



# Renal tubular injury induced by glyphosate combined with hard water: the role of cytosolic phospholipase A<sub>2</sub>

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**Background:** The combined effects of glyphosate and hard water on chronic kidney disease of unknown etiology (CKDu) have attracted much interest, but the mechanisms remain unknown. Cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) plays a key role in the acute and chronic inflammatory reactions. This study explored the effect of glyphosate combined with hard water on renal tubules and the possible targets and mechanisms involved.

**Methods:** *In vivo* experiments were conducted to investigate the synergistic effects and potential mechanisms of glyphosate and hard water on renal tubular injury in mice.

**Results:** Administration of glyphosate in mice resulted in elevated levels of β<sub>2</sub>-microglobulin (β<sub>2</sub>-MG), albumin (ALB), and serum creatinine (SCr) compared to control mice. This increase was more pronounced when glyphosate was combined with hard water. In the glyphosate-treated mice, small areas of the kidney revealed fibroblast proliferation and vacuolar degeneration, particularly at the higher dose of 400 mg/kg glyphosate. However, the combination of glyphosate and hard water induced an even greater degree of pathological changes in the kidney. Immunofluorescence and western blot analyses showed that glyphosate and hard water had a coordinated effect on calcium ions (Ca<sup>2+</sup>)-activated phospholipase A<sub>2</sub> and the activation may play a key role in inflammation and renal tubular injury. Exposure to glyphosate alone or glyphosate plus hard water increased the levels of oxidative stress markers and inflammatory biomarkers, namely, thromboxane A<sub>2</sub> (TX-A<sub>2</sub>), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), nitric oxide synthase (NOS), and nitric oxide (NO). Parameters of oxidative stress, including the levels of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were decreased. Further analysis showed that the levels of these biomarkers were significantly different between the mice treated with glyphosate plus hard water and the mice treated with glyphosate alone.

**Conclusions:** These findings suggested that hard water combined with glyphosate can induce renal tubular injury in mice, and this may involve mitogen-activated protein kinases (MAPK)/cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>)/arachidonic acid (AA) and its downstream factors.

**Keywords:** Glyphosate; hard water; renal tubule; calcium sensitive receptor; cytosolic phospholipase A<sub>2</sub>

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## Introduction

The recent emergence of chronic kidney disease of unknown etiology (CKDu) has become a global public health crisis. In India and Egypt, the prevalence of CKDu is 16% and 27%, respectively (1,2). According to the available data, the disease now affects approximately 2.5 million people worldwide (3). Although the disease progresses slowly, it often develops into end-stage renal disease, leading to the premature death of patients. Therefore, it is particularly important to explore the etiology of CKDu. Several studies have shown that exposure to glyphosate, hard water, and other environmental factors are considered key etiological factors for the prevalence of CKDu (4,5).

Glyphosate has been widely used as a high-efficiency, low-toxicity, broad-spectrum herbicide in the world. The rate of glyphosate application has escalated dramatically since its introduction in the 1970s. Today, a reported one million tons are used annually. Due to the promotion of genetically engineered crops, the agricultural use of glyphosates has increased considerably. As a result, its concentrations in aquatic environments have risen markedly, along with increased detection of both glyphosate and its major metabolite in the human body (6-8). Animal and epidemiology studies have revealed that glyphosate has a high tendency to persist and accumulate in the body. Even low concentrations of glyphosate in the human body can lead to liver and renal dysfunction (9,10). Our previous studies demonstrated that glyphosate-induced nephrotoxicity was dependent on the activation of N-methyl-D-aspartate receptors (NMDARs) expressed in the proximal tubular epithelium. This resulted in the elevation of cytoplasmic calcium ions ( $\text{Ca}^{2+}$ ) and oxidative damage (11).

Hard water is another environmental factor intricately linked to the incidence of CKDu in endemic areas. Hard water is widely distributed in Asia, and can measure more than 250 mg/L (12). Continuous intake of high  $\text{Ca}^{2+}$  will increase intestinal  $\text{Ca}^{2+}$  absorption. To normalize blood calcium levels, the body will send hormonal signals to increase renal calcium reabsorption. The calcium-sensing receptor (CaSR) plays an important role in this process. Ferrè and colleagues (13) demonstrated that increased levels of plasma of  $\text{Ca}^{2+}$  and magnesium ( $\text{Mg}^{2+}$ ) binds and activates the CaSR in proximal tubules. This stimulates both the influx and release of  $\text{Ca}^{2+}$  from intracellular storage sites, leading to reduced parathyroid hormone secretion and calcium resorption in the renal tubule. While hard water may be involved in renal injury through the above

mechanisms, to date, there has been insufficient research in this area. In addition, the role of the CaSR in renal tubular injury induced by hard water and glyphosate is still unclear. Therefore, exploring the effect of glyphosate combined with hard water on renal tubular injury and the possible mechanisms may lead to improved understanding of the risk factors leading to CKDu, and hence provide support for reducing the use of glyphosates, hence reducing the incidence of CKDu.

Cytosolic phospholipase  $\text{A}_2$  (cPLA<sub>2</sub>) is a highly conserved lipid-modifying enzyme. It cleaves arachidonic acid (AA) from glycerophospholipids by catalyzing the hydrolysis of the bond at the sn-2 position (14,15). Elevated cytosolic  $\text{Ca}^{2+}$  and phosphorylated mitogen-activated protein kinase (MAPK) are necessary for cPLA<sub>2</sub> to produce free AA, which is then oxidized by lipoxygenase and cyclooxygenase to produce proinflammatory eicosanoids, such as prostaglandins (PGs) and leukotrienes (LTs) (16-18). Previous evidence have implicated these eicosanoids in a host of inflammatory disorders, and thus cPLA<sub>2</sub> may play an important role in the development of inflammatory diseases (19-21). Furthermore, our previous study (11) demonstrated that oral exposure of glyphosate at a dose of 400 mg/kg in mice resulted in intracellular calcium ( $[\text{Ca}^{2+}]_i$ ) overload and overproduction of reactive oxygen species (ROS). This suggested that cPLA<sub>2</sub> may be involved in renal tubular damage induced by glyphosate. Further studies are warranted to determine the specific mechanisms

In this study, we investigated the effects of glyphosate and hard water on the renal tubule and examined the potential role of cPLA<sub>2</sub> in this process. We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-7739>).

## Methods

### Primary materials

Roundup (41% isopropylamine salt) was purchased from Monsanto (St, Louis, MO, USA). Anhydrous calcium chloride and magnesium chloride hexahydrate (analytical reagent grade) were obtained from Sinopharm Chemical Reagent Co., Ltd.

### Animals

Animal experiments were approved by the Fudan University Animal Care and Use Committee, in accordance with the

Guide for the Care and Use of Laboratory Animals issued by the Ministry of Health of the People's Republic of China.

Male Institute of Cancer Research (ICR) mice, 6 weeks old and weighing 28–30 grams, were acquired from Shanghai SLAC Laboratory Animal Co., Ltd. The animals were housed under a 12-h light-dark cycle at 25 °C and humidity of 45%±5% with free access to standard food and distilled water. All mice were adapted in the housing for 1 week prior to treatment. After 7 weeks of age, mice were divided randomly into six groups (8 animals per group): control group, hard water group, 100 mg/kg glyphosate group, 100 mg/kg glyphosate plus hard water group, 400 mg/kg glyphosate group, and 400 mg/kg glyphosate plus hard water group. Mice in the glyphosate groups were administered glyphosate at a daily dose of 100 or 400 mg/kg via oral gavage for 90 days. Mice in glyphosate plus hard water group were administered glyphosate at a daily dose of 100 or 400 mg/kg and 250 mg/L hard water via oral gavage for 90 days. Mice in the control group were administered distilled water via oral gavage for 90 days. For the duration of the experiment, all mice were given free access to hard water (250 mg/L) or distilled water. For administration, all mice were given to the mice once per day in a volume of 0.1 mL/10 g of body weight. The body weight of all mice was measured daily. Urine was collected using metabolic cages at 30 and 90 days. At the end of the exposure period, mice were sacrificed under anesthesia with an intraperitoneal injection of pentobarbital sodium. Blood samples and renal tissues were collected. The left kidney was harvested and stored at -80 °C for enzyme-linked immunosorbent assay (ELISA) and western blot analysis. The right kidney was fixed in 4% paraformaldehyde solution and embedded in paraffin blocks.

