

LABORATORY STUDY

Quercetin ameliorates methotrexate-induced renal damage, apoptosis and oxidative stress in ratsMustafa Erboğa¹, Cevat Aktas¹, Zeynep Fidanol Erboğa¹, Yeliz Bozdemir Donmez¹, and Ahmet Gurel²¹Department of Histology and Embryology, Faculty of Medicine, University of Namik Kemal, Tekirdag, Turkey and ²Department of Biochemistry, Faculty of Medicine, University of Namik Kemal, Tekirdag, Turkey**Abstract**

Background: In the present study, the protective and therapeutic effects of quercetin (QE) on renal injury induced by methotrexate (MTX) have been examined. **Materials and methods:** A total of 24 male rats were divided into the following three groups: control group, MTX group, and MTX + QE group. Rats in MTX group received 20 mg/kg of single dose of MTX, while those in MTX + QE group received 20 mg/kg of single dose MTX, in addition to 15 mg/kg of QE administered 30 min prior to MTX and in the following 5-day period as a single daily dose. At the end of the experimental period, renal tissues were removed for histopathological and biochemical assessments. **Results:** Light microscopic examination showed a disruption of the renal structure in rats in MTX group in the form of tubular degeneration and dilation, with shedding of the tubular epithelial cells into the lumen. QE treatment was associated with less marked degenerative changes, with a similar histological appearance to that of controls. Furthermore, QE treatment resulted in decreased the number of apoptotic cells. Biochemical assessments showed significantly higher malondialdehyde (MDA) levels in MTX group as compared to control and MTX + QE groups. superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) levels showed a significant decrease in MTX group as compared to controls. However, QE significantly suppressed MDA level, compensated deficits in the anti-oxidant defenses [reduced SOD, GSH-Px, and CAT levels] in kidney tissue resulted from MTX administration. **Conclusions:** In conclusion, renal toxic effects of MTX may be alleviated by QE.

Keywords

Apoptosis, methotrexate, nephrotoxicity, oxidative stress, quercetin

History

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Introduction

Methotrexate (MTX), a folic acid antagonist, is not only used as a chemotherapeutic agent for the treatment of leukemia, osteosarcoma, head and neck tumors, lung cancer, and certain other types of cancer, but also has anti-inflammatory, anti-proliferative, immunosuppressive, and anti-psoriatic effects, with appropriate clinical indications.¹

Despite this wide range of indications, MTX therapy is associated with a number of adverse reactions, particularly pneumonia, nephrotoxicity, and hepatotoxicity that may necessitate dose reductions or discontinuation of the treatment.^{2,3} In previous studies, oxidative injury mostly due to reactive oxygen species has been implicated in the majority of such adverse effects, particularly hepatotoxicity and nephrotoxicity.^{3,4}

One of the most common effects observed after MTX use is the functional impairment in kidneys.⁵ Administration of MTX to rats has been found to decrease glutathione (GSH) levels in kidneys and small intestines, and increase the levels

of myeloperoxidase and malondialdehyde (MDA), which are indicators of the inflammatory response and lipid peroxidation, respectively.³

Quercetin (QE) is an anti-oxidant compound and represents an important member of the flavonoids. QE has several biological functions such as anti-oxidant, anti-mutagenic, anti-cancer, anti-viral, anti-thrombotic, anti-ischemic, anti-inflammatory, and anti-allergic effects, as well as preventive effects on arteriosclerosis and coronary heart disease, and stimulation of cellular immunity.^{6,7} Studies at a cellular level found inhibitory effects of QE on colon, stomach, prostate, breast, cervix, ovarian, lung, and liver cancer cells. Also, it has been found to exert anti-aggregating effects on platelets and anti-hypertensive effects.⁸ In a study by Ishikawa et al. involving renal glomerular cell cultures, QE showed anti-apoptotic effects as well.⁹

Agents with potent anti-oxidant effects have been tested in terms of their efficacy to prevent MTX-related side effects, with significant success in some of these studies.^{10,11} However, the results of these studies with these agents have not been adequately reflected in the clinical studies due a number of factors such as the high cost of such chemicals or absence of licensed preparations.

