500 rpm for 10 min. The supernatant layer was separated and absorbance was observed with a spectrophotometer at 532 nm.13

\[
\text{MDA} = \frac{Y \times 100 \times V_t}{1.56 \times 10^5 \times W_t \times V_u}
\]

\(V_t\): total volume of assay (4 mL), \(W_t\): weight of tissue (g), \(V_u\): volume of aliquot, and \(Y\): absorbance, 1.56 × 105 = molar extinction coefficient.

**Estimation of GSH Level.** Tissue homogenate (1 mL) was precipitated with 1 mL of triclyceric acid (10%). 5,5 Dithio-bis-2-nitrobenzoic acid (DTNB) reagent was made by dissolving 29.78 mg of DTNB in 25 mL of methanol. Afterward, 4 mL of sodium phosphate buffer solution (0.1 M, pH 7.4) and 0.5 mL of DTNB reagent were added and absorbance was observed at 412 nm.14

\[
\text{GSH} = \frac{Y - 0.00314}{0.0314} \times \frac{\text{DF}}{B_i \times V_u}
\]

\(Y\): absorbance, DF: dilution factor (1), \(B_i\): tissue homogenate (mL), and \(V_u\): volume of aliquot.

**Estimation of Total Protein Contents.** Reagents 1 and 2 were prepared first. Reagent 1 contained solution A (48 mL) (2% Na2CO3 in 0.1 N NaOH), solution B (1 mL) (1% C6H5KNaO6 in H2O), solution C (1 mL) (0.5% CuSO4·5H2O) in water. Reagent 2 contained 2 N folin-phenol and H2O (1:1). Tissue homogenate (0.2 mL) was added in reagent 1 (4.5 mL) and incubated for 10 min. Then, reagent 2 (0.5 mL) was added in the mixture and incubated again for 30 min. Absorbance of the mixture was recorded at 660 nm. Various concentrations of bovine serum albumin were employed for the development of the regression line.

\[
Y = 0.00007571x + 0.00004762
\]

**Enzyme-Linked Immunosorbent Assay (ELISA).** Hsp-70 (CAT no. E-EL-H1863, Elabscience Biotechnology Inc.) and Bcl-2 (CAT no. E-EL-H0114, Elabscience Biotechnology Inc.) levels were determined by the ELISA method according to the manufacturer protocol. Briefly, antigen (tissue homogenate) was added to a well plate, pre-coated with a rat Bcl-2 primary antibody. Biotinylated detection antibody specific for rat Bcl-2 and Avidin-horseradish peroxidase conjugates were added sequentially to the well plate and incubated. Free components were washed away. Afterward, substrate solution was added. The reaction was stopped by adding the stop solution. The absorbance was estimated by a micro plate ELISA reader (Biobase-EL 10A) at a wavelength of 450 nm.

**Estimation of Caspase-3 Activity.** One unit of caspase-3 activity is the quantity of enzyme that is needed to cleave 1.0 nM of the colorimetric substrate Ac-DEVD-pNA per hour at 37 °C under saturated substrate concentrations. Caspase-3 activity (CAT no. E-CK-A311, Elabscience) was estimated by a colorimetric method according to the manufacturer’s...
protocol. Briefly, 200 μL of cold lysis buffer was added in 50 mg of the renal tissue in order to prepare the tissue homogenate. 2X reaction working solution (50 μL) was added into the tubes and then separately added 45 μL of sample homogenate in sample tubes, and 45 μL lysis working solution in blank tubes. Finally, 5 μL of Ac-DEVD-pNA was added and thoroughly mixed. The mixture was incubated for 4 h at 37 °C. Absorbance was observed at 405 nm by a spectrophotometer (CE-7400S, CECIL instruments). Caspase-3 activity was calculated by using the formula

\[
\text{Caspase 3 activity } = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{negative control}} - \text{OD}_{\text{blank}}}
\]