#### ***Measurement of cytokine expression in the serum and urine***

Urine and serum were gathered through deposition and centrifugation, and then stored at -80 °C.  $\beta_2$ -microglobulin ( $\beta_2$ -MG), albumin (ALB), and serum creatinine (SCr) levels were measured using an ELISA kit in accordance with the manufacturer's instructions (Yuanmin Biotechnology Co., Ltd, Shanghai, China). The absorbance at 450 nm (OD450) was detected using a microplate reader. Samples were assayed in triplicate and the concentrations of  $\beta_2$ -MG, ALB, and SCr were calculated using the averaged absorbance readings and a standard curve.

#### ***Periodic acid-Schiff staining and immunohistochemistry***

The kidney tissues were fixed in a 4% paraformaldehyde buffer. Tissue samples were cleared in xylene and embedded in paraffin followed by dehydration in graded alcohols. Sections of the kidney paraffin blocks (4  $\mu$ m) were stained with periodic acid-Schiff (PAS) stain and visualized with a microscope (Olympus, Tokyo, Japan). For each sample, ten random non-overlapping fields of view (400 $\times$  magnification) were examined. The area of kidney injury was quantified using the ImageJ software.

Immunohistochemistry was used to detect N-methyl-D-aspartate receptor 1 (NMDAR1) expression. Tissue slides were immersed in sodium citrate (pH 6), followed by blocking in 10% goat serum. After several washes, sections were incubated with a rabbit monoclonal anti-NMDAR1 primary antibody (Shanghai Yuanmin Biotechnology Co., Ltd, 1:10) at 4 °C overnight. Samples were then incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody for 50 minutes at room temperature. Following this, slides were stained with 3,3'-diaminobenzidine (1:50) for 5 minutes and counterstained with hematoxylin for 3 minutes. Images were observed under a microscope at 200 $\times$  magnification. Cells staining yellow was considered as positive NMDAR1 expression.

#### ***Electron microscopic examination***

Small pieces of renal cortex tissue (approximately 1 mm<sup>3</sup>) were immersed in 0.1 M sodium phosphate buffer containing 2.5% glutaraldehyde, and post-fixed in 1% osmium tetroxide solution. The tissue specimens were dehydrated in a graded ethanol water series and embedded in Agar-100 resin. Ultrathin sections were doubly stained with uranyl acetate and lead citrate solutions and observed using a HT7700 transmission electron microscope (Hitachi, Japan).

#### ***Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis of apoptosis***

Apoptosis was measured by using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis according to the manufacturer's instructions (Shanghai Yuanmin Biotechnology Co., Ltd). The kidney samples were fixed in formalin, processed, and embedded with paraffin. After deparaffinization and rehydration, the

sections were permeabilized with proteinase K solution, and subsequently incubated with the TUNEL reaction mixture at 37 °C for 2 hours in a humidified chamber. The reaction was halted with 4'-6-Diamidino-2-phenylindole (DAPI). Finally, fluorescence images were analyzed using an Olympus microscope at 200× magnification. Five different fields of view were examined for each kidney section. Cells with green fluorescence were interpreted as TUNEL-positive.

#### ***Immunohistochemistry analysis for CaSR***

Renal sections were fixed with a 4% paraformaldehyde solution for 4–6 hours at 4 °C, then transferred into a 30% sucrose solution overnight. Samples were embedded in optimal cutting temperature (OCT) compound, cut into serial 8 µm thick sections, placed on glass slides, and fixed in acetone for 10 minutes. Subsequently, slides were washed three times in phosphate-buffered saline (PBS) and blocked with 5% goat serum for 30 minutes at room temperature, followed by overnight incubation at 4 °C with primary antibodies against the CaSR (1:100, Proteintech Group). Slides were then incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:1,000, CST) for 1 hour at room temperature in the dark. After treatment with DAPI (Beyotime, Shanghai, China), the sections were washed an additional three times. Slides were then visualized with a laser confocal microscope (Olympus, Tokyo, Japan). The CaSR and nucleus were stained green and blue, respectively.

#### ***Flow cytometric analysis of renal tissue***

Tissue samples (30 g) were minced and digested with type II collagenase. Isolated cells were collected and washed with 1 mL PBS. The supernatant was removed after centrifugation and re-suspended in 0.1 mL PBS. The suspension was then transferred to 1.5 mL centrifuge tubes and blocked with 3% bovine serum albumin (BSA) for 30 minutes. Subsequently, 100 µL of primary antibodies against CaSR (Cell Signaling) and phosphorylated cPLA<sub>2</sub> (p-cPLA<sub>2</sub>, Ser505, Cell Signaling) were added at a 1:100 dilution and incubated at 37 °C for 2 hours. After washing, Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody was added and incubated at room temperature for 30 minutes. The cells were washed twice, resuspended in the flow medium in the dark, and analyzed with flow cytometry (CytoFLEX, Beckman).

#### ***Identifying the biomarkers of inflammation and oxidative stress in renal tissue***

Frozen kidney tissue samples were homogenized with PBS on ice and centrifuged at 4 °C for 10 minutes. The supernatants were collected and the levels of oxidative stress markers and inflammatory biomarkers [thromboxane A<sub>2</sub> (TX-A<sub>2</sub>), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), nitric oxide synthase (NOS), and nitric oxide (NO), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured using ELISA kits (Yuanmin Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's instructions.

#### ***Western blot analysis***

Frozen kidney tissue samples (30 mg) were homogenized in cold lysis buffer containing Phenylmethanesulfonyl fluoride (PMSF, Beyotime, Shanghai, China) for 90 seconds. After centrifugation for 10 minutes at 14,000 g at 4 °C, the supernatant was collected. Protein concentration was detected using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Equal amounts of protein were mixed with sample buffer and electrophoretically separated on a 5% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Samples were then transferred onto polyvinylidene fluoride (PVDF) membranes by wet electrophoresis (350 mA for 75 minutes). The membrane was then blocked with 5% skimmed milk powder for 2 hours at room temperature followed by overnight incubation at 4 °C with primary antibodies against total cPLA<sub>2</sub> (t-cPLA<sub>2</sub>, 1:1,000, Cell Signaling), p-cPLA<sub>2</sub> (Ser505, 1:1,000, Cell Signaling), CaSR (1:1,000, Cell Signaling), and β-actin (1:5,000, Sigma). After washing with TBST, the membranes were incubated with anti-mouse or anti-rabbit secondary antibody (1:2,000, Cell Signaling) for 2 hours at room temperature. Finally, visualization was performed using electrochemiluminescence (ECL) chemiluminescence solution and β-actin was used as an internal control.

#### ***Statistical analysis***

The data showed normal distribution and were expressed as the means ± standard deviation (SD) and analyzed using the SPSS version 22.0 software (IBM, Armonk, USA) and GraphPad Prism 7.00 (GraphPad Software Inc., San Diego,

CA, USA). Mean comparison was determined using single-factor analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. The interaction between glyphosate and hard water was analyzed by ANOVA. All experiments were repeated in triplicates and a P value of less than 0.05 was considered statistically significant.

## Results

### *General signs and renal function parameters*

Control mice maintained a stable weight, while mice exposed to glyphosate alone or in combination with hard water showed a slight decrease in body weight. However, there was no statistically significant differences in body weight gain among the various groups examined (*Figure 1A*). Glyphosate alone or in combination with hard water had no effect on the kidney weight or kidney weight index compared to control mice (*Figure 1B*). To evaluate the role of glyphosate and hard water in renal function, the levels of  $\beta_2$ -MG and ALB in mice after 30 and 90 days of exposure were measured. As shown in the *Figure 1C* and *D*,  $\beta_2$ -MG and ALB levels in the glyphosate and glyphosate plus hard water groups were significantly higher than in the control mice. Further analysis showed that the levels of  $\beta_2$ -MG and ALB in the 400 mg/kg glyphosate group were higher than those in the 100 mg/kg glyphosate group. Mice exposed to glyphosate plus hard water showed significantly higher levels of  $\beta_2$ -MG and ALB compared with mice treated with glyphosate alone. However, there was no statistical significance between mice treated with hard water alone and mice in the control group. Similar results were observed with SCr levels (*Figure 1E*), indicating that the combined effect of glyphosate and hard water was more remarkable than that of glyphosate alone.