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Thus, we aimed to examine the anti-apoptotic and anti-oxidant effects of QE in MTX-induced nephrotoxicity in a rat model considering its low acquisition cost, wide availability, and proven *in vivo* and *in vitro* anti-oxidant effects.

Materials and methods

Animals

Twenty-four healthy Sprague Dawley male rats (8–10 weeks old) were utilized in this study. All animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health. Our study was approved by Namik Kemal University, Local Animal Ethics Committee and ethical rules were observed during our study (Permission number: NKU HADYEK 2014/04-3, 03.07.2014).

Experimental protocol

The rats were randomly allotted into one of three experimental groups: control, MTX, and MTX + QE; each group contains eight animals. Rats in the control group only received physiological saline through the intraperitoneal (i.p.) route. Rats in MTX group received a single 20 mg/kg i.p. dose of MTX (50 mg flakon[®], Koçak Farma, Tekirdağ, Turkey) dissolved physiological saline. Those rats in MTX + QE group received a single dose of MTX on day 1 (20 mg/kg, in physiological saline, i.p.) in addition to a daily dose of QE (5 mg/kg, in physiological saline) administered 30 min prior to MTX and in the following 5-day period as a single daily dose.

At the end of the experiments, all rats were sacrificed by decapitation under ketamine-xylazine anesthesia. After bilateral nephrectomies were carried out, the right kidney was put into Bouin’s solution for histopathological examination and left kidney was preserved at -80°C until analysis of MDA, superoxide dismutase (SOD), catalase (CAT), and GSH-px levels was performed.

Dosage of MTX was determined from those described by relevant literatures.^{11–13} The dose of QE was decided based on a recent study reported by Aktöz et al.¹⁴

Histopathologic evaluation

Biopsies from the renal tissues of the rats were harvested and tissue fragments were fixed in Bouin’s solution, embedded in paraffin, sectioned at 5 μm thickness and then stained with H&E. The preparations were evaluated by light microscope and photographed (Olympus CX41 microscope, Olympus, Tokyo, Japan).

Renal tissue injury index was performed quantitative analysis, the assessment was expressed as the sum of the individual score grades from 0 (no findings), 1 (mild), 2 (moderate), to 3 (severe) for each of the following parameters from kidney sections: tubular degeneration, tubular dilatation, tubular cell swelling, cellular vacuolization and glomerular congestion and degeneration.

TUNEL assay

Apoptosis was evaluated by the terminal dUTP nick end-labeling (TUNEL) assay. The TUNEL method, which detects

fragmentation of DNA in the nucleus during apoptotic cell death *in situ*, was employed using an apoptosis detection kit (ApopTag[®] Peroxidase In Situ Apoptosis Detection Kit, Cat. No. S7100).

Three randomly selected slides, each of five different fields at high-power lens visual fields ($\times 400$), and the apoptotic cells were identified by the intense dark nuclear staining. Total cell population and TUNEL positive cells were counted. TUNEL positive cells were expressed as a percentage of total cells.¹⁵

Lipid peroxidation and anti-oxidant enzyme analysis

The protein content of the supernatant was determined using the Lowry method.¹⁶ MDA (nmol/mg protein), SOD (U/mg protein), CAT (U/mg protein), and GSH-Px (U/mg protein) levels were determined according to the methods of Buege and Aust,¹⁷ Sun et al.,¹⁸ Aebi,¹⁹ and Paglia and Valentine,²⁰ respectively.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (S0064 Minitab Release 13, License number: WCP1331.00197). All data were presented in mean \pm SD. Differences in measured parameters among the three groups were analyzed with a nonparametric test (Kruskal–Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann–Whitney *U*-test. These differences were considered significant when probability was less than 0.05.

Results

Histopathologic findings

The control group showed regular morphology with no evidence of histopathological changes (Figure 1a). MTX treatment showed prominent degenerative changes with tubular degeneration, tubular dilatation, tubular cell swelling, cellular vacuolization, glomerular congestion and degeneration in the renal cortical tissues (Figure 1b). On the contrary, QE cure was able to restore MTX-induced histopathological changes (Figure 1c).

In addition, renal tissue injury index was higher in MTX group comparing to MTX + QE group significantly, and it was higher in MTX + QE group comparing to control group significantly (Figure 1d).