Estimation of Percentage DNA Strand Breakage. The percentage DNA strand breakage was calculated by the process of followed by Imtiaz et al. The renal tissue was first homogenized in Tris-EDTA buffer (pH 8.0) (1:10 volume). Tissue homogenates were centrifuged for 20 min at 27,000 rpm for the isolation of the chromatin pellet and supernatant, containing the fragmented chromatin material. The pellet was again suspended in Tris-EDTA buffer (pH 8.0). Equal volumes of the pellet suspension and supernatant (0.5 mL) were taken in test tubes, afterward recently prepared diphenylamine solution (1.5 mL) was put into test tubes. Reaction mixtures were incubated for 20 h at 37 °C. Absorbance of reaction mixtures were recorded at 620 nm by a spectrophotometer. The percentage DNA strand breakage was estimated by using the formula

\[
\% \text{ DNA strand breakage } = \frac{\text{absorbance of supernatant}}{\text{absorbance of supernatant} + \text{absorbance of pellet}} \times 100
\]

Estimation of mRNA Expression by RT-PCR. RNA extraction was done from renal tissues by the Trizol method. Renal tissue (100 mg) was treated with 1 mL of Trizol (lot no. 00675453, Thermo Fisher Scientific). Isolated mRNA was transcribed into the first strand cDNA by using the RevertAid First Strand cDNA synthesis kit (K1622, Thermo Fisher Scientific). The reaction was carried out in thermal cycler PCR (T100, BIO-RAD) according to manufacturer’s protocols: the mixture was incubated at 42 °C for 60 min and the reaction was terminated by heating at 70 °C for 5 min. The amplification and quantification of cDNA was carried out in real-time PCR (CFX96 Real Time System, BIO-RAD) by using a Maxima SYBR Green/ROX qPCR Master Mix (2X) (lot no. 00798833 Thermo Fisher Scientific) according to the manufacturer’s protocols. Briefly, 1 μL of cDNA was mixed with 5 μL of the master mix (2X), 2 μL of nuclease-free H2O, and 1 μL of forward and reverse primers each. Primer sequences of targeted and reference genes is given in Table 1. GAPDH was employed as an internal reference gene. The reaction was carried out as follows: 95 °C for 5 min followed by 40 cycles (denaturation for 15 s at 95 °C, annealing for 20 s at 60 °C, and extension for 15 s at 72 °C). PCR data were analyzed by the Livak–Schmittgen method by comparing threshold cycle CT value with realplex software. This method makes the comparison between two values in the exponent that represent the normalized expression values for a gene of interest (GOI) in sample type A relative to sample type B. A GOI in both sample types A and B are normalized using a reference gene (REF) and then compared to one another in the exponent.

Relative quantification was calculated according to the following formula,

\[
R = 2^{-(\Delta C_{\text{A}} - \Delta C_{\text{B}})}
\]

Statistical Analysis. All numerical values were expressed as mean ± SEM. One-way ANOVA followed by Bonferroni or Dunnett’s post-test were applied for statistical analysis using GraphPad Prism version 5. p < 0.05 was set as a statistically significant value.

CONCLUSIONS
The results of the current study have shown that TQ may provide protection against the hazardous effects of low level chronic HgCl2 exposure. Unlike classical chelators for xenobiotics, TQ molecules has potential to go intracellularly where it may neutralize hazardous effects produced by HgCl2. TQ at a dose of 5 mg/kg produced the best results. TQ amplified the natural defensive mechanisms to cope with the HgCl2-induced oxidative stress level and reduced the cell death. TQ, as an antioxidant, improved the redox balance that is majorly disturbed with xenobiotic exposure. The results of the current study have shown that a TQ upregulated Nrf2 expression, which in turn potentiate SOD and GSH thus maintains a redox balance. Nrf2 was also found to be involved in the upregulation of metallothionein and hsp expression. Hsp-70 has a critical role in the control of HgCl2-induced apoptosis. Briefly, TQ may potentiate adaptive responses and assist the cells to cope with stress situation and prevent apoptosis.

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Author Contributions
U.S. and M.S.H.A. supervised the project and designed the research. S.S. performed the experiment and prepared the manuscript. M.Q. did qRT-PCR. Z.C. performed the analysis.

Notes
The authors declare no competing financial interest.

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