### *Synergistic effect of glyphosate and hard water on renal biomarkers*

As shown in *Figure 2*, solid line indicates the groups treated with hard water and dashed line indicates the groups without hard water. The two lines are nonparallel to each other and exhibit a rising trend with the increase of dosage, indicating that glyphosate and hard water appeared to have a synergistic effect on the levels of  $\beta_2$ -MG, ALB, and SCr in mice. Glyphosate alone elevated the levels of  $\beta_2$ -MG, ALB, and SCr, and this effect was amplified when the dose was increased. When glyphosate was combined with hard

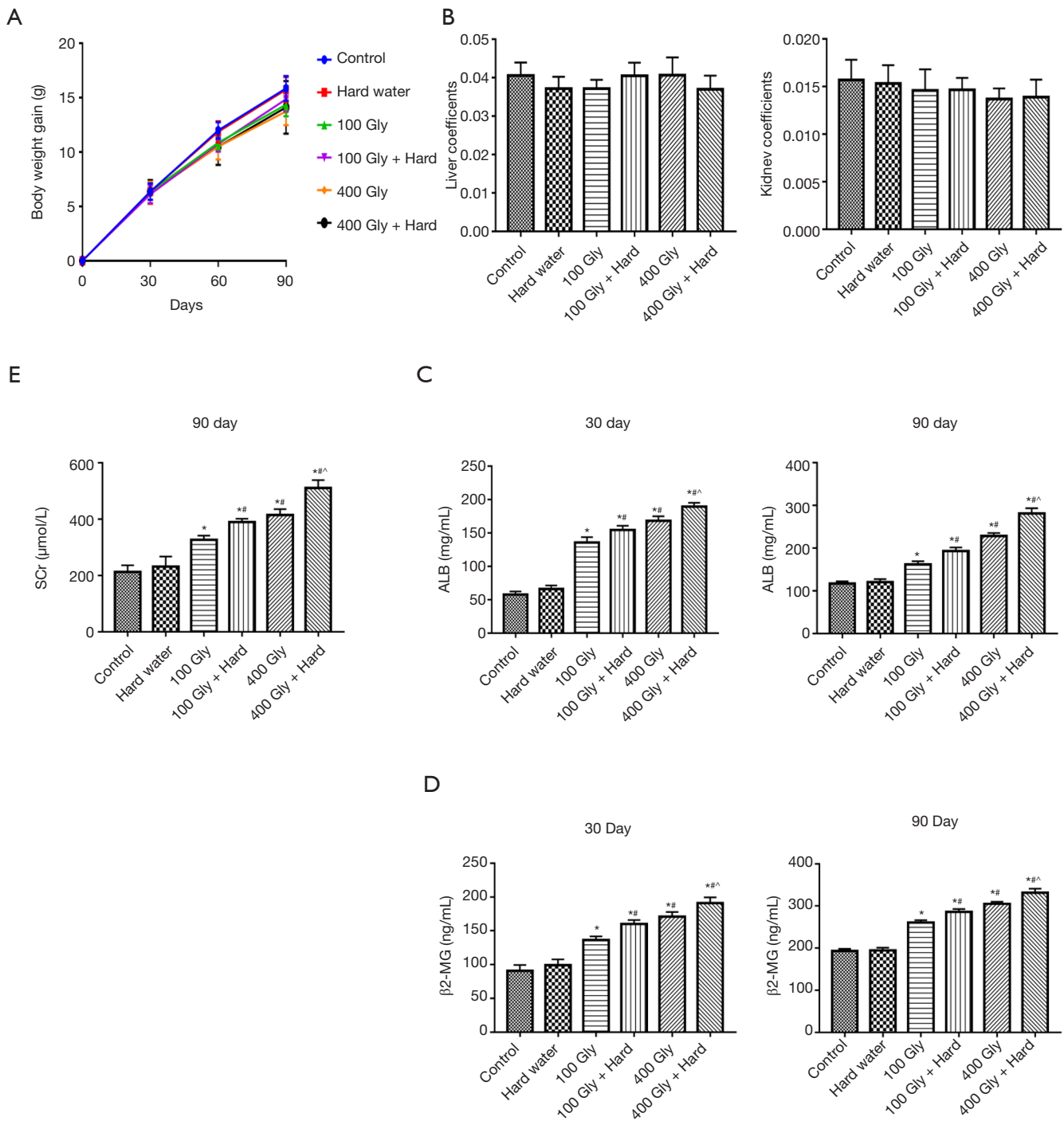
water, the levels of  $\beta_2$ -MG, ALB and SCr were significantly increased after 30 and 90 days.

### *Histopathological changes in the kidneys of mice*

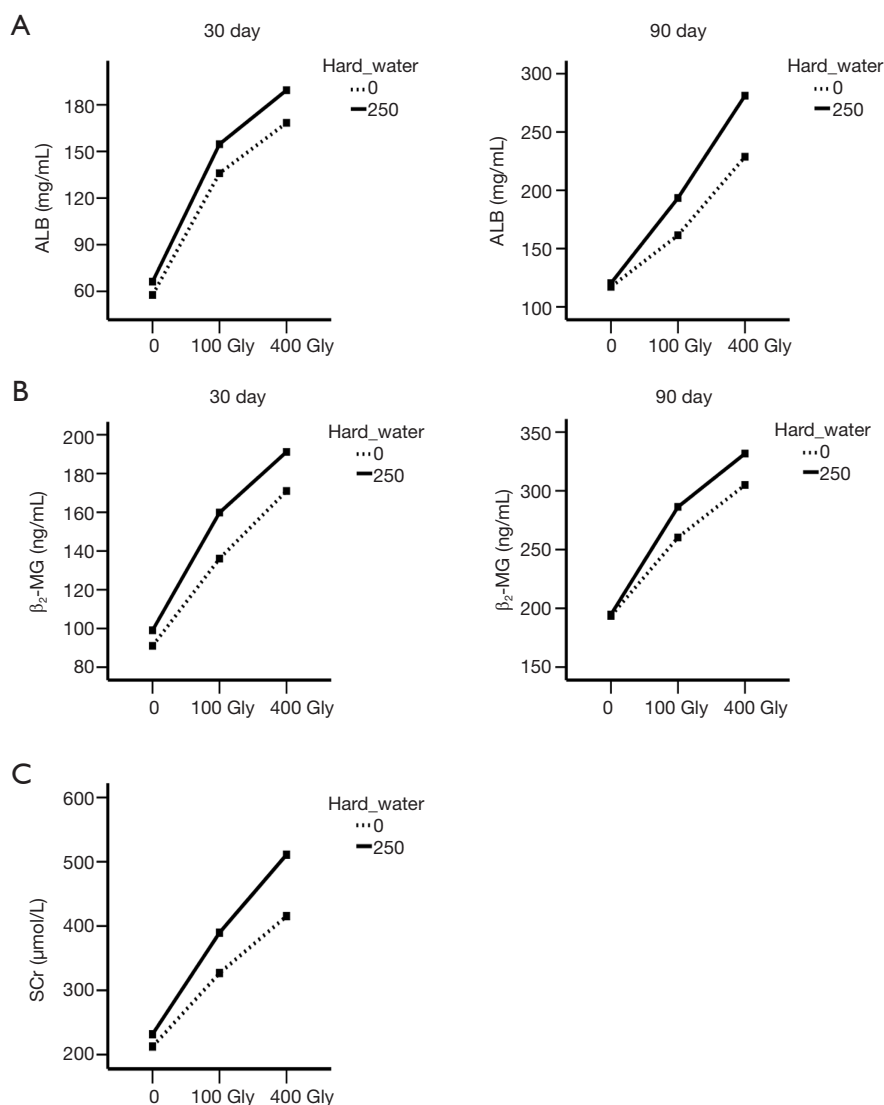
Histopathological examinations were performed to investigate the combined effect of glyphosate and hard water on renal injury in mice. Under light microscopy, the kidneys from control mice showed complete renal glomerulus and tubule morphology. The epithelial cells of the tubule were arranged in order, with close intercellular adhesion. Slight edema and inflammatory cell infiltration were observed. After treatment with glyphosate, the epithelial cells of the renal tubule showed swelling, caryolysis, and exfoliation, accompanied by proliferation of interstitial fibroblasts and infiltration of inflammatory cells. The cytoplasmic vacuolization of tubular epithelial cells and fibroblast proliferation were more pronounced in mice administered 400 mg/kg glyphosate compared to mice given 100 mg/kg glyphosate. In mice treated with glyphosate combined with hard water, more severe tissue injury was observed, including vacuolar degeneration of renal tubular epithelial cells and increased fibroblast density in the mesenchyme. This was particularly pronounced in the 400 mg/kg glyphosate plus hard water group (*Figure 3*).