TUNEL findings

TUNEL method was used in order to determine apoptosis in renal tissues of all groups. In control group, only a few TUNEL positive cells were observed (Figure 2a). After the MTX treatment a great number of TUNEL positive apoptotic cells were observed on the wall of tubules and glomeruli (Figure 2b). QE therapy was seen to decrease the number of apoptotic cells (Figure 2c).

TUNEL positive cells were counted and apoptotic index values were calculated for all groups. The percent of TUNEL positive cells increased in the renal tissue after MTX administration as compared with control group and it was found statistically to decrease significantly in MTX + QE

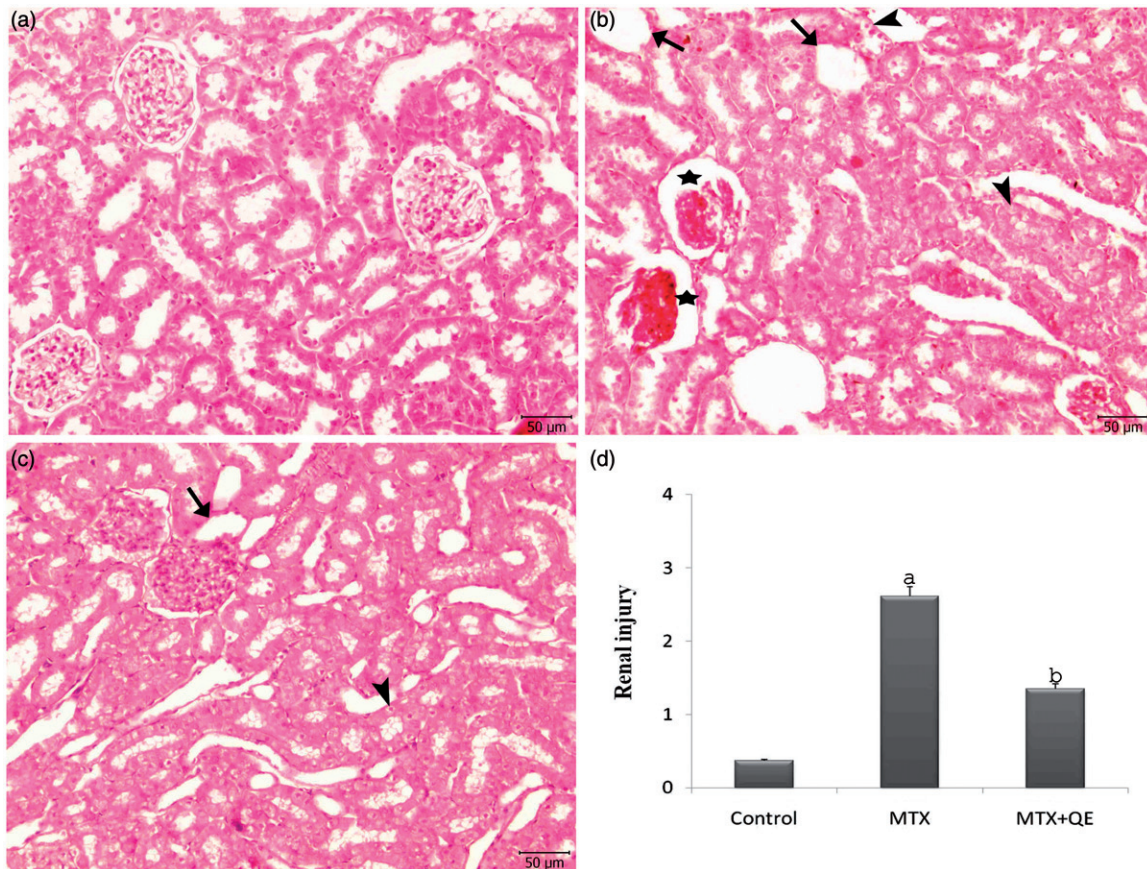


Figure 1. Light microscopy of renal cortical tissues in different groups for H&E. (a) In control, normal renal tissue morphology was seen. (b) After MTX, severe tubular and glomerular damage was noted. (c) QE treatment prevented tubular and glomerular damage compared with alone MTX group. (d) Renal injury degree was significantly decreased in the MTX + QE group when compared to MTX group. Asterisk: glomerular congestion and degeneration, Arrowhead: tubular cell swelling, Arrow: tubular dilatation. (H&E, scale bar, 50 μ m). ^a $p < 0.001$ compared to control group, ^b $p < 0.01$ compared to MTX group.

group as compared with the values of MTX group (Figure 2d).

