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RESEARCH ARTICLE

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Activation of the *N*-methyl-D-aspartate receptor is involved in glyphosate-induced renal proximal tubule cell apoptosis

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Abstract

Glyphosate-based herbicides have been used worldwide for decades and have been suggested to induce nephrotoxicity, but the underlying mechanism is not yet clear. In this study, we treated a human renal proximal tubule cell line (HK-2) with glyphosate for 24 hours at concentrations of 0, 20, 40 and 60 μM. Glyphosate was found to reduce cell viability and induce apoptosis and oxidative stress in a dose-dependent manner. Because the chemical structures of glyphosate and those of its metabolite AMPA are similar to glycine and glutamate, which are agonists of the *N*-methyl-D-aspartate receptor (NMDAR), we investigated the potential role of the NMDAR pathway in mediating the proapoptotic effect of glyphosate on proximal tubule cells. We found that NMDAR1 expression, as well as intracellular Ca^{2+} ($[Ca^{2+}]_i$) and reactive oxygen species (ROS) levels, increased after glyphosate treatment. Blocking NMDAR attenuated glyphosate-induced upregulation of $[Ca^{2+}]_i$ and ROS levels as well as apoptosis. Meanwhile, inhibition of $[Ca^{2+}]_i$ reduced glyphosate-induced ROS and apoptosis, and inhibition of ROS alleviated glyphosate-induced apoptosis. In mice exposed to 400 mg/kg glyphosate, the urine low molecular weight protein levels started to increase from day 7. Upregulation of apoptosis and NMDAR1 expression in renal proximal tubule epithelium and an imbalance of oxidant and antioxidative products were observed. These results strongly suggest that activation of the NMDAR1 pathway, together with its downstream $[Ca^{2+}]_i$ and oxidative stress, is involved in glyphosate-induced renal proximal tubule epithelium apoptosis.

KEYWORDS

apoptosis, glyphosate, kidney, NMDA receptor, oxidative stress

1 | INTRODUCTION

Glyphosate [*N*-(phosphonomethyl) glycine] is a broad-spectrum herbicide used worldwide (NAWQA, 2012; Swanson, Leu, Abrahamson, & Wallet, 2014). Since its introduction for weed control in the agricultural industry in 1974 (Duke & Powles, 2008), particularly after the development of glyphosate-resistant products such as glyphosate-resistant soybean, canola and corn, the total acreage treated with glyphosate has rapidly increased. Currently, the use of glyphosate-based herbicides for agricultural production is widespread in both industrialized and developing countries (Duke, 2015; Myers et al., 2016). It has been reported that glyphosate-containing products are currently manufactured by more than 90 producers in 20 countries (WHO, 2017).

Glyphosate is an herbicide whose mode of action is through the specific inhibition of 5-enolpyruvylshikimate-3-phosphate synthase in the shikimate aromatic amino acid biosynthesis pathway. Because 5-enolpyruvylshikimate-3-phosphate synthase is an enzyme present only in plants and some bacteria but not in vertebrates, glyphosate

was initially considered nontoxic (Williams, Watson, & DeSesso, 2012), with an estimated lethal dose (LD_{50}) higher than 5000 mg/kg (EPA, US, 1993; Mensink & Janssen, 2018). However, given the widespread use of glyphosate-based herbicides, residues of glyphosate and its major metabolite AMPA have been found in surface waters, soil (Huang, Pedersen, Fischer, White, & Young, 2004; Struger et al., 2008) and some plants such as legume, cereal, rice, wheat seed, canola seed and various types of animal fodder (Arregui et al., 2004; Cessna et al., 1994; Cessna, Darwent, Townley-Smith, Harker, & Kirkland, 2000; Cetin, Sahan, Ulgen, & Sahin, 2017; Cuhra, 2015; FAO/WHO, 2007). Glyphosate is primarily absorbed through the skin, intestine and respiratory tract, and has been detected in many human urine samples, not only in those of farmers but also in those of the general public (Acquavella et al., 2004; Arbuckle, Lin, & Mery, 2001; Niemann, Sieke, Pfeil, & Solecki, 2015; Krüger et al., 2014). Therefore, the effects of glyphosate on human health have attracted extensive attention.

Glyphosate-based herbicides may have neurotoxicity, cardiovascular toxicity, hepatotoxicity, reproductive and developmental toxicity, and nephrotoxicity (Cattani et al., 2017; Gress, Lemoine, Seralini, & Puddu, 2015; Milesi et al., 2018; Tang, Hu, Li, Win-Shwe, & Li, 2017; Wunnapuk et al., 2014); however, the underlying mechanisms are not fully understood. There are some epidemiological studies suggesting a potential relationship between glyphosate-based herbicides (together with other factors) and chronic kidney disease of unknown etiology (CKDu) (Jayasumana, Gunatilake, & Senanayake, 2014; Jayasumana et al., 2015; Peraza et al., 2012). Several studies using models of acute or subchronic exposure as well as long-term exposure have indicated that the kidney may be one of the main targets of glyphosate, and the renal proximal tubule may be a main target site for glyphosate-based herbicides (Dedeke, Owagboriaye, Ademolu, Olujimi, & Aladesida, 2018; Hamdaoui et al., 2016; Mesnage et al., 2015; Tizhe et al., 2014; Wunnapuk et al., 2014). However, some studies have suggested that the formulation of herbicides is more toxic than glyphosate alone (Dedeke et al., 2018; Gasnier et al., 2009). Therefore, whether glyphosate alone could impair renal function and how glyphosate targets the renal proximal tubule should be explored.

The toxic mechanism of glyphosate-based herbicides may include altering cellular Ca^{2+} homeostasis, which has been suggested by several studies. For example, acute exposure to the glyphosate product Roundup increases Ca^{2+} influx in neural and testicular cells (Cattani et al., 2014; De Liz et al., 2013). Altered Ca^{2+} homeostasis can induce oxidative stress through the upregulated generation of reactive oxygen species (ROS) (Milić et al., 2018). *N*-Methyl-D-aspartate receptor (NMDAR), originally identified in the central nervous system, is a heterotetrameric amino acid receptor that functions as a calcium-dependent membrane channel (Davies, Evans, Francis, & Watkins, 1979; Mandir et al., 2000). NMDAR is also expressed in the subapical proximal kidney tubule, and both glycine and glutamate molecules may lead to channel opening and calcium influx (Gabbai, 2018). However, the role of NMDAR in the effects of glyphosate on the kidney has not been investigated.

In this study, we used a cell culture model and an animal exposure model to investigate the influences of glyphosate on renal proximal tubule cells and the potential role of NMDAR in response to glyphosate exposure.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Glyphosate (*N*-(phosphonomethyl) glycine [96%]), glyphosate monoisopropylamine salt solution (40% wt. in H_2O), dimethyl sulfoxide, hydrogen peroxide and pentobarbital sodium were purchased from Millipore Sigma (St. Louis, MO, USA). RPMI 1640 medium, phosphate-buffered saline (PBS), trypsin-EDTA and penicillin/streptomycin were purchased from Corning (Manassas, VA, USA). Fetal bovine serum was purchased from Gibco (Melbourne, Vic., Australia). An NMDAR inhibitor (MK-801), intracellular calcium chelator (BAPTA-AM) and ROS scavenger, *N*-acetylcysteine (NAC), were purchased from Selleck (Houston, TX, USA). An Apoptosis Inducers Kit and ionomycin were obtained from Beyotime (Shanghai, China), 3,3′-diaminobenzidine was purchased from Dako Corp. (Carpinteria, CA, USA), and normal rabbit IgG was purchased from Abcam (Cambridge, MA, USA).