### *Effects of glyphosate and hard water on kidney cell apoptosis and ultrastructure*

A few TUNEL-positive cells were observed in the kidneys of control mice and mice given hard water. However, the percentage of TUNEL-positive cells increased in glyphosate-treated mice. When glyphosate and hard water were administered together, more apoptotic cells were observed compared with mice treated with glyphosate alone. There was no significant difference between the control group and the group given hard water alone (*Figure 4A*). Electron microscopy observations revealed specific ultrastructural changes in the renal tubular epithelial cells following treatment with glyphosate. The renal tubules of mice in the control group and the hard water group showed a normal appearance. In glyphosate group, mitochondria were partially damaged, appeared mildly swollen and irregularly shaped. After 90 days of administering glyphosate and hard water together, the renal cortex of the mice were examined by electron microscopy and intracellular edema was observed. This was manifested as mitochondrial swelling and fracture, destruction



**Figure 1** The effects of glyphosate and hard water on the kidneys of ICR mice. (A) Body weight gain; (B) liver and kidney coefficient; (C) ALB levels; (D)  $\beta_2$ -MG levels; (E) SCr levels. \*,  $P < 0.05$  compared with controls; #,  $P < 0.05$  compared with 100 mg/kg glyphosate; ^,  $P < 0.05$  compared with 400 mg/kg glyphosate. ICR, Institute of Cancer Research; Gly, glyphosate; ALB, albumin;  $\beta_2$ -MG,  $\beta_2$ -microglobulin; SCr, serum creatinine.



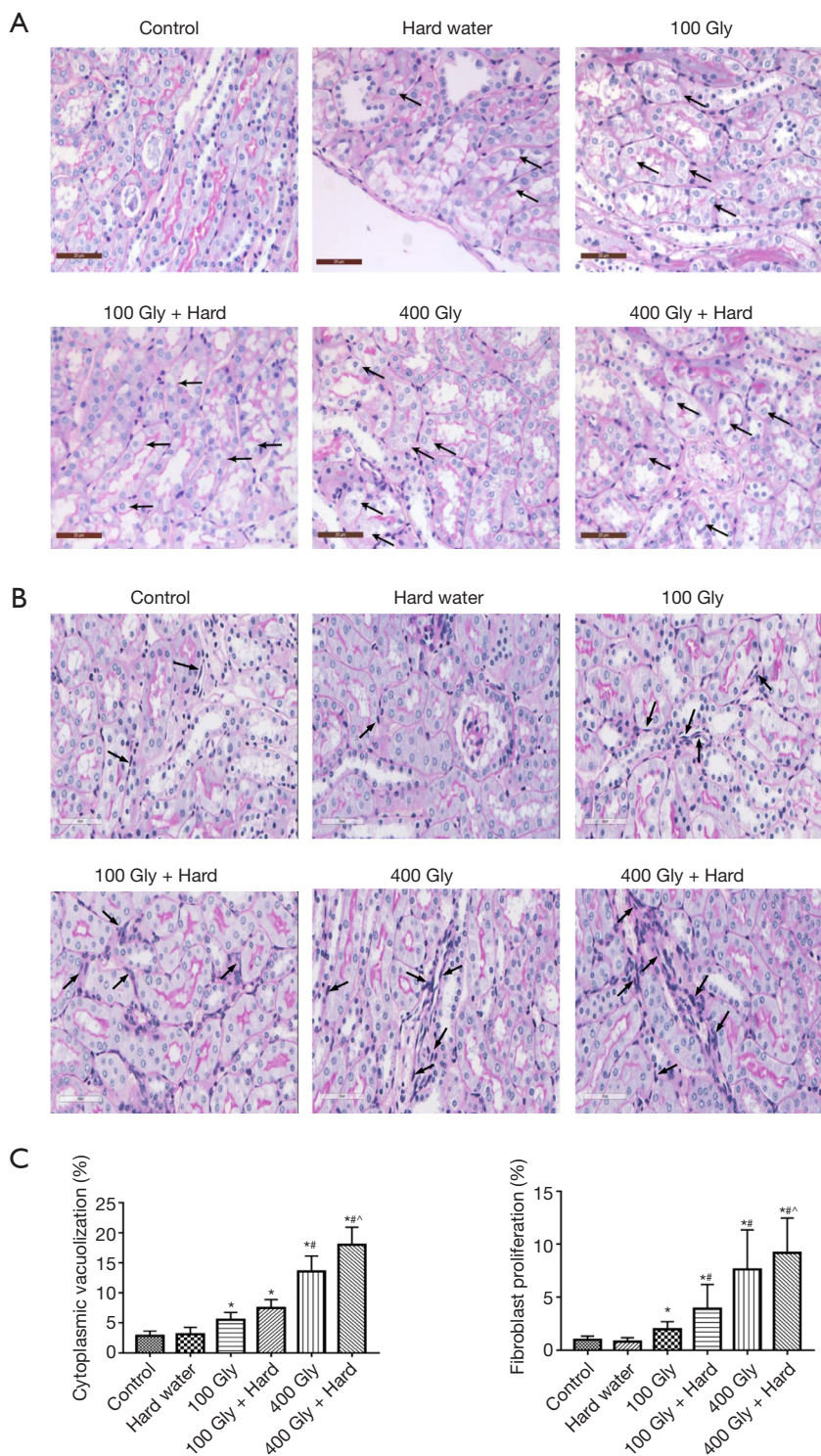
**Figure 2** Interaction plots between the two factors, glyphosate and hard water. (A) The effect of glyphosate and hard water on ALB levels; (B) the effect of glyphosate and hard water on  $\beta_2$ -MG levels; (C) the effect of glyphosate and hard water on SCr. Gly, glyphosate; ALB, albumin;  $\beta_2$ -MG,  $\beta_2$ -microglobulin; SCr, serum creatinine.

of the mitochondrial double membrane structure and mitochondrial cristae disappeared. Rough endoplasmic reticulum expansion and degranulation were also observed (Figure 4B).

#### **Effects of glyphosate and hard water on renal CaSR and cPLA<sub>2</sub>**

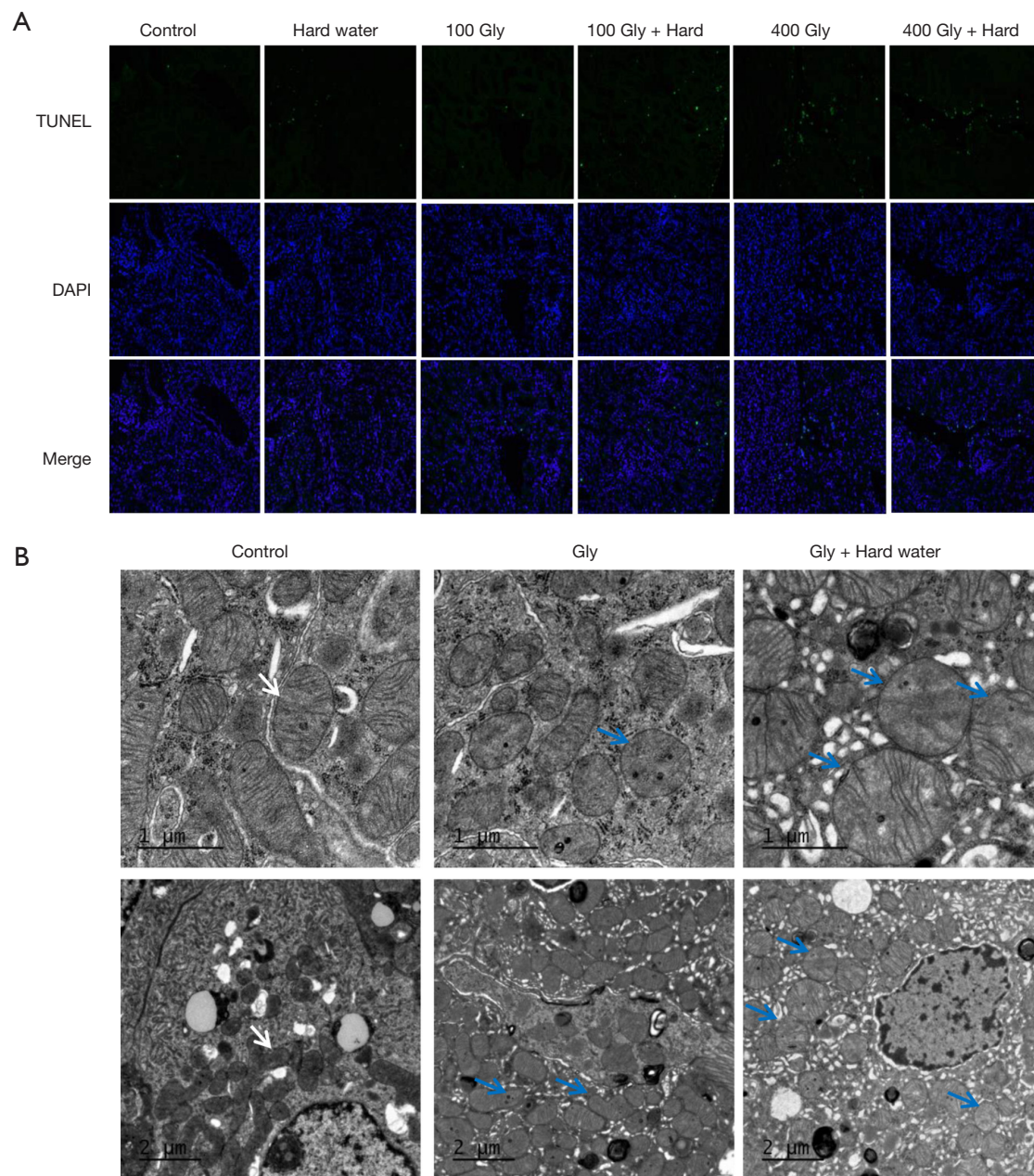
As has been reported previously (11), NMDAR1-positive expression were detected in kidneys from glyphosate-treated mice compared to control mice (Figure 5A). Increasing

