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# Nrf2/ARE is the potential pathway to protect Sprague–Dawley rats against oxidative stress induced by quinocetone

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ABSTRACT

3-methyl-2-quinoxalin benzenevinylketo-1, 4-dioxide (Quinocetone, QCT) is a newly used veterinary drug which has been proven to promote feed efficiency and growth of animals; however, its potential toxicity can't be ignored. Therefore, the present study was aimed to investigate the nephrotoxicity of QCT and the oxidative stress induced by it. Sprague–Dawley rats (SD rats) were randomly divided into four groups with doses of 2400, 800, 50 and 0 mg/kg/day with administration of QCT for 4 weeks. Results proved that QCT could induce nephrotoxicity and this phenomenon had dose dependent manner. Simultaneously, this phenomenon was accompanied by intracellular reactive oxygen species (ROS) accumulation, enhanced lipid peroxidation and inhibited antioxidant system, i.e. glutathione S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GSH). Additionally, the higher expression of Nrf2 in QCT treated groups illustrated that QCT-induced oxidative stress would be partly mitigated by the induction of phase II detoxifying enzymes *via* increasing Nrf2 expression.



## 1. Introduction

3-methyl-2-quinoxalin benzenevinylketo-1,4-dioxide (Quinocetone, QCT) is a newly used veterinary drug in P.R. China which has been proven to promote feed efficiency and growth of animals (Li et al., 2007). Furthermore, QCT can inhibit many kinds of intestinal pathogens, such as *Staphylococcus aureus, Esherichia coli, Clay burt's coli*, *Dysentery bacterium*, etc. (Zhou, 2005). Nevertheless, recent studies found that QCT induced a lot of adverse effects both *in vivo* and *in vitro*. In Vero cells, the cell viability result indicated that QCT could lead to severe inhibitory effects in both dose and time dependent manner and meanwhile comet assay illustrated that QCT enhanced DNA damage (Chen et al., 2009). Similarly in human hepatoma cells, the cell viability test and comet assay indicated that QCT inhibited cell proliferation and induced significant DNA fragment migration (Jin et al., 2009). On the other hand, *in vivo* tests indicated that, when feeding QCT (1800 mg/kg) to Wistar rats, body weights, feed efficiency, fetal body lengths, tail lengths, litter weights and number of viable fetuses all significantly decreased (Wang et al., 2012). Evaluating sub-chronic oral toxicological test in Wistar rats with dose of 1800 mg/kg/day, researchers discovered that QCT could induce renal damage (Wang et al., 2010).

Previous literatures mainly mentioned about the adverse effects of QCT. However, related toxicological mechanisms are not yet clear. Some members of QdNOs (i.e. olaquindox, mequindox and cyadox) have been proven to induce DNA damage *in vitro*. It was associated with oxidative stress due to excessive ROS generation (Huang et al., 2010). As the member of QdNOs, in our prior study, we already demonstrated that QCT would induce ROS generation and oxidative DNA damage as well. Simultaneously, we found the antioxidant supplementation could attenuate this phenomenon (Wang et al., 2011). In this study, we aimed to find out the cell defense system to protect itself from oxidative damage.

*Abbreviations:* QCT, quinocetone; ROS, reactive oxygen species; SD rats, Sprague–Dawley rats; QdNOs, quinoxaline-1,4-dioxides family; GST, glutathione S-transferase; HO-1, heme oxygenase-1; GPx, glutathione peroxidase; GCL, glutamate cysteine ligase; PRX I, peroxiredoxin I; H-QCT, high QCT group (2400 mg/kg/day); M-QCT, media QCT group (800 mg/kg/day); L-QCT, low QCT group (50 mg/kg/day); BUN, blood urea nitrogen; Cr, creatinine; MFD, mean fluorescence density; GSH, glutathione reductase; MDA, malonaldehyde; SOD, superoxide dismutase; CAT, catalase; OTM, olive tail moment; TBS, tris base solution; BSA, bovine serum albumin; PI, propidium iodide; FITC, fluorescein isothiocyanate; SEM, standard error of the mean; ANOVA, one-way analysis of variance for homogeneity; Keap1, kelch-like ECH-associated protein 1; ARE, antioxidant response element.

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The Nrf2-Keap1 system is a major cellular defense mechanism against oxidative stress. Nrf2 activates genes that encode phase II detoxifying enzymes and antioxidant enzymes, such as GST, HO-1, GPx, GCL and PRX I, which play crucial roles in cellular defense by improving the removal of ROS (Pedruzzi et al., 2012). In addition, a recent study showed that ginsenoside Rb1 attenuated acute renal injury induced by intestinal ischemia reperfusion by activating the Nrf2/ARE pathway (Sun et al., 2012).

Consequently, this time we focused on considering nephrotoxicity of QCT and the oxidative stress induced by it. Simultaneously, we discussed the cell defense system which could mitigate or repair ROS-induced cell damage.

## 2. Materials and methods

### 2.1. Materials

QCT ($C_{18}H_{14}N_2O_3$, purity > 98%) was provided by Hubei Zhongmu Anda Pharmaceutical Co., Ltd. (Wuxue, Hubei, China). The chemical structures of QCT and its metabolisms had been reported in detail (Shen et al., 2010).