2.2 | Cell culture and treatment

The human renal proximal tubular epithelial cell line HK-2 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were used in this study. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Beyotime) in a humidified incubator with 5% CO_2 at 37°C. Glyphosate monoisopropylamine salt solution (40% wt. in H_2O) was used to prepare a glyphosate stock solution (200 mM), which was then was diluted in complete medium to the final concentrations. For glyphosate treatment, HK-2 cells were treated with different doses (0, 20, 40, 50, 60, 70, 80, 90 or 100 μM) of glyphosate for 24 hours. For intervention experiments, HK-2 cells were pretreated with MK-801 (100 μM for 12 hours), BAPTA-AM (2 μM for 12 hours) or NAC (2 mM for 12 hours) followed by treatment with 40 μM glyphosate.

2.3 | Cytotoxicity assay

Cell viability and death were evaluated by Cell Counting Kit-8 (CCK-8) and lactate dehydrogenase (LDH) cytotoxicity assay kit (Dojindo, Shanghai, China) following the manufacturer's instructions. To determine cell viability, HK-2 cells were seeded into a 96-well plate (1×10^4 cells/mL) and then treated with various concentrations of glyphosate for 24 hours. Cell-free medium and cell treated with H_2O served as the blank and solvent controls, respectively. A mixture of 10 μL of CCK-8 solution and 90 μL of culture medium was added to each well and then incubated at 37°C for 2 hours. The optical density values at 450 nm were measured with a Synergy™ HT Microplate

Reader (Bio Tek, Winooski, VT, USA). During the detection of glyphosate cytotoxicity, cell supernatants from each well were collected and then incubated with the LDH assay solutions for 30 minutes at 25°C. Cell-free medium and cells treated with H_2O served as the blank and solvent controls, respectively; cells treated with lysis buffer and lysis buffer alone served as the positive and positive blank controls, respectively. The optical density values at 490 nm were detected using a Synergy™ HT Microplate Reader.

2.4 | Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis assay

Cell apoptosis was measured using an Annexin V-fluorescein isothiocyanate apoptosis detection kit (BD Biosciences; San Diego, CA, USA). Glyphosate- or vehicle-treated HK-2 cells were harvested and suspended in binding buffer. Annexin V-fluorescein isothiocyanate (5 μL) was added to the cells, followed by the addition of propidium iodide (PI; 5 μL). H_2O served as the solvent control, and apoptosis inducer A (Apopida) in the Apoptosis Inducer Kit served as the positive control. Cells without Annexin V and PI were used as negative controls. Subsequently, the cells were labeled for 15 minutes at 37°C. The fluorescence intensity of Annexin V and PI was recorded using a Becton-Dickinson FACSCalibur flow cytometer. Data from 10 000 events per sample were analyzed using FlowJo™ software (BD Biosciences).

2.5 | Cellular reactive oxygen species measurement

Intracellular ROS production was measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Millipore Sigma) using flow cytometry. HK-2 cells were incubated with 100 nM DCFH-DA for 30 minutes at 37°C. Cells were then harvested and resuspended in basal medium. H_2O and hydrogen peroxide were used as the solvent and positive controls, respectively. Cells without the DCFH-DA probe were used as a negative control. The fluorescence intensity of 3×10^4 cells per sample was acquired using a Becton-Dickinson FACSCalibur flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. FACS data were analyzed using FlowJo software (BD Biosciences).

2.6 | Detection of intracellular Ca^{2+} levels

The intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration was analyzed using a Fluo-4/AM fluorescent probe (Invitrogen, Carlsbad, CA, USA). The cells were incubated with 2 μM Fluo-4/AM in Hanks' balanced salt solution at 37°C for 30 minutes and then suspended in Hanks' balanced salt solution and incubated at 37°C for an additional 20 minutes. H_2O and ionomycin (5 μM) were used as the solvent and positive controls, respectively. Cells without Fluo-4/AM probe were used as a negative control. Cell analysis was performed on a BD FACSCalibur flow cytometer (BD Biosciences) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The $[Ca^{2+}]_i$ value is represented by the mean fluorescent intensity.

2.7 | Western blotting

Untreated and treated cells were washed three times with ice-cold PBS and then lysed by RIPA lysis buffer (Beyotime) containing 1% protease inhibitors (Millipore Sigma). The cell lysate was centrifuged and the supernatant was collected. The total cellular protein concentration was determined using a BCA assay kit (Beyotime). Equal amounts of total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through a 12% gel and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk in PBS + Tween 20 (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl and 0.1% Tween 20; pH 7.5) at room temperature for 2 hours. Subsequently, the membranes were washed with PBS + Tween 20 and incubated with primary antibodies (1:1000) against human NMDAR1, Bcl-2, Bax, Bcl-xl, Bad, cleaved caspase-3 or β-tubulin overnight at 4°C. The membranes were then incubated with antirabbit horseradish peroxidase-conjugated IgG antibodies (1:2000) for 2 hours at room temperature. The protein bands were visualized using an ECL detection kit (Pierce Chemical, Dallas, TX, USA). β-Tubulin served as an internal loading control.

2.8 | Animals and treatment

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. The use of animals in the present study also met the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985, updated in 2011; https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf). Adult male ICR mice (aged 8 weeks) were obtained from the Shanghai Jiesijie Laboratory Animal Company (Shanghai, China) with license number SCXK (Hu)2013-0006. The mice were provided food and water ad libitum and maintained in a controlled environment at a temperature of 24 ± 1°C, humidity of 45% ± 5% and 12-hour light/dark cycle. The animals were randomly assigned to the control group or glyphosate exposure group (six mice per group). The mice in the glyphosate exposure group were 400 mg/kg body weight/day glyphosate via oral gavage for a period of 28 days, while those in the control group were administered distilled water. For administration, glyphosate was diluted in an aqueous suspension and given to the mice once per day in a volume of 0.1 mL/10 g of body weight. Body weight and food intake were measured daily. Urine was collected once a week via metabolic cages. At the end of the exposure period, mice were anesthetized by an intraperitoneal injection of pentobarbital sodium, after which blood was collected from the orbital venous plexus to prepare serum. Then, the animals were killed by cervical dislocation, and the kidney tissue was dissected and washed with saline solution. The kidney samples were fixed by immersion in a 4% paraformaldehyde solution (4%

paraformaldehyde in 0.01 M phosphate buffer, pH 7.4) for 24 hours at 4°C for histology, immunohistochemistry and TUNEL examinations. The remaining samples were snap-frozen in liquid nitrogen and maintained at −80°C for subsequent laboratory analysis.

2.9 | Urine biomarker measurement

Urine and serum were frozen and stored at −80°C immediately after collection and centrifugation. Urine creatinine, uric acid, urea and serum creatinine levels were determined using the respective assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing City, China). Urine β_2-microglobulin and albumin levels were measured using enzyme-linked immunosorbent assay kits (Abcam, Cambridge, MA, USA).

2.10 | Superoxide dismutase, catalase, glutathione peroxidase and malondialdehyde measurements

At the end of glyphosate treatment, the culture supernatants of treated and untreated HK-2 cells (in vitro experiment) or renal tissue (in vivo experiment) were freshly collected on ice for measurements of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) activities using the corresponding assay kits (Beyotime). SOD activities were examined with the xanthine oxidase method; CAT activities were tested with the colorimetric method; GSH-Px activities were examined using the ultraviolet spectrophotometric method; and MDA levels were examined with the thiobarbituric acid method. The experiments were performed by following the manufacturer's instructions.

2.11 | Hematoxylin and eosin staining

The kidneys fixed for histological examination were dehydrated and embedded in paraffin. Sections for histology (5 μm) were sliced, mounted on poly-L-lysine-coated slides, deparaffinized with xylene, and stained with hematoxylin and eosin. A trained pathologist blinded to the treatments evaluated the tissue slides using an optical microscope (Olympus, Tokyo, Japan).