evidence has shown that the CaSR is substantially expressed within the renal tissue (22,23). This study confirmed that the CaSR is intensely expressed on proximal tubules and thick ascending limb of the mouse kidney (Figure 5B). The effect of Ca<sup>2+</sup> cations in hard water on the renal CaSR was examined. The kidneys of mice treated with hard water demonstrated higher expression of the CaSR compared to control mice. The levels of CaSR expression in the kidneys of glyphosate-treated mice was not statistically different to that observed in control mice (Figure 5C). cPLA<sub>2</sub> is activated by phosphorylation at serine-505 by MAPKs (24). This results



**Figure 3** PAS-stained sections. (A) Vacuolar degeneration in epithelium cells, bar =20  $\mu$ m, black arrows show cytoplasmic vacuolization; (B) proliferation of interstitial fibroblasts, bar =60  $\mu$ m, black arrows show fibroblast hyperplasia; (C) quantification of pathology. \*, P<0.05 compared with controls; #, P<0.05 compared with 100 mg/kg glyphosate; ^, P<0.05 compared with 400 mg/kg glyphosate. Gly, glyphosate; PAS, periodic acid-schiff.

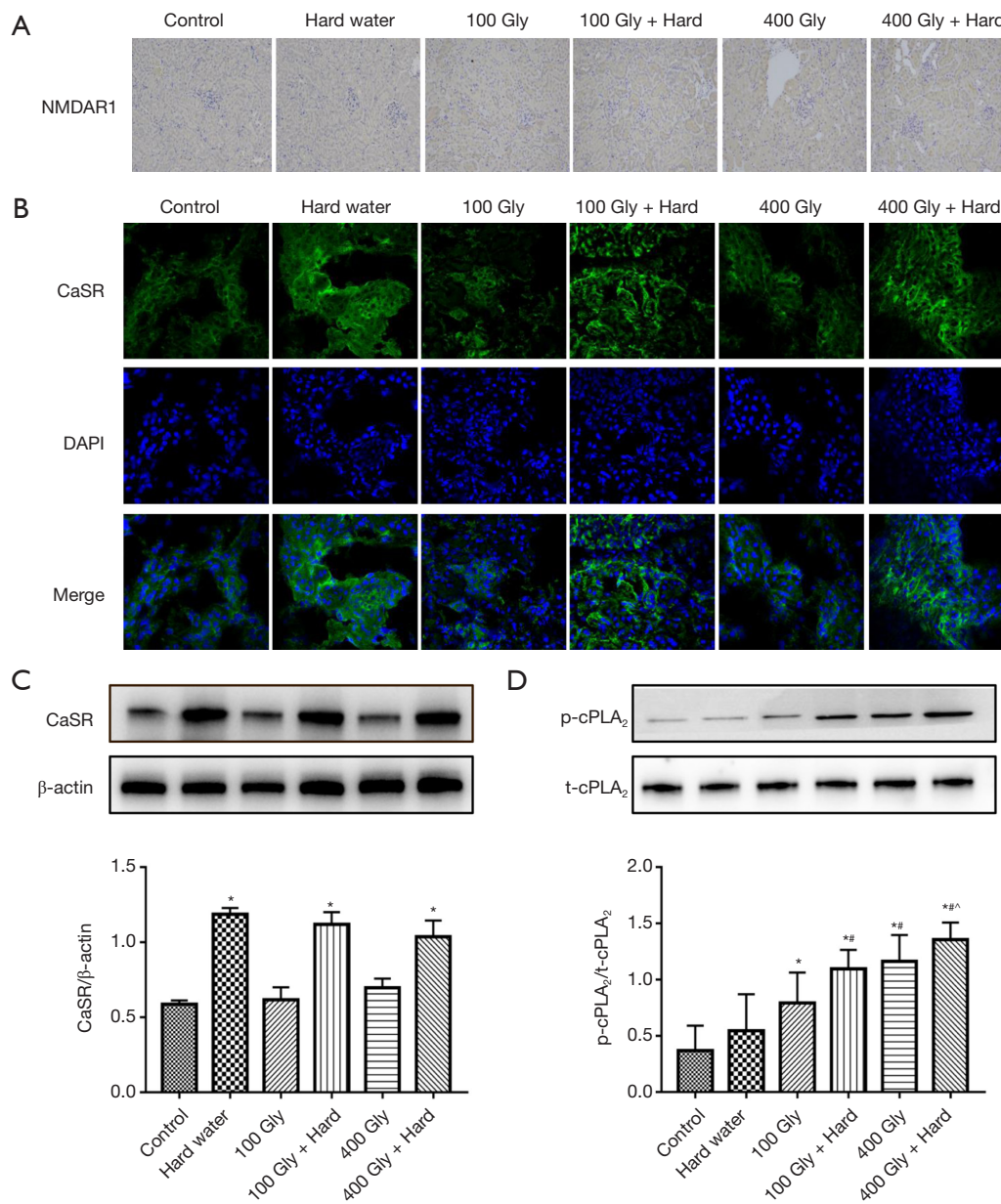




**Figure 4** The detection of renal tubular epithelial cell death. (A) TUNEL staining. Green cells indicate TUNEL-positive cells, 200× magnification; (B) ultrastructural analysis of kidney tissue. In the control group, mitochondrial morphology was normal with preserved membranes and cristae (arrow). Mitochondrial swelling and cristae rupture (blue arrow) were observed in glyphosate and glyphosate plus hard water groups. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; Gly, glyphosate.

in the release of AA and its metabolites cascade, which are associated with inflammatory reactions and oxidative stress (25,26). Western blot analyses were performed to determine the levels of t-cPLA<sub>2</sub> and p-cPLA<sub>2</sub> protein expression in the kidneys of the mice. The levels of p-cPLA<sub>2</sub> protein in

the kidneys of glyphosate-treated mice was significantly higher than the levels observed in control mice (Figure 5D). However, the increase in p-cPLA<sub>2</sub> protein levels was more pronounce in mice treated with glyphosate and hard water together. The treatment of mice with hard water alone did



**Figure 5** The levels of CaSR and cPLA<sub>2</sub> expression in the renal tissue. (A) immunohistochemical staining of NMDAR1 in kidneys (yellow staining indicates NMDAR1-positive expression), 200× magnification; (B) immunocytochemical localization of CaSR in mouse renal tissue, 400× magnification; (C) protein expression of CaSR; (D) protein expression of t-cPLA<sub>2</sub> and p-cPLA<sub>2</sub>. \*, P<0.05 compared with controls; #, P<0.05 compared with 100 mg/kg glyphosate; ^, P<0.05 compared with 400 mg/kg glyphosate. Gly, glyphosate; CaSR, calcium-sensing receptor; p-cPLA<sub>2</sub>, phosphorylated-cPLA<sub>2</sub>; t-cPLA<sub>2</sub>, total cPLA<sub>2</sub>.

not lead to any alterations in p-cPLA<sub>2</sub> protein expression compared with the control group. The results showed that the protein levels of t-cPLA<sub>2</sub> were similar in all 6 treatment groups, suggesting a regulatory effect of glyphosate and hard water together on cPLA<sub>2</sub> phosphorylation.