Lipid peroxidation and anti-oxidant enzyme assay

In our study performed on rats, SOD, CAT and GSH-Px levels from anti-oxidant defense systems were determined spectrophotometrically. However, MDA levels occurring as a result of lipid peroxidation and used as an important parameter in introducing oxidative damage were measured by the same method. MDA levels of renal tissue were significantly increased in the renal tissue of rats treated with MTX. However, this elevation was significantly suppressed when QE treatment followed MTX administration (Figure 3). Renal tissue of rats showed significantly reduction of SOD, CAT and GSH-Px levels after MTX treatment. These anti-oxidant enzymes level was higher in MTX + QE group comparing to MTX group significantly (Figure 3).

Discussion

MTX is an effective chemotherapeutic agent in cancer treatment, as well as its use in other indications such as rheumatoid arthritis, psoriasis, Crohn's disease, and ulcerative colitis.^{21–23} Despite its widespread use, it is also associated with a number of serious side effects, particularly nephrotoxicity.²⁴

In previous experimental studies, MTX has been administered to rats at varying doses and routes of administration. In our study, MTX was given with a single i.p. dose of 20 mg/kg as previously described in certain tissue toxicity studies.^{11,25}

Although the exact mechanisms of MTX-associated nephrotoxicity have not been clearly understood, recent studies suggest that it may be due to oxidative stress.²⁶ QE is a very potent anti-oxidant providing protection against oxidative injury through prevention of the formation of hydroxyl radicals via Fe and Cu. Previous experimental work on nephrotoxicity confirmed the QE's efficacy on reducing the oxidative injury.^{27,28} Similarly, we also preferred QE for our study based on its proven anti-oxidant and anti-apoptotic efficacy.

Reduction in GSH levels due to MTX treatment results in a weakening of the anti-oxidant defense system protecting cells against the effects of reactive oxygen species such as superoxide anion, hydroxyl radicals, hydrogen peroxide, and hydrochloride.²⁹ Also, MTX has been found to lead to tissue injury through lipid peroxidation.³ Again, MTX was found to increase MDA levels, and certain anti-oxidant compounds could reduce MDA levels.^{3,5,29} In a 2011 study by Asvadi et al.¹² rats had increased levels of MDA upon administration of MTX, while SOD and GSH-Px were found to decrease. Similarly other studies found increased levels of MDA and decreased levels of anti-oxidant enzymes SOD, CAT, and