2.12 | Immunohistochemistry

The kidney samples fixed for immunohistochemical examination were dehydrated and embedded with paraffin, and 5-μm thick slices were cut from the embedded blocks. Then, those slides were deparaffinized and incubated with rabbit monoclonal anti-NMDAR1 primary antibodies (1:10) at 4°C overnight, washed in PBS and incubated with goat antirabbit IgG-horseradish peroxidase secondary antibodies (1:200) for 50 minutes at room temperature. Next, 50 mL of 3,3′-diaminobenzidine (1:50) was added to each kidney section, which was stained for 5 minutes. After the slides were washed, they were counterstained with hematoxylin for 3 minutes. The slides were then mounted and observed under a microscope. NR1 expression was quantified using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA). We used two negative controls: PBS treatment in place of primary antibody and an isotype-matched non-specific antibody (normal rabbit IgG). Brain tissues from mice were used as a positive control.

2.13 | TUNEL assay

Apoptotic cells were detected with a TUNEL assay kit according to the manufacturer's instructions. 4′-6-Diamidino-2-phenylindole was used as for counterstaining. The kidney samples fixed for TUNEL examination were dehydrated and embedded with paraffin. Five-micron sections were cut from the embedded blocks. Then, the sections were deparaffinized, rehydrated and treated with Protease K, after which they were incubated with the TUNEL reaction mixture in a humidified chamber at 37°C for 2 hours. The sections were washed with PBS (pH 7.4) to terminate the reaction and then treated with 4′-6-diamidino-2-phenylindole. Sections treated with DNase before TUNEL examination served as a positive control, and sections without terminal deoxynucleotidyl transferase were used as a negative control. From each section, five randomly selected fields (200× magnification) were photographed with a fluorescence microscope (Eclipse CI-S; Nikon Corporation, Tokyo, Japan). The number of TUNEL-positive cells in each field was counted using Image-Pro Plus 6.0 software (Media Cybernetics Inc.) and divided by the field area. The average apoptotic cell density of the five fields was then obtained for each group.

2.14 | Data analysis

Unless specified otherwise, data are presented as the means ± SEM. The Shapiro-Wilk method was used to test the normality of the variables. One-way ANOVA followed by the multiple range least significant difference test was used to assess differences among different groups. We used the Mann-Whitney *U*-test to perform the nonparametric test. All statistical analyses were performed with SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered statistically significant. GraphPad Prism (v.4.0; GraphPad Software Inc., San Diego, CA, USA) was used to prepare all the figures.

3 | RESULTS

3.1 | Glyphosate induces cytotoxicity and apoptosis in HK-2 cells

HK-2 cells were exposed to glyphosate for 24 hours at concentrations of 0, 20, 40, 50, 60, 70, 80, 90 or 100 μM. We quantified the number of viable cells using the CCK-8 assay. The results showed that the number of viable cells was significantly reduced in the glyphosate-exposed group at concentrations ≥40 μM (Figure 1A). Meanwhile, the results from the LDH release assay also demonstrated that glyphosate treatment increased LDH release and induced toxicity effects at high concentrations (40-100 μM) (Figure 1B). In addition, we

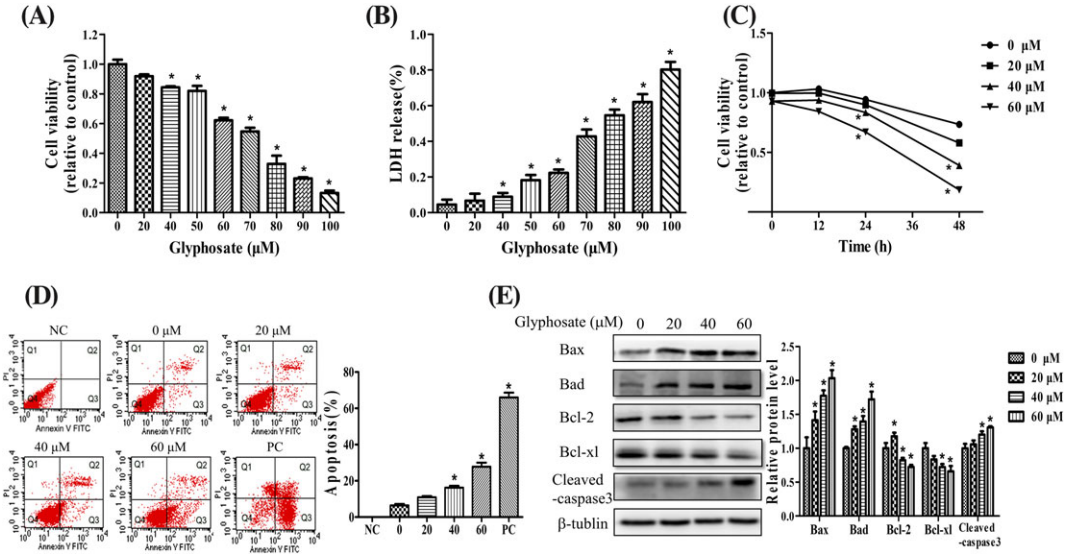

FIGURE 1 Glyphosate treatment induces cytotoxicity and apoptosis in HK-2 cells. A, Cell viability after different doses of glyphosate treatment for 24 h was measured using the CCK-8 assay. B, Release of LDH after 24 h of glyphosate treatment. C, Cell viability after 12, 24 and 48 h was measured using the CCK-8 assay. Fold changes were calculated relative to control cells. D, Percentage of apoptosis detected after glyphosate treatment for 24 h. Apoptosis data are from Q2 + Q3. E, Representative immunoblotting images of apoptosis proteins and quantitation of the expression levels by western blot. β-Tubulin was used as a loading control. Data are shown as the mean ± SEM. $^*P < 0.05$ compared with controls; $n = 3$. FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase; NC, negative control; PC, positive control

prolonged the exposure times and found that the cytotoxic effect of glyphosate on HK-2 cells was dose- and time-dependent. The cell viabilities of both the 40 and 60 μM groups were reduced but still greater than 50% after 24 hours (Figure 1C). Therefore, we conducted subsequent experiments with four doses (0, 20, 40 and 60 μM) and 24-hour exposure periods.

Apoptosis rates were examined after cells were exposed to glyphosate (0, 20, 40 and 60 μM) for 24 hours (Figure 1D). The results revealed that, in comparison with the control group, the glyphosate exposure groups showed increased percentages of apoptotic cells (Annexin V+, PI+/−), with significant differences occurring at concentrations greater than 40 μM. Furthermore, we investigated the expression of apoptosis-related proteins using Western blotting. As shown in Figure 1E, in comparison to the control group, the 20 and 40 μM glyphosate groups exhibited upregulated proapoptotic proteins (Bax and Bad) while the 40 and 60 μM groups exhibited downregulated antiapoptotic proteins (Bcl-2 and Bcl-xl). Meanwhile, the cleaved caspase-3 levels were significantly upregulated at both 40 and 60 μM.

3.2 | Glyphosate induces oxidative stress and increases NMDAR1 expression and calcium influx in HK-2 cells

In comparison with the control group, the 40 and 60 μM groups showed significantly increased cellular ROS levels (Figure 2A). Moreover, the glyphosate exposure groups showed significant reductions in SOD, CAT and GSH-Px levels and a significant increase in the MDA level (Figure 2B).

Western blotting was used to detect expression changes in NMDAR1 after glyphosate exposure. As shown in Figure 2C, glyphosate exposure increased NMDAR1 expression in a dose-dependent manner. Because NMDAR is involved in calcium influx and calcium homeostasis, we further determined the $[Ca^{2+}]_i$ levels after glyphosate exposure using flow cytometry-based measurement with the Ca^{2+}-sensitive probe Fluo-4/AM. It was found that glyphosate exposure increased $[Ca^{2+}]_i$ levels (Figure 2D).

3.3 | Effects after pretreatment with MK-801

To examine the role of NMDAR in glyphosate-induced calcium influx and apoptosis, we pretreated HK-2 cells with the NMDAR inhibitor MK-801 (100 μM for 12 hours) followed by treatment with 40 μM glyphosate for 24 hours. MK-801 attenuated the upregulation of $[Ca^{2+}]_i$ in the HK-2 cells after 24 hours of glyphosate treatment (Figure 3A). Meanwhile, inhibition of NMDAR attenuated the ROS increase in these cells (Figure 3B) and significantly decreased glyphosate-induced apoptosis (Figure 3C).