We further analyzed the expression of CaSR and p-cPLA<sub>2</sub> using flow cytometry studies. The proportion of cells in the kidney expressing the CaSR was significantly increased in mice that were given hard water compared to control mice. However, there were no significant differences

between mice treated with glyphosate and control mice (Figure 6A). In the kidney of mice treated with glyphosate, the proportion of cells expressing the p-cPLA<sub>2</sub> protein was significantly increased compared to the control mice (Figure 6B). This increase was significantly amplified in mice treated with both glyphosate and hard water.

#### ***Effects of glyphosate and hard water on renal inflammation and oxidative stress in mice***

The levels of TX-A<sub>2</sub>, LTB<sub>4</sub>, PGE<sub>2</sub>, NOS, and NO were significantly increased in the groups exposed to glyphosate compared with the control group, with 400 mg/kg glyphosate resulting in a more pronounced increase compared to 100 mg/kg glyphosate. The combination of glyphosate with hard water resulted in higher levels of TX-A<sub>2</sub>, LTB<sub>4</sub>, PGE<sub>2</sub>, NOS, and NO compared to mice given glyphosate alone, with the 400 mg/kg glyphosate plus hard water group showing the most significant increase. Parameters of oxidative stress, such as SOD and GSH-Px, were measured. The levels of SOD and GSH-Px were relatively high in the control group and the hard water group. In contrast, the levels of SOD and GSH-Px in the 100 mg/kg glyphosate group decreased and further significant decreases were observed in mice treated with 400 mg/kg glyphosate. When glyphosate and hard water were given together, the SOD and GSH-Px levels were remarkably decreased compared with mice given glyphosate alone (Figure 7).

#### ***The synergistic effect of glyphosate and hard water on inflammation and oxidative stress***

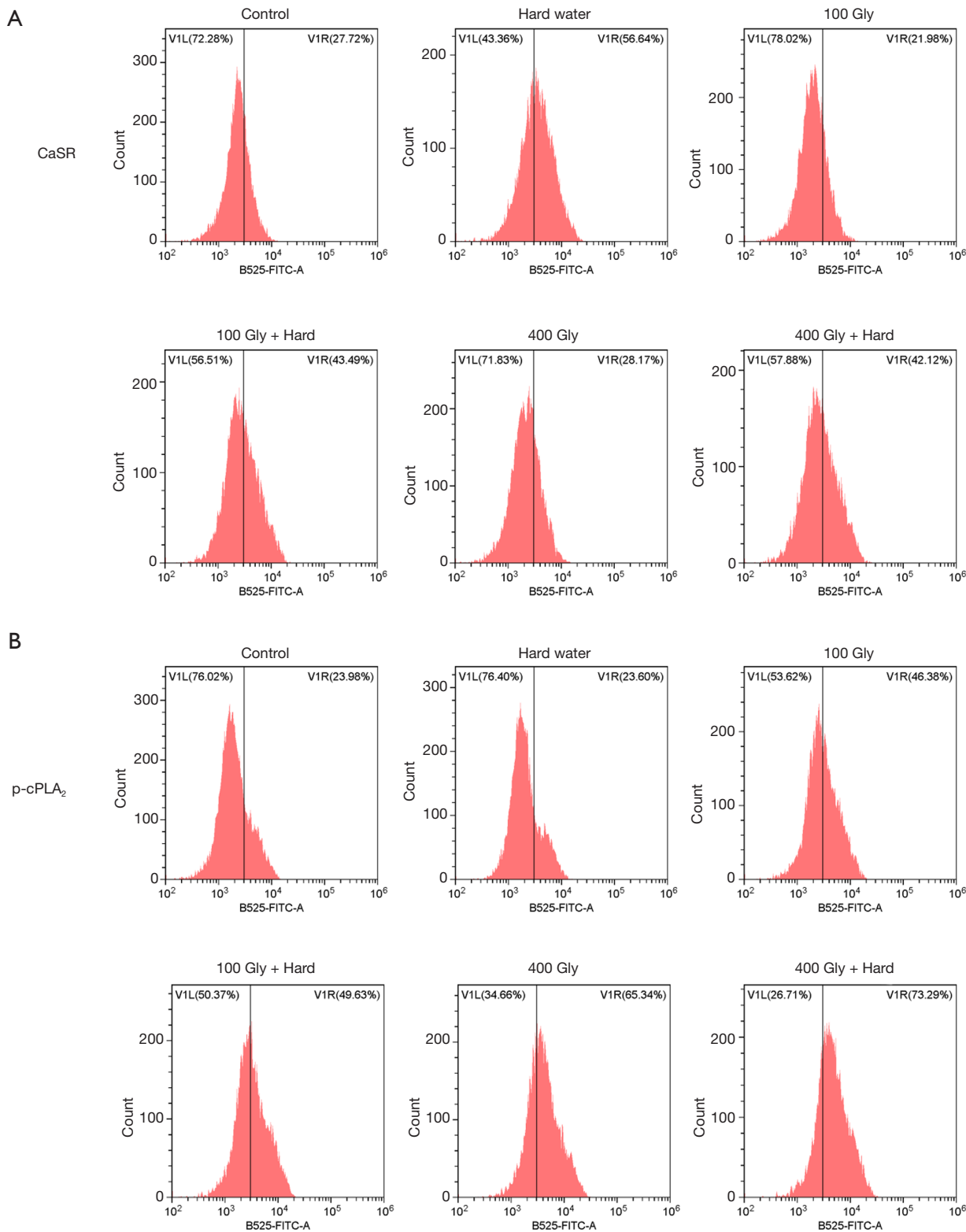
The results of the analysis of variance demonstrated an interaction between glyphosate and hard water (Figure 8). The interaction plots showed that treatment with glyphosate alone significantly increased the levels of TX-A<sub>2</sub>, LTB<sub>4</sub>, PGE<sub>2</sub>, NOS, NO and the effect was more pronounced with the increased dosage. When hard water was given alone, there was no significant difference in each index. Furthermore, glyphosate and hard water showed a synergistic effect on the activation of inflammation markers. No interaction between SOD and GSH-Px was observed.

## **Discussion**

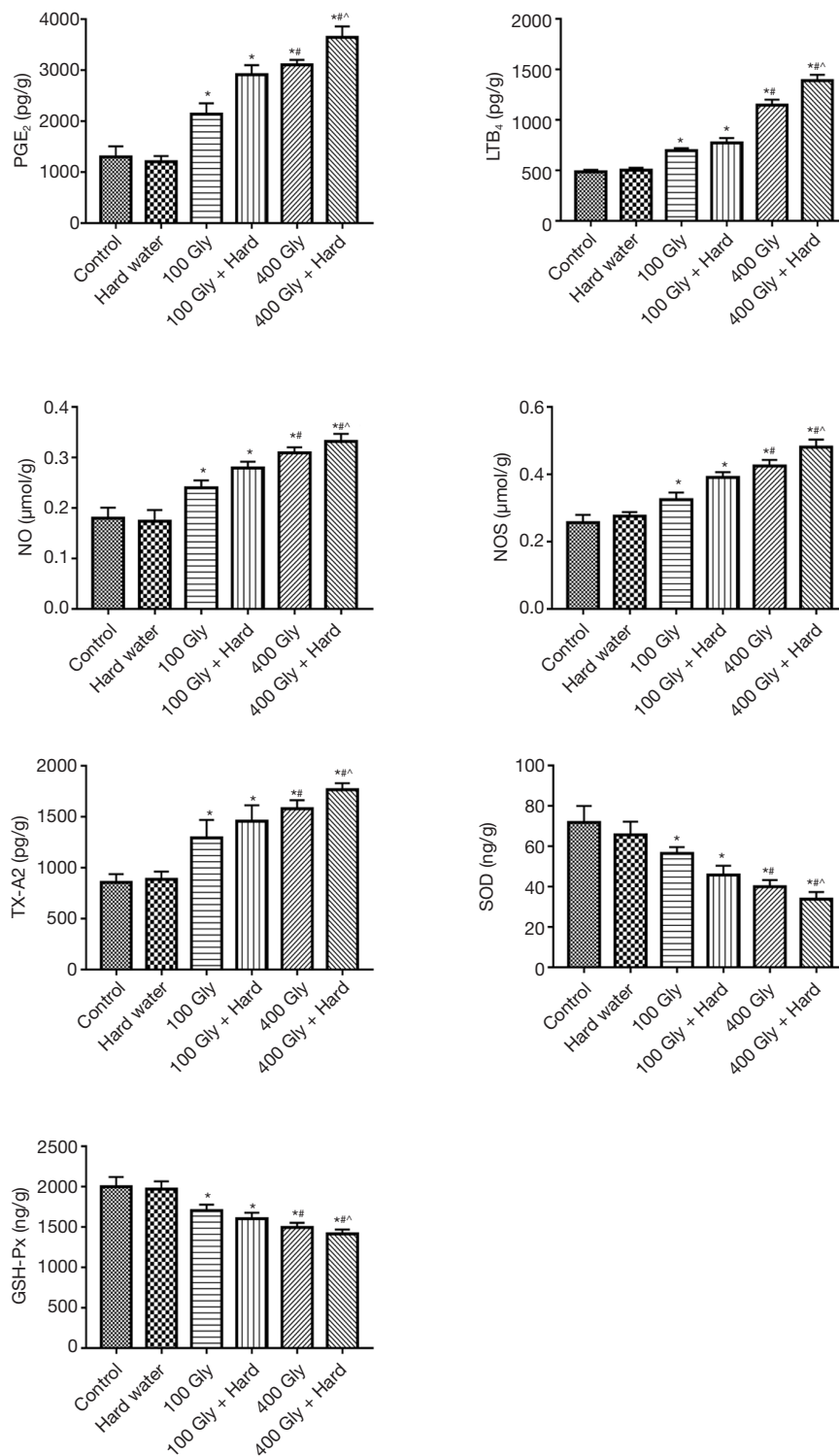
The interaction between glyphosate and hard water has been a major focus of recent research, especially in relation to its impact on CKDu (27,28). Although glyphosate