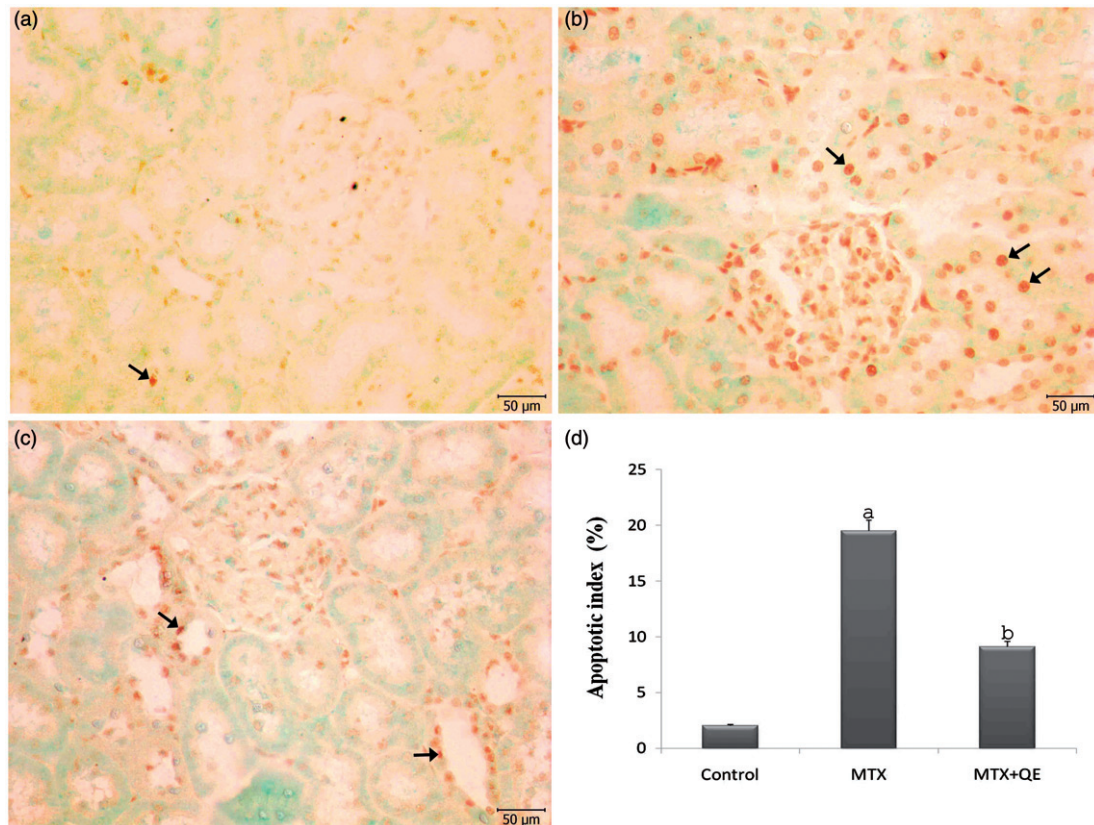


Figure 2. TUNEL staining of renal cortical tissues in different groups. (a) In control group, a few TUNEL positive cells were observed in the renal cortical tissues. (b) The TUNEL positive cells were significantly higher in the renal cortical tissues of the MTX treated group. (c) QE treatment markedly decreased the number of TUNEL positive cells. (d) The apoptotic index was significantly decreased in the MTX+QE group when compared to MTX group. (Arrow: TUNEL positive cells), (TUNEL, scale bar, 50 μm). ^a $p < 0.001$ compared to control group, ^b $p < 0.01$ compared to MTX group.