### 2.2. Animals and treatment

A total of 40 male SD rats were procured from Sino-British Sippr/BK (Shanghai, China) and used after 1 week acclimatization. In order to ensure the health of each animal, the body weights and detailed physical examinations were recorded twice during the acclimation period. An independent cage was applied for every single animal. Feed and tap water were provided freely during the non-exposure periods. A Specific-Pathogen Free (SPF) level room in which the light–dark cycle (12–12 h, lights on 7:00–19:00), ventilation (air-exchange rate of 18 times per hour), temperature (23 ± 2 °C) and relative humidity (55 ± 5%) were strictly controlled was supplied for the animals during the study. The cages and the chip bedding were exchanged twice a week. The use of animals in this study was in accordance with "Guidelines for the Care and Use of Laboratory Animals, 1996" that prepared by National Institute of Health.

### 2.3. Study design

The SD rats were randomly divided into four groups and treated with QCT by gavage. The doses of QCT used in the present study were calculated according to the $LD_{50}$ given in the previous study (Wang et al., 2010). They were 2400 mg/kg/day (H-QCT), 800 mg/kg/day (M-QCT), 50 mg/kg/day (L-QCT) and 0 mg/kg/day (control) respectively. Clinical observations were recorded daily, food consumption and body weights were measured weekly. Serum from blood samples was collected and stored at −80 °C after the 4-week administration. In each group, sections of one side kidney were placed in 10% neutral buffered formalin and stained with hematoxylin and eosin (H&E). Sections of another side kidney were washed with ice cold isotonic saline. Finally all sections were stored at −80 °C after been drying out (by blotting between two pieces of filter paper).

### 2.4. BUN and Cr levels in serum

Serum was centrifuged at 3500*g* for 10 min at 4 °C (Eppendorf centrifuge 5804R, Germany). Then the BS-200 automatic biochemistry analyzer (Mindary Co., Ltd.) was used to measure the BUN and Cr levels in serum.

### 2.5. ROS in renal cells

Dihydroethidium (Invitrogen, Corporation) was used to measure ROS in the renal cells (Carter et al., 1994). 10 μm cross-sections of unfixed, frozen kidney tissues were incubated with 5 μmol dihydroethidium (diluted in PBS) at 37 °C for 15 min. Using a Nikon 2000S fluorescence microscope (Nikon, Melville, NY) to observe the slides that were washed twice by ice-cold PBS. Three samples of kidney in each group were detected and the MFD was calculated as fluorescence intensity per unit area and assayed using Image-pro Plus 5.0 (Media Cybernetics, Corporation).

### 2.6. Antioxidant capacity biomarkers in blood

Carefully weighing the sections of kidneys which had been stored at −80 °C, then the sections were homogenized in ice cold 50 mmol/l phosphate buffer (containing 0.1 mmol/l EDTA). In order to remove all the cell debris and nuclei, the homogenate was centrifuged at 3500*g* for 10 min at 4 °C (Eppendorf centrifuge 5804R, Germany). The final supernatant was stored at −20 °C for kinds of biochemical assays.

Then the protein concentrations of the tissue homogenate samples were measured according to the prior study (Lowry et al., 1951). The levels of GSH and MDA and the activities of SOD, GPx, CAT and GST were measured using commercial assay kits (Nanjing Jiancheng Institute, China). All the procedures were performed in accordance with the manufacturer's instructions. The levels of GSH and MDA were expressed as mg/g protein and nmol/mg protein respectively. Besides, the activities of SOD, GPx, CAT and GST were all expressed as units/mg protein.

### 2.7. Comet assay in vivo

The Comet assay was mainly performed according to our prior study (Wang et al., 2011). The isolation of nephric cells was basically conducted (Sasaki et al., 1997). Each cell suspension was chosen three slides to prepare. The microscope slides were first layered with 0.8% normal melting-point agarose (Sigma, USA) and the cell suspensions that embedded in a layer of 0.5% of low melting-point agarose (Sigma, USA) were placed onto it. Then the slides were immersed in a lysing solution for 1 h at 4 °C, afterwards they were removed and placed on a horizontal gel electrophoresis unit (filled with freshly prepared alkaline electrophoresis buffer, 1 mM EDTA and 300 mM NaOH, pH > 13). In this unit, DNA was unwound and shown as single-strand breaks. Next, electrophoresis was conducted for 20 min at 0–4 °C by applying an electric current of 0.7 V/cm (25 V/300 mA). After electrophoresis, the slides were immediately neutralized with 0.4 M Tris (pH 7.5) and then they were air-dried and stored at room temperature until scored for DNA migration. After being stained with ethidium bromide, 150 randomly selected cells were analyzed under a Nikon 2000S fluorescence microscope (Nikon, Melville, NY) (magnification 400×) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. The microscope was connected through a camera to a computer-based image analysis system (Comet Assay IV software, Perspective Instruments). Three samples of kidney in each group were detected. The OTM (Olive Tail Moment) and Tail DNA% were calculated by Comet Assay Software Project (CASP) 1. 2. 2 (University of Wroclaw, Poland). OTM was first presented by Olive, which was calculated by multiplying the percent of DNA (fluorescence) in the tail by the length of the tail. And the tail length is measured between the edge of Comet head and the end of the Comet tail (Olive et al., 1990; Nesslany et al., 2007).

### 2.8. Nrf2 expression by confocal microscopy

The Nrf2 expression in kidney was determined with primary antibody rabbit polyclonal IgG Nrf2 [Cat No. sc-13032] and goat anti-rabbit–FITC-conjugated secondary antibody and confocal microscopy (Sriram et al., 2009). Briefly, at first the renal sections