alone and hard water alone may not cause an epidemic of chronic kidney disease, when combined together, they could form complexes with nephrotoxic metals and cause renal damage (29). A multivariable analysis performed by Lunyera *et al.* (27) demonstrated that exposure to hard water and glyphosate together was associated with higher odds of developing CKDu. The adjusted odds ratios for drinking hard water together with exposure to intermediate or high concentrations (median 3.2 µg/L) of glyphosate were 2.5 (95% confidence interval: 1.1–5.7) and 5.5 (95% confidence interval: 2.9–10.3), respectively. Soft water with traces or no detectable level of glyphosate was used as a reference. A significant positive association between hard water consumption and the occurrence of CKDu has been previously reported (30). One study demonstrated that 96% of patients who developed CKDu had been exposed to hard or very hard water over a 5-year period (31). While there has been much evidence in the literature demonstrating that CKDu is affected by hard water and a number of other factors, the details and mechanisms of this relationship are not well understood (32,33). There has been much research examining the relationship between glyphosate and CKDu. While glyphosate is widely used and generally considered to be safe for humans and animals, several animal studies have shown evidence of neurotoxicity, endocrine disruption, cell damage, and immune suppression due to glyphosate (34). In addition, glyphosate-induced toxicity to the renal tubules has been reported, suggesting that the kidney is an important target organ of glyphosate (35). In fact, Wunnapuk and colleagues (36) have demonstrated a link between glyphosate and renal injury in rats. Furthermore, our previous studies confirmed that acute glyphosate exposure can cause epithelial cell damage in renal tubules mediated by changes to the NMDAR, calcium influx, and oxidative stress (11).

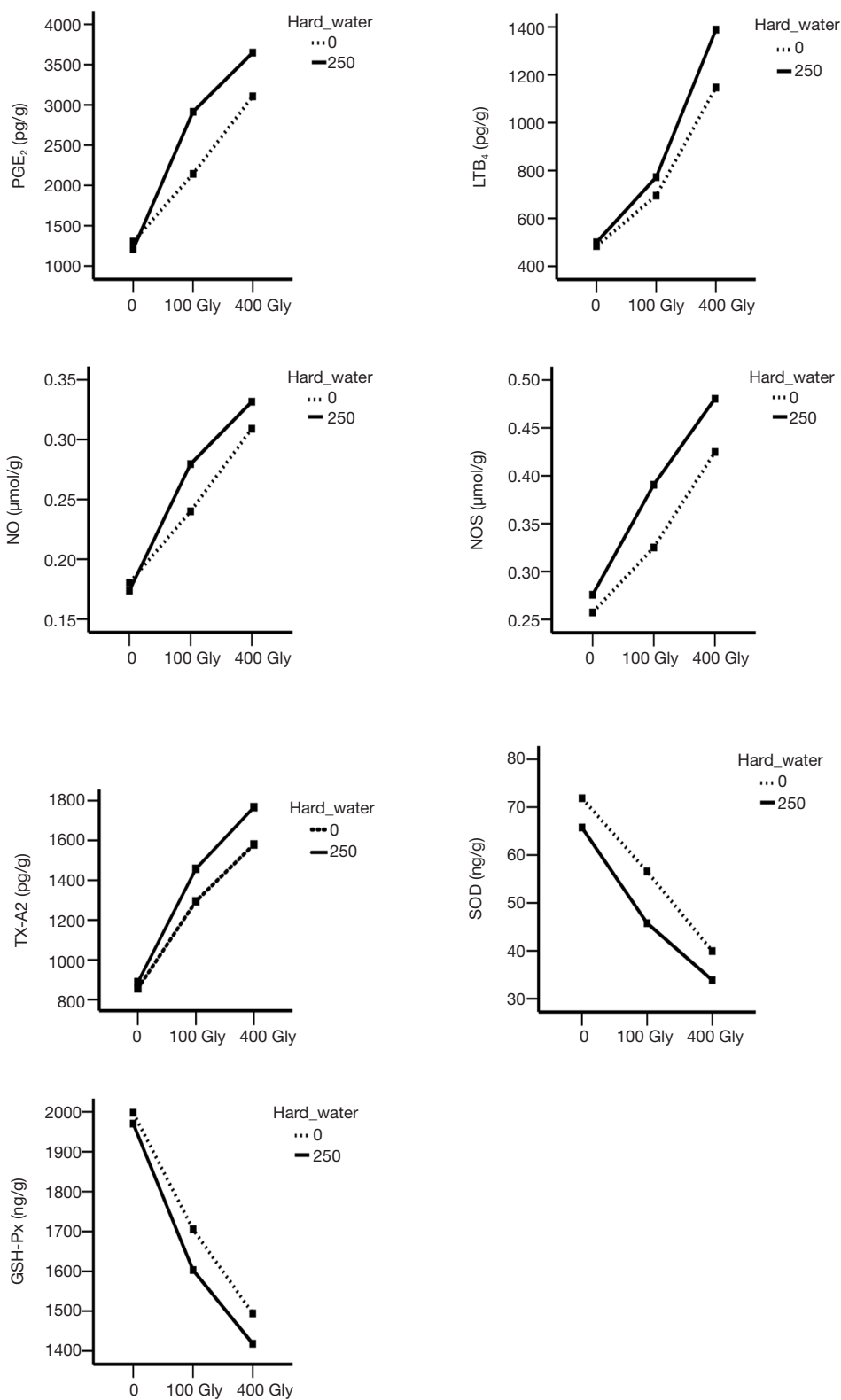
This study explored the role of hard water and glyphosate in renal tubular injury. The results demonstrated that hard water alone did not cause any direct health risks to the mice. Therefore, we hypothesized that hard water acts synergistically to enhance renal tubular injury induced by glyphosate. Well-water used in CKDu-endemic areas contain higher levels of Ca<sup>2+</sup> compared to Mg<sup>2+</sup> (29 vs. 13 mg/L) (37). Similar findings have been reported in China, with calcium salt content accounting for about 70% of the total hardness in the water. The CaSR is a homodimeric G-protein-coupled receptor, highly expressed in parathyroid glands and kidneys, and plays a substantial role in systemic calcium metabolism. In the



**Figure 6** The ratio of protein expression of CaSR and p-cPLA<sub>2</sub>. (A) Flow cytometric analysis of CaSR; (B) flow cytometric analysis of p-cPLA<sub>2</sub>. Gly, glyphosate; CaSR, calcium-sensing receptor; p-cPLA<sub>2</sub>, phosphorylated-cPLA<sub>2</sub>.



**Figure 7** The effects of glyphosate and hard water on oxidative stress and inflammatory indexes in renal tissue. \*, P<0.05 compared with controls; #, P<0.05 compared with 100 mg/kg glyphosate; ^, P<0.05 compared with 400 mg/kg glyphosate. Gly, glyphosate; TX-A<sub>2</sub>, thromboxane A<sub>2</sub>; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; NOS, nitric oxide synthase; NO, nitric oxide; GSH-Px, glutathione peroxidase.