3.4 | Effects after pretreatment with BAPTA-AM and *N*-acetylcysteine

To examine the relationship between glyphosate-induced calcium influx and apoptosis, we pretreated with 2 μM BAPTA-AM (an intracellular calcium chelator) for 12 hours to decrease $[Ca^{2+}]_i$. Cells treated with BAPTA-AM and 40 μM glyphosate had lower $[Ca^{2+}]_i$ levels than cell exposed to glyphosate alone (Figure 4A). BAPTA-AM

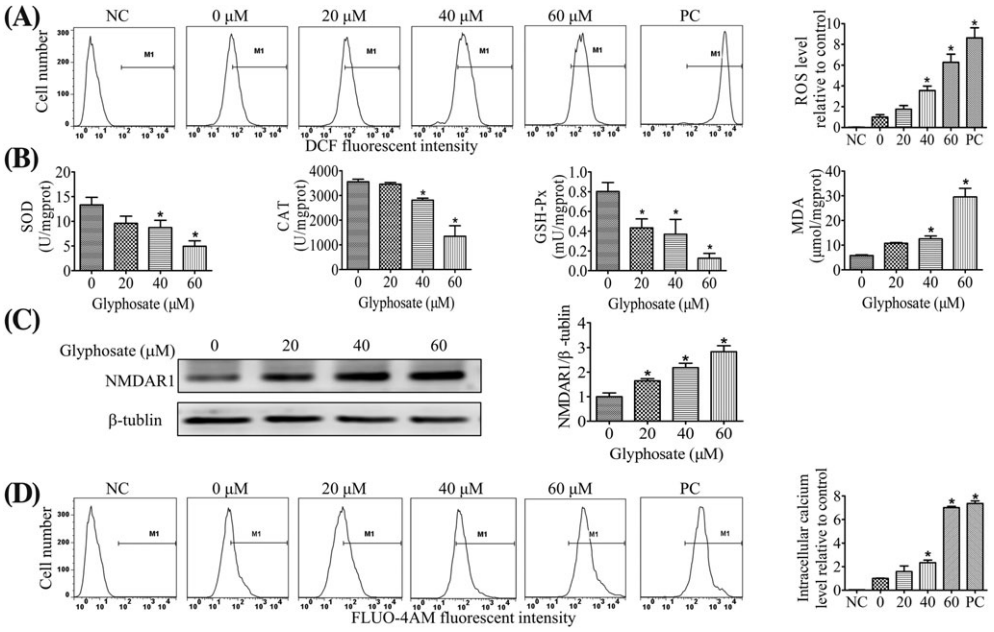

FIGURE 2 Glyphosate induces oxidative stress and increases NMDAR1 expression and calcium influx in HK-2 cells. A, Intracellular ROS production. Representative histograms of DCF fluorescence intensity are presented. NC indicates a negative control, and PC indicates a positive control. Percentage of cells located at M1 phase was quantitated. B, SOD, CAT, GSH-Px and MDA activities. C, Expression of NMDAR1 detected by immunoblotting. Protein expression levels were normalized against those of β-tubulin. D, Level of $[Ca^{2+}]_i$ analyzed by flow cytometry. NC indicates a negative control, and PC indicates a positive control. All data are presented as the mean ± SEM (n = 3). *P < 0.05 compared with controls cells. CAT, catalase; DCF, 2′,7′-dichlorofluorescein; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; mgprot, mg protein; NC, negative control; NMDAR1, *N*-methyl-D-aspartate receptor 1; PC, positive control; ROS, reactive oxygen species; SOD, superoxide dismutase

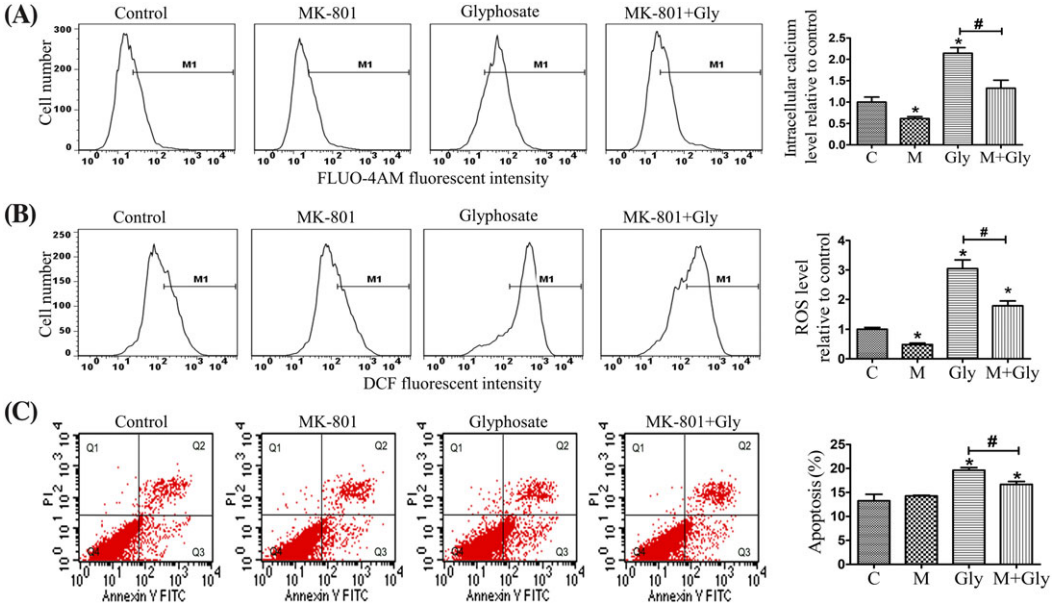

FIGURE 3 Inhibitory effects of MK-801 on glyphosate-induced elevation of $[Ca^{2+}]_i$, ROS levels and apoptotic rates. A, $[Ca^{2+}]_i$ levels. B, ROS levels. C, Apoptosis. All data are presented as the mean ± SEM (n = 3). *P < 0.05 compared with control cells. C, control; DCF, 2′,7′-dichlorofluorescein; FITC, fluorescein isothiocyanate; M, MK-801; Gly, glyphosate; M + Gly, MK-801 + glyphosate; ROS, reactive oxygen species

pretreatment also significantly alleviated glyphosate-induced apoptosis and ROS (Figure 4B and 4C).

To determine the role of ROS in glyphosate-induced apoptosis of HK-2 cells, we pretreated cells with 2 mM NAC (a ROS scavenger) for 12 hours before glyphosate treatment. NAC pretreatment reduced the ROS levels elevated by glyphosate (Figure 4D). Moreover, glyphosate-induced apoptosis of HK-2 cells was also alleviated by NAC (Figure 4E).

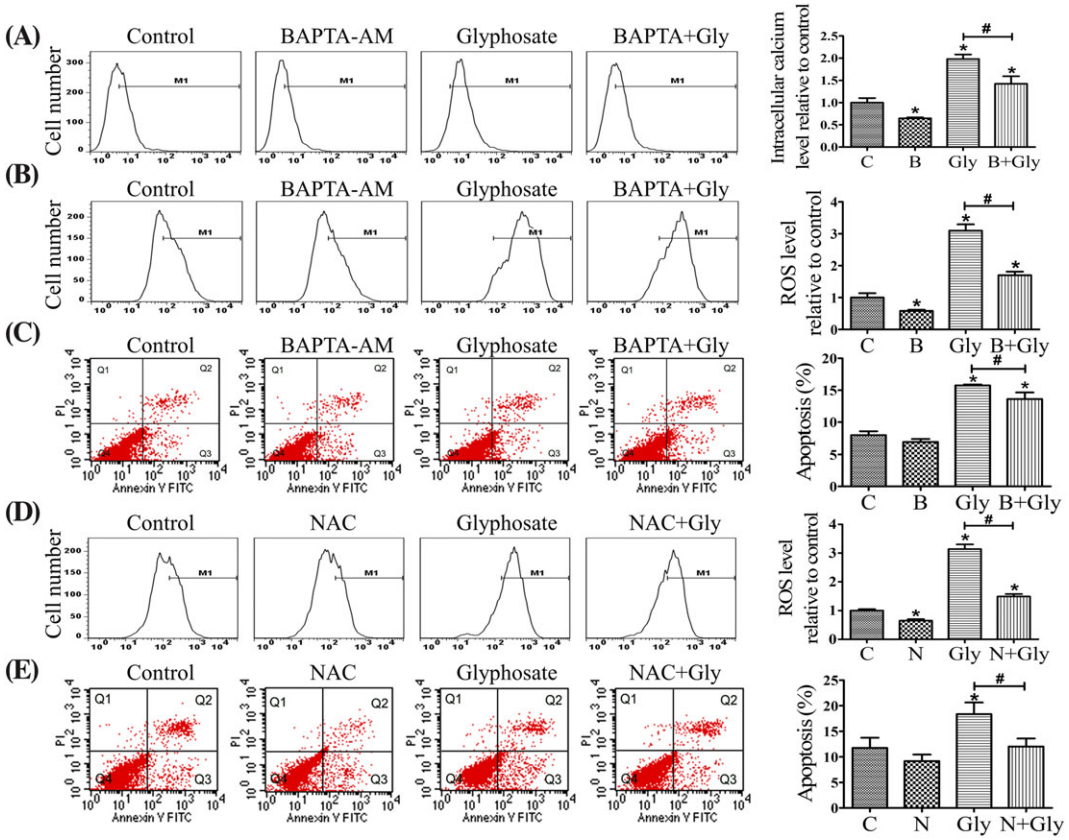

FIGURE 4 Effects of BAPTA-AM and NAC on glyphosate-induced elevation of $[Ca^{2+}]_i$, ROS levels and apoptosis rates. A, $[Ca^{2+}]_i$ level. B, ROS levels. C, Apoptosis rates in the BAPTA-AM-treated groups. D, ROS levels. E, Apoptosis rates in the NAC-treated groups. All data are presented as the mean ± SEM (n = 3). *P < 0.05 compared with control cells. B + Gly, BAPTA-AM+glyphosate; B, BAPTA-AM; C, control; FITC, fluorescein isothiocyanate; Gly, glyphosate; N + Gly, NAC + glyphosate; N, NAC; NAC, *N*-acetylcysteine; ROS, reactive oxygen species

3.5 | NMDAR1 expression and kidney damage caused by glyphosate

To investigate the effect of glyphosate on the kidney in vivo, we administered glyphosate solution to ICR mice for 28 days at a daily dose of 400 mg/kg. There was no significant difference between the control and glyphosate exposure groups in terms of body weight gain and organ coefficients of liver and kidney (Figure 5A and 5B). However, histological examination identified exfoliation of renal tubular cells (arrowhead) in the kidney (Figure 5C). The TUNEL assay also confirmed the increase in renal tubular cell apoptosis in glyphosate-treated mice (Figure 5D).

No significant changes in urine creatinine, uric acid, urea nitrogen, serum creatinine and blood urea nitrogen levels were observed (results not shown). However, an increase in urine albumin level was observed on days 7 and 14, but this increase disappeared later on. Urine β_2-microglobulin levels were increased on days 7, 21 and 28 (Table 1).

In addition, in comparison with the control group, the glyphosate-treated group showed significant reductions in the levels of SOD, CAT and GSH-Px, and a significant increase in MDA level in the kidney (P < 0.05) (Figure 5E). We also found that the average optical density for NMDAR1 was increased in kidneys from the glyphosate-treated group (P < 0.05) (Figure 5F).

4 | DISCUSSION

Several studies have linked kidney damage to exposure to glyphosate-based herbicides, but the underlying mechanism remains unclear. In this study, using a cell culture model, we found that glyphosate could directly affect the human renal proximal tubular epithelial cell line HK-2 through the NMDAR1/calcium influx/oxidative stress pathway. Moreover, we confirmed that glyphosate exposure in an animal model could induce NMDAR1, increase oxidative stress and damage renal proximal tubular epithelial cells.

Although glyphosate has low oral acute mammalian toxicity, a considerable number of animal and epidemiological studies have demonstrated the toxicity of commercial glyphosate products (Cattani et al., 2017; Gress et al., 2015; Milesi et al., 2018; Tang et al., 2017; Wunnapuk et al., 2014). The kidney may be the main target of glyphosate-based herbicides. Some epidemiological studies have suggested a potential relationship between glyphosate-based herbicides (together with other factors) and CKDu in some farm workers in Sri Lanka and sugarcane workers in Central America (Jayasumana et al., 2014, 2015; Peraza et al., 2012). Metabolic studies suggested that the concentration of glyphosate and its metabolic products in kidney and liver tissues were 10-100-fold (or even greater) higher than that in most other tissues (BfR, 2015). Acute administration of a commercial

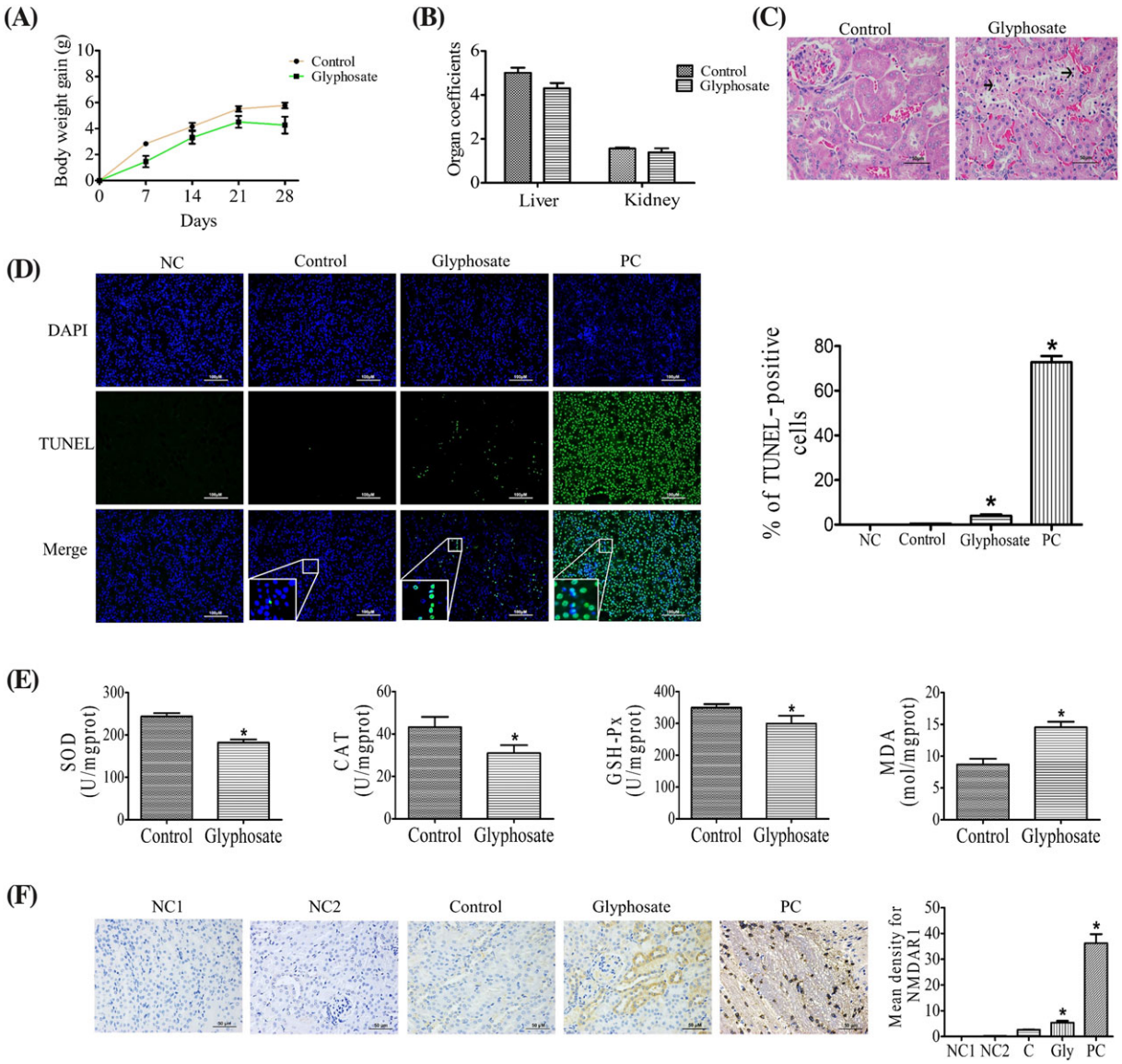

FIGURE 5 Effects of glyphosate on kidneys from 8-week-old ICR male mice. A, Body weight gain. B, Organ coefficient of liver and kidney. C, Histological analysis (at 400× magnification), black arrows indicating tubular cell damage. D, TUNEL assay (at 200× magnification) to detect apoptotic cells. Green cells indicate TUNEL-positive apoptotic cells. (Lower left inset: higher magnification of TUNEL-positive apoptotic cells.) *P < 0.05 compared with control cells. E, SOD, CAT, GSH-Px and MDA activities. F, Immunohistochemical analysis of NMDAR1 expression in kidney. Immunoreactivity was quantified with ImageJ software by measuring the integrated optical density. Representative images of positive immunohistochemical staining for NMDAR1 (yellow staining indicates NMDAR1-positive expression), magnification: 400×. Data are shown as the mean ± SEM. *P < 0.05 compared with controls; n = 3. NC, negative control; C, control; CAT, catalase; Gly, glyphosate; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; mgprot, mg protein; NMDAR1, *N*-methyl-D-aspartate receptor 1; PC, positive control; SOD, superoxide dismutase

product of glyphosate at high doses or subchronic oral exposure for 8 weeks caused renal toxicity in rats (Tizhe et al., 2014). A long-term and low-dose administration regimen (for approximately 2 years) induced gene expression changes in liver and kidney, and the affected genes were related to inflammatory responses and oxidative stress (Mesnage et al., 2015). The renal proximal tubule may be a main target. In an acute exposure model (Wunnapuk et al., 2014), necrotic and apoptotic cells were shown to be primarily located in the tubular epithelium of the proximal straight tubule at 48 hours after exposure. Tubular necrosis was also shown in 12-week and 60-day exposure models (Dedeke et al., 2018; Hamdaoui et al., 2016). However, glyphosate-based herbicides such as Roundup contain a mixture of 15% polyoxyethylene amine with other unspecified surfactants (Howe et al., 2004); as such, its formulation was reported to be more toxic than glyphosate alone (Dedeke et al., 2018; Gasnier et al., 2009). Therefore, whether glyphosate has renal toxicity remains a question. Currently, commercial formulations containing glyphosate as the active ingredient are heavily used worldwide; thus, determining the renal toxicity of glyphosate can lay the foundation for assessing the risk of many glyphosate-based herbicides aside from their formulations.

TABLE 1 Results of urine biomarkers in two groups ($\bar{X} \pm S$)[a]

		Days				
		0	7	14	21	28
β_2-MG	Control	0.076 ± 0.020	0.068 ± 0.053	0.091 ± 0.019	0.077 ± 0.027	0.066 ± 0.079
	Glyphosate	0.076 ± 0.021	0.142 ± 0.041	0.152 ± 0.091	0.143 ± 0.049	0.235 ± 0.206
	t	0.003	−2.503	−1.32	−2.66	−3.324
	P	0.998	0.0368	0.235	0.026	0.021
ALB	Control	0.104 ± 0.034	0.085 ± 0.010	0.107 ± 0.027	0.108 ± 0.020	0.118 ± 0.050
	Glyphosate	0.105 ± 0.074	0.174 ± 0.076	0.188 ± 0.039	0.130 ± 0.022	0.089 ± 0.029
	t	−0.016	−2.284	−3.916	−1.676	1.196
	P	0.988	0.0518	0.004	0.128	0.262

ALB, albumin (mg/L); β_2-MG, β_2-macroglobulin (mg/L).

[a]Data are presented as the means ± SEM. *t*- and *P*-values were calculated using the Mann-Whitney *U*-test by comparing variables between the control group and glyphosate group.

In the current in vitro study, we used the human renal proximal tubular epithelial cell line HK-2 as the experimental model. This cell line (Ryan et al., 1994) is widely used to study human renal proximal tubule epithelial cell biology and pathology (Maggiorani et al., 2015). We found that glyphosate does induce cytotoxicity, although its acute toxicity might be low (because it was only observed at concentrations ≥40 μM). In our previous study, we observed cytotoxicity in HK-2 cells induced by cadmium exposure at a concentration of 20 μM (Ge et al., 2018). Furthermore, our in vivo experiment also indicated injury to renal tubular cells. In our mice model, we administered glyphosate daily at 400 mg/kg for 28 days. This dosage was determined according to the following data: the FAO/WHO estimate that the acceptable daily intake for humans of the sum of glyphosate, *N*-acetyl-glyphosate, AMPA and *N*-acetyl-AMPA is up to 1 mg/kg (FAO-WHO, 2016). We considered the safety factors as 10 × 10 × 4, in which the first factor of 10 represents species differences from animal extrapolation to humans, the second factor of 10 represents intraspecific species variation, and the factor of 4 represents the sensitivity for children because glyphosate has been reported to have developmental toxicity (Cattani et al., 2017). Based on the above assumption, if kidney damage is not observed when mice are dosed at 400 mg/kg, then glyphosate should be considered safe. Glyphosate tissue retention time is relatively short, but elimination of glyphosate from bone could be slower due to reversible binding of the phosphonic acid moiety to calcium ions in the bone matrix (such as residual glyphosate in soil) (Sprankle, Meggitt, & Penner, 1975; Williams, Kroes, & Munro, 2000); thus, bone might be a storage site during subacute or chronic glyphosate exposure. Therefore, any glyphosate that reaches the kidney directly after exposure might be metabolized quickly, but the kidney could be exposed to glyphosate continuously released from bone to induce chronic injury. Our in vivo experiment also confirmed pathological chronic injury with loss of tubular cells due to increased apoptosis. In addition, it has been reported that urinary glyphosate concentrations of occupationally exposed farmers without protective equipment were much higher than those of the farmers who only encountered environmental exposure; the highest urine concentrations of glyphosate in the occupationally exposed farmers may be 150 times higher than the average concentration in farmers (Acquavella et al., 2004; Arbuckle et al., 2001). Therefore, we estimate that the exposure level in the unprotected working population might be higher than expected.

To confirm the renal effects of glyphosate, reasonable mechanisms underlying such effects should be elucidated. Induction of oxidative stress seems to be the main mechanism for glyphosate-based herbicides (Hamdaoui et al., 2016; Milesi et al., 2018). In our cell culture experiment, glyphosate exposure increased the production of ROS. In both the in vitro and in vivo experiments, the MDA activity increased, but the activities of the major endogenous antioxidant enzymes SOD, CAT and GSH-Px decreased in the exposure groups compared to the control group. The SOD converts superoxide anions into H_2O_2 via a dismutation reaction, CAT metabolizes H_2O_2 to water and oxygen, and GSH-Px reduces H_2O_2 and other organic hydroperoxides (Andreoli, 1991). The increase in ROS and MDA and the decrease in CAT, SOD and GSH-Px indicated a disturbance in the pro-oxidant/antioxidant balance. To explore how glyphosate induced these imbalances, we focused on the role of NMDAR because the structure of glyphosate is similar to that of glycine and glutamate (which are agonists of NMDAR1) (Cattani et al., 2017). Therefore, NMDAR might be a potential candidate for glyphosate targets. NMDAR was originally identified in the central nervous system, but it has also been expressed outside of the central nervous system, including the kidney (Gabbai, 2018). Because NMDAR is a ligand of glutamate, which is an excitatory neurotransmitter that plays an integral role in synaptic plasticity (Willard & Koochekpour, 2013), a large number of studies have been conducted to investigate the roles of NMDAR in physiological and pathological processes within the nervous system. In the kidney, the functions of NMDAR are related to renal blood flow, filtration and reabsorption in the proximal tubule (Deng & Thomson, 2009). NMDAR has various subunits, among which the NMDAR1 subunit is essential for channel activity (Ishii et al., 1993) and can be detected in renal tubular epithelial cells (Leung et al., 2002). Because NMDAR has been reported to transduce the binding of glutamate and glycine by coupling with the opening of a calcium-permeable ion channel (Mandir et al., 2000), the activation of NMDAR may increase $[Ca^{2+}]_i$ levels and induce Ca^{2+} uptake into the mitochondria. The increase in $[Ca^{2+}]_i$ may promote ATP synthesis and increase the

metabolic rate, which would consume more oxygen and increase ROS generation (Brookes, Yoon, Robotham, Anders, & Sheu, 2004), ultimately leading to apoptosis (Görlach, Bertram, Hudecova, & Krizanova, 2015). NMDAR has been reported to mediate some renal diseases, such as hyperhomocysteinemia-induced glomerulosclerosis (Zhang & Shim, 2010), gentamicin nephrotoxicity (Leung et al., 2002) and lipopolysaccharide-induced renal insufficiency (Lin, Hung, Huang, & Ma, 2015). Those studies found that antagonists of NMDAR play a protective role. However, whether NMDAR expressed in renal tubular epithelium responds to glyphosate exposure remains unclear. In this study, using the human renal tubular epithelial cell line HK-2, we demonstrated increased expression of NMDAR1 protein upon glyphosate exposure. In the animal tests, we observed a clear upregulation of NMDAR1 in renal tissue, particularly in tubular epithelial cells by immunohistochemical staining. Accompanied by the increase in NMDAR1 upon glyphosate exposure, $[Ca^{2+}]_i$, oxidative stress markers and apoptosis were all increased. Blocking NMDAR not only ameliorated glyphosate-induced increases in $[Ca^{2+}]_i$ and ROS levels but also attenuated apoptosis. Therefore, our results indicated that the increase in ROS levels and apoptosis induced by glyphosate was a result of NMDAR1 activation in renal tubular epithelial cells.

In the animal study, we monitored several parameters of proteinuria weekly. Although we did not find any differences in the urinary levels of creatinine, urea nitrogen or uric acid between the control and exposure groups, the levels of urea β_2-microglobulin increased after 7 days of glyphosate exposure, while urea albumin increased after 7 and 14 days of exposure but returned to normal levels after 21 and 28 days of exposure. β_2-Microglobulin is a small protein that is abundant in blood, filtered by the glomerulus and nearly completely reabsorbed by tubules. The increase in β_2-microglobulin in the urine indicated an impairment of tubular reabsorption (Dieterle et al., 2010). Albumin is not freely filtered by glomerulus. An increase in urinary albumin has been used as a marker of glomerular disease and may indicate impaired reabsorption by proximal tubules (Yu et al., 2010). The present results indicated that impaired renal function might occur at the early stage of glyphosate exposure. We speculated that the reversal of urea albumin levels at days 21 and 28 of exposure might be compensatory changes in renal function. However, pathological changes in renal tubular tissue still existed at day 28. These results suggest difficulty in the early diagnosis of kidney injury because of this compensatory response.

In conclusion, we demonstrated that glyphosate, the active ingredient of glyphosate-based herbicides, could injure renal tubule epithelial cells via the NMDAR1/$[Ca^{2+}]_i$/ROS pathway both in vitro and in vivo. These findings provide a theoretical basis and reference data to assess the risk of glyphosate and to explore the etiology of CKDu.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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REFERENCES

Acquavella, J. F., Alexander, B. H., Mandel, J. S., Gustin, C., Baker, B., Chapman, P., & Bleeke, M. (2004). Glyphosate biomonitoring for farmers and their families: results from the Farm Family Exposure Study. *Environmental Health Perspectives*, *112*, 321–326. https://doi.org/10.1289/ehp.6667

Andreoli, S. P. (1991). Reactive oxygen molecules, oxidant injury and renal disease. *Pediatric Nephrology*, *5*, 733–742. https://doi.org/10.1007/BF00857888

Arbuckle, T. E., Lin, Z., & Mery, L. S. (2001). An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population. *Environmental Health Perspectives*, *109*, 851–857. https://doi.org/10.2307/3454830

Arregui, M. C., Lenardon, A., Sanchez, D., Maitre, M. I., Scotta, R., & Enrique, S. (2004). Monitoring glyphosate residues in transgenic glyphosate-resistant soybean. *Pest Managment Science*, *60*, 163–166. https://doi.org/10.1002/ps.775

BfR (Bundesinstitut für Risikobewertung) (2015). BfR-contribution to the EU-approval process of glyphosate is finalised. Retrieved from https://mobil.bfr.bund.de/cm/349/bfr-contribution-to-the-eu-approval-process-of-glyphosate-is-finalised.pdf

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., & Sheu, S. S. (2004). Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *American Journal of Physiology. Cellular Physiology*, *287*, C817–C833. https://doi.org/10.1152/ajpcell.00139.2004

Cattani, D., Cesconetto, P. A., Tavares, M. K., Parisotto, E. B., De Oliveira, P. A., Rieg, C., … Zamoner, A. (2017). Developmental exposure to glyphosate-based herbicide and depressive-like behavior in adult offspring: Implication of glutamate excitotoxicity and oxidative stress. *Toxicology*, *387*, 67–80. https://doi.org/10.1016/j.tox.2017.06.001

Cattani, D., de Liz, O. C. V., Heinz, R. C., Domingues, J. T., Dal-Cim, T., Tasca, C. I., … Zamoner, A. (2014). Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: involvement of glutamate excitotoxicity. *Toxicology*, *320*, 34–45. https://doi.org/10.1016/j.tox.2014.03.001

Cessna, A. J., Darwent, A. L., Kirkland, K. J., Townleysmith, L., Harker, K. N., & Lefkovitch, L. P. (1994). Residues of glyphosate and its metabolite AMPA in wheat seed and foliage following preharvest applications. *Canadian Journal of Plant Science*, *74*, 653–661. https://doi.org/10.4141/cjps94-117

Cessna, A. J., Darwent, A. L., Townley-Smith, L., Harker, K. N., & Kirkland, K. J. (2000). Residues of glyphosate and its metabolite AMPA in canola seed following preharvest applications. *Canadian Journal of Plant Science*, *80*, 425–431. https://doi.org/10.4141/P99-062

Cetin, E., Sahan, S., Ulgen, A., & Sahin, U. (2017). DLLME-spectrophotometric determination of glyphosate residue in legumes. *Food Chemistry*, *230*, 567–571. https://doi.org/10.1016/j.foodchem.2017.03.063

Cuhra, M. (2015). Review of GMO safety assessment studies: glyphosate residues in Roundup Ready crops is an ignored issue. *Environmental Sciences Europe*, *27*. https://doi.org/10.1186/s12302-015-0052-7

Davies, J., Evans, R. H., Francis, A. A., & Watkins, J. C. (1979). Excitatory amino acid receptors and synaptic excitation in the mammalian central nervous system. *Journal de Physiologie*, *75*, 641–654.

De Liz, O. C. V., Cattani, D., Heinz, R. C., Pierozan, P., Zanatta, L., Benedetti, P. E., … Zamoner, A. (2013). Roundup disrupts male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells. *Free Radical Biology and Medicine*, *65*, 335–346. https://doi.org/10.1016/j.freeradbiomed.2013.06.043

Dedeke, G. A., Owagboriaye, F. O., Ademolu, K. O., Olujimi, O. O., & Aladesida, A. A. (2018). Comparative assessment on mechanism underlying renal toxicity of commercial formulation of roundup herbicide and glyphosate alone in male albino rat. *International Journal of Toxicology*, *37*, 285–295. https://doi.org/10.1177/1091581818779553

Deng, A., & Thomson, S. C. (2009). Renal NMDA receptors independently stimulate proximal reabsorption and glomerular filtration. *American Journal of Physiology. Renal Physiology*, *296*, F976–F982. https://doi.org/10.1152/ajprenal.90391.2008

Dieterle, F., Perentes, E., Cordier, A., Roth, D. R., Verdes, P., Grenet, O., … Maurer, G. (2010). Urinary clusterin, cystatin C, beta 2-microglobulin and total protein as markers to detect drug-induced kidney injury. *Nature Biotechnology*, *28*, 463–U114. https://doi.org/10.1038/nbt.1622

Duke, S. O. (2015). Perspectives on transgenic, herbicide-resistant crops in the United States almost 20 years after introduction. *Pest Management Science*, *71*, 652–657. https://doi.org/10.1002/ps.3863

Duke, S. O., & Powles, S. B. (2008). Glyphosate: a once-in-a-century herbicide. *Pest Management Science*, *64*, 319–325. https://doi.org/10.1002/ps.1518

EPA, US (1993). Reregistration Eligibility Decision (RED) for Glyphosate. Retrieved from https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/red_PC-417300_1-Sep-93.pdf

FAO/WHO. (2007). Pesticide Residues in Food 2006: Report of the Joint Meeting of the FAO Panel of Experts on Pesticide Residues in Food and the Environment and the WHO Core Assessment Group, Rome, Italy, 3–12 October 2006. World Health Organization; Food and Agriculture Organization of the United Nations. Retrieved from http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/JMPRrepor2006.pdf

FAO-WHO. (2016). Joint FAO/WHO Meeting on Pesticide Residues (JMPR). Pesticide residues in food—2016, Retrieved from http://www.fao.org/3/a-i5693e.pdf.

Gabbai, F. B. (2018). The role of renal response to amino acid infusion and oral protein load in normal kidneys and kidney with acute and chronic disease. *Current Opinion in Nephrology and Hypertension*, *27*, 23–29. https://doi.org/10.1097/MNH.0000000000000380

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M. C., & Seralini, G. E. (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology*, *262*, 184–191. https://doi.org/10.1016/j.tox.2009.06.006

Ge, Z., Diao, H., Ji, X., Liu, Q., Zhang, X., & Wu, Q. (2018). Gap junctional intercellular communication and endoplasmic reticulum stress regulate chronic cadmium exposure induced apoptosis in HK-2 cells. *Toxicology Letters*, *288*, 35–43. https://doi.org/10.1016/j.toxlet.2018.02.013

Görlach, A., Bertram, K., Hudecova, S., & Krizanova, O. (2015). Calcium and ROS: A mutual interplay. *Redox Biology*, *6*, 260–271. https://doi.org/10.1016/j.redox.2015.08.010.

Gress, S., Lemoine, S., Seralini, G. E., & Puddu, P. E. (2015). Glyphosate-based herbicides potently affect cardiovascular system in mammals: review of the literature. *Cardiovascular Toxicology*, *15*, 117–126. https://doi.org/10.1007/s12012-014-9282-y

Hamdaoui, L., Naifar, M., Mzid, M., Ben, S. M., Chtourou, A., Makni-Ayadi, F., … Rebai, T. (2016). Nephrotoxicity of Kalach 360 SL: biochemical and histopathological findings. *Toxicology Mechanisms and Methods*, *26*, 685–691. https://doi.org/10.1080/15376516.2016.1230918

Howe, C. M., Berrill, M., Pauli, B. D., Helbing, C. C., Werry, K., & Veldhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry*, *23*, 1928–1938. https://doi.org/10.1897/03-71

Huang, X., Pedersen, T., Fischer, M., White, R., & Young, T. M. (2004). Herbicide runoff along highways. 1. Field observations. *Environmental Science & Technology*, *38*, 3263–3271. https://doi.org/10.1021/es034847h

Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., … Et, A. (1993). Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits. *Journal of Biological Chemistry*, *268*, 2836–2843.

Jayasumana, C., Gunatilake, S., & Senanayake, P. (2014). Glyphosate, hard water and nephrotoxic metals: are they the culprits behind the epidemic of chronic kidney disease of unknown etiology in Sri Lanka? *International Journal of Environmental Research and Public Health*, *11*, 2125–2147. https://doi.org/10.3390/ijerph110202125

Jayasumana, C., Paranagama, P., Agampodi, S., Wijewardane, C., Gunatilake, S., & Siribaddana, S. (2015). Drinking well water and occupational exposure to Herbicides is associated with chronic kidney disease, in Padavi-Sripura, Sri Lanka. *Environmental Health*, *14*, 6. https://doi.org/10.1186/1476-069X-14-6

Krüger, M., Schledorn, P., Schrödl, W., Hoppe, H.-W., Lutz, W., & Shehata, A. (2014). Detection of glyphosate residues in animals and humans. *Journal of Environmental & Analytical Toxicology*, *4*(2), 1. https://doi.org/10.4172/2161-0525.1000210

Leung, J. C., Travis, B. R., Verlander, J. W., Sandhu, S. K., Yang, S. G., Zea, A. H., … Silverstein, D. M. (2002). Expression and developmental regulation of the NMDA receptor subunits in the kidney and cardiovascular system. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *283*, R964–R971. https://doi.org/10.1152/ajpregu.00629.2001

Lin, C., Hung, S., Huang, H., & Ma, M. (2015). Blockade of the N-methyl-D-aspartate glutamate receptor ameliorates lipopolysaccharide-induced renal insufficiency. *PLoS ONE*, *10*, e0132204. https://doi.org/10.1371/journal.pone.0132204

Maggiorani, D., Dissard, R., Belloy, M., Saulnier-Blache, J., Casemayou, A., Ducasse, L., … Buffin-Meyer, B. (2015). Shear stress-induced alteration of epithelial organization in human renal tubular cells. *PLoS ONE*, *10*, e0131416. https://doi.org/10.1371/journal.pone.0131416

Mandir, A. S., Poitras, M. F., Berliner, A. R., Herring, W. J., Guastella, D. B., Feldman, A., … Dawson, V. L. (2000). NMDA but not non-NMDA excitotoxicity is mediated by poly(ADP-ribose) polymerase. *Journal of Neuroscience*, *20*, 8005–8011. https://doi.org/10.1523/JNEUROSCI.20-21-08005.2000

Mensink, D. H., & Janssen, D. P. (2018). Glyphosate. Environmental Health Criteria No. 159. Geneva: World Health Organization. Retrieved from http://www.inchem.org/documents/ehc/ehc/ehc159.htm.

Mesnage, R., Arno, M., Costanzo, M., Malatesta, M., Séralini, G., & Antoniou, M. N. (2015). Transcriptome profile analysis reflects rat liver and kidney damage following chronic ultra-low dose Roundup exposure. *Environmental Health*, *14*, 70. https://doi.org/10.1186/s12940-015-0056-1

Milesi, M. M., Lorenz, V., Pacini, G., Repetti, M. R., Demonte, L. D., Varayoud, J., & Luque, E. H. (2018). Perinatal exposure to a

glyphosate-based herbicide impairs female reproductive outcomes and induces second-generation adverse effects in Wistar rats. *Archives of Toxicology*, *92*, 2629–2643. https://doi.org/10.1007/s00204-018-2236-6

Milić, M