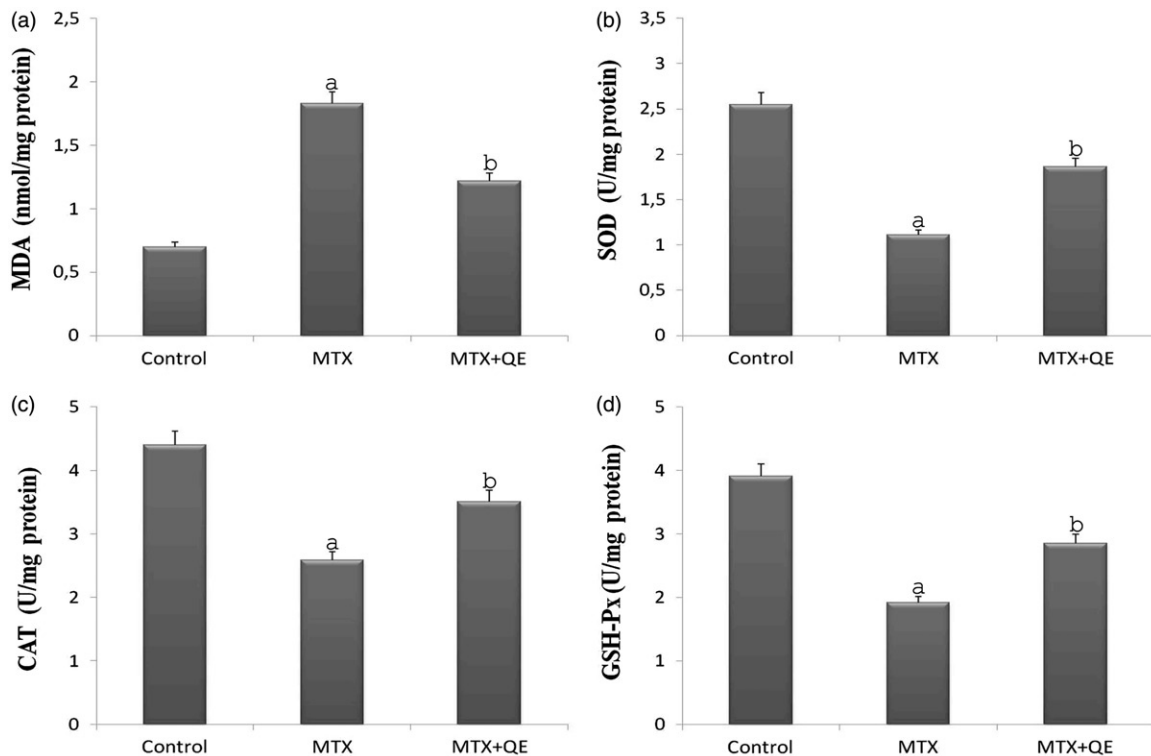


Figure 3. Renal tissue MDA, SOD, CAT, and GSH-Px levels in control, MTX, and MTX + QE groups. Tissue MDA levels were significantly increased, SOD, CAT, and GSH-Px levels were significantly decreased in MTX rats in comparison to control rats. Treatment of QE significantly decreased the elevated tissue MDA levels and increased of reduced SOD, CAT, and GSH-Px levels in the renal tissues. ^a $p < 0.001$ compared to control group, ^b $p < 0.01$ compared to MTX group.

GSH-Px with administration of MTX. Anti-oxidant molecules in the same studies resulted in a reduction of oxidative injury when coadministered with MTX.^{13,30} Caffeic acid phenylester, a potential free radical scavenger, has been found to exert protective effects through the regulation of oxidation/anti-oxidation balance.³¹ Similar findings have been observed with the use of taurine, which corrected the oxidative tissue injury caused by MTX as detected by biochemical assays.³² Consistent with previous studies, MTX significantly increased the renal MDA level, while it in caused a decline in SOD, CAT, and GSH-Px. However, the anti-oxidant molecule utilized in our study, i.e., QE decreased MDA levels, which were increased by MTX, and in increased the concentrations of anti-oxidants such as SOD, CAT, and GSH-Px.

High dose intake of MTX is known to result in the development of acute renal failure.²⁴ Although the mechanism underlying the renotoxic effects of MTX has not been fully elucidated, its precipitation in renal tubules, or direct toxic effect of the original molecule or its metabolite has been implicated.³³ Also, high dose MTX may precipitate in renal tubules, causing acute renal failure.³⁴ Significant pathological changes in the renal tubules and glomeruli have been reported to occur in response to MTX use.^{4,5} Vardi et al.¹³ in their study showed significant structural damage including glomerulosclerosis and apoptosis upon MTX administration. Kose et al.³⁵ reported MTX-related effects such as tubular desquamation and brush-border injury in the proximal tubules. Other studies showed degeneration and dilation in proximal and distal tubular epithelial cells in addition to glomerular degenerative changes.^{25,36} In the present study, in line with these previous reports, QE was able to alleviate the histopathological injury caused by MTX in renal tissues.

MTX causes the formation of DNA defects leading to apoptosis through the inhibition of purine and pyrimidine.³⁷ Also, activation of caspase represents another mechanism through which MTX may be causing apoptosis.³⁸ In studies by Vardi et al.¹³ and Kocaman and Colakoglu³⁹ an increased number of apoptotic cells have been detected with MTX administration. However, Asvadi et al.¹² observed a decrease in the number of apoptotic cells after an increase caused by apoptosis by the use of pentoxifylline, a molecule with anti-oxidant properties. Also, QE was found to possess apoptosis-reducing properties in heavy metal renal injury due to lead.⁴⁰ Again, Ozyurt et al.⁴¹ were able to reduce the radiation-induced apoptotic changes by QE. Similar to previous publications, we have been able to show that MTX was associated with increased apoptosis in renal tissues, and that QE reduced apoptotic changes induced by MTX.

Conclusions

Our results demonstrate that oxidative stress plays an essential role in MTX-induced renal toxicity in rats. The findings of this study show that QE is capable of reducing MTX-induced renal oxidative injury through its anti-oxidant and anti-apoptotic effects, which were evaluated both histologically and biochemically. However, further research will be necessary to understand the mechanisms by which QE is able to prevent renal damage.

Declaration of interest

The authors report no conflicts of interest.

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