**Figure 8** The synergistic effect of glyphosate and hard water on inflammatory biomarkers. Gly, glyphosate; TX-A<sub>2</sub>, thromboxane A<sub>2</sub>; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; NOS, nitric oxide synthase; NO, nitric oxide; GSH-Px, glutathione peroxidase.

kidney, CaSR modulates electrolyte and water excretion, thereby regulating the function of different tubular segments. In particular, the CaSR reduces passive and active calcium reabsorption in distal tubules to maintain physiological ionized calcium concentrations in the serum. Further, it is reported that CaSR agonists such as  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  act through the heterotrimeric G-proteins Gq, Gi and G12/13. Activation of G(q) stimulates phospholipase C, and inositol 1,4,5-trisphosphate, releasing calcium from intracellular stores (38-40). In physiological situations, the activity of CaSR is partially activated by the presence of an agonist. The degree correlated with the concentration and time of agonist. The elevated calcium levels stimulate the renal CaSR, thus promoting migration of newly synthesized receptors to the cell membrane (41). In this study, the expression of CaSR protein in the kidney tissue increased following exposure to hard water alone or hard water combined with glyphosate. However, expression of the CaSR in mice treated with glyphosate alone was not statistically different compared to the control mice. This suggested that continuous exposure to hard water may activate the renal CaSR, which may be one of the key mechanisms of kidney damage induced by combined exposure to hard water and glyphosate.

The CaSR has been shown to interact with MAPK, cPLA<sub>2</sub> and the epidermal growth factor. Of the phospholipase A2 (PLA2) enzymes, cytosolic phospholipase A2 (cPLA2) has a unique set of biochemical properties, which is regulated by calcium and MAPKs, releasing arachidonic acid. The metabolism of arachidonic acid by cyclooxygenase (COX) enzymes or 5-lipoxygenase (5-LO) generates eicosanoids, lipid mediators capable of triggering cellular responses, which contributes to inflammation (42,43). Glyphosate-induced cytotoxicity has been observed in HK-2 cells and it has been shown that the renal effect of glyphosate relies on activation of the NMDAR, which results in intracellular calcium overload and increased oxidative stress. Exposure of neurons from different brain regions to NMDA has been reported to activate cPLA<sub>2</sub> through the  $\text{Ca}^{2+}$  mechanism (44). Our results showed that levels of p-cPLA<sub>2</sub> were increased in mice treated with glyphosate compared with control mice, and protein levels of p-cPLA<sub>2</sub> was higher at the 400 mg/kg dose compared to the 100 mg/kg dose. Moreover, the increase was more apparent when hard water was offered in combination with glyphosate. These data suggested that cPLA<sub>2</sub> is involved in the interaction between glyphosate and the  $\text{Ca}^{2+}$  cations in the hard water, presumably through activation of the

NMDAR and the CaSR.

To explore the effects of cPLA<sub>2</sub> and its downstream pathways on renal function, biomarkers related to renal inflammation, including TX-A<sub>2</sub>, LTB<sub>4</sub>, PGE<sub>2</sub>, NOS, and NO, were assessed. Exposure to glyphosate (100 and 400 mg/kg) significantly increased the levels of TX-A<sub>2</sub>, LTB<sub>4</sub>, PGE<sub>2</sub>, NOS, and NO, and the increase was even higher when used in combination with hard water. Active cPLA<sub>2</sub> is responsible for the delivery of AA, with the ensuing release of TXA<sub>2</sub>, LTB<sub>4</sub> and PGE<sub>2</sub>, which are associated with diverse physiological effects, as well as inflammatory responses and tumor dissemination (45,46). Prostaglandins (PGs) are biosynthesized by the COX enzymes, either constitutively or in response to cell specific trauma, stimuli, or signaling molecules (47,48). PGE<sub>2</sub> is the most abundantly detected PG in various tissues (49). It has been shown to induce chronic inflammation and various autoimmune diseases through T helper 1 (Th1)-cell differentiation, Th17-cell proliferation, and IL-22 production from Th22 cells via the prostaglandin E receptor 2 and prostaglandin E receptor 4 (50,51). Additionally, PGE<sub>2</sub> was found to exacerbate acute inflammation by inducing vascular hyperpermeability and recruiting neutrophils via the prostaglandin E receptor 3 in mast cells, resulting in swelling (52). TXA<sub>2</sub>, also synthesized from AA via the actions of COX enzymes, can promote the synthesis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1B (IL-1B) (53,54). Consistent with the above findings in animal models, Nakanishi and colleagues reported that the microsomal prostaglandin E synthase-1 knockout mice were generally protected against a variety of inflammatory disease phenotypes, including collagen-induced arthritis, lipopolysaccharide (LPS)-induced bone loss, and antigen-induced paw edema (55). In addition to the above pro-inflammatory metabolites, LTB<sub>4</sub> is another class of molecules that is rapidly generated from AA through the sequential action of 5-LO in response to various stimuli (56). LTB<sub>4</sub> is well known to induce polymorphonuclear neutrophils and phagocytosis of macrophages (57). Extensive attention has been given to the role of LTB<sub>4</sub> in acute and chronic inflammatory diseases, such as infectious diseases, allergies, autoimmune diseases, and metabolic diseases (58). More importantly, increases in NO production by inducible NOS was accompanied by cPLA<sub>2</sub> activation (59). Through the reaction of NO with a superoxide anion, peroxynitrite is produced, which is cytotoxic and causes severe damage to other molecules in the cell (60,61). Meanwhile, previous studies have highlighted the effect of cPLA<sub>2</sub> on oxidative

stress. Our results demonstrated lower levels of SOD and GSH-Px in mice exposed to glyphosate plus hard water, indicating that the endogenous antioxidant protective pathways of the mouse kidney were inadequate or overwhelmed. Free radical scavengers including catalase, SOD, and GSH-Px protect cells from ROS (62,63). Several studies shown that cPLA<sub>2</sub> can generate large amounts of superoxide through the “cPLA<sub>2</sub>-AA-NADPH oxidase” pathway, thereby disturbing the oxidant-antioxidant balance, and evoking inflammatory responses (64,65). This is consistent with our observations.

The effects of glyphosate and hard water on the changes in the biomarkers of renal function were examined. Urine  $\beta_2$ -MG, ALB and serum creatinine levels in mice administered glyphosate were higher than those in the control group, but lower than the levels observed in mice treated with the combination of glyphosate plus hard water. The increase in the levels of  $\beta_2$ -MG in the urine indicated an impairment of tubular reabsorption, while the increase in the levels of ALB reflected the impairment of glomerular function. Our results demonstrated that prolonged exposure to glyphosate led to renal impairment, and the combined effect of hard water was more pronounce. Combination treatment of glyphosate and hard water enhanced cellular edema, and interstitial fibroblast proliferation. Similarly, the TUNEL assay confirmed the increase in renal tubular cell apoptosis in mice exposed to glyphosate and hard water. cPLA<sub>2</sub> has been demonstrated to be involved in various cytotoxic reactions, and its downstream product AA may stimulate apoptosis through activation of sphingomyelinase (66). Several recent reports also suggested that the release of AA parallels the reduction in cell viability and DNA fragmentation (67,68). Moreover, continuous chronic inflammations promote the transformation of renal interstitial cells to fibroblasts (69,70). As noted by Montford and colleagues (71), cPLA<sub>2</sub> was highly expressed in patients with chronic nephritis and in animal models of glomerulonephritis. In this current study, there was no significant difference in renal function index and pathological results between the control mice and mice given hard water, which indicated that hard water, at the concentration used in this study, did not cause renal tubular damage.

This study demonstrated the possible combined effect of glyphosate and hard water on renal damage. Both glyphosate and hard water are risk factors for renal disease. This study is limited by the short study timeframe and future studies examining the effects of a lifetime exposure

to glyphosate would be insightful. We merely performed the *in vivo* experiment, and the *in vitro* experiment will be performed in our following experiments. Overexpression and downregulation experiments of phospholipase A<sub>2</sub> are required to verify the observations in this study

## Conclusions

In conclusion, we demonstrated that the CaSR may be activated after continuous exposure to hard water, leading to renal tubular epithelial cells injury. Glyphosate exerts a synergistic effect though the MAPK/cPLA<sub>2</sub>/AA signaling pathway. The findings of this study provide a theoretical basis and reference point for evaluating the risk of glyphosate and hard water. Furthermore, cPLA<sub>2</sub> may be a therapeutic target for renal damage induced by glyphosate and hard water.

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*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were approved by the Fudan University Animal Care and Use Committee, in accordance with the Guide for the Care and Use of Laboratory Animals issued by the Ministry of



Health of the People's Republic of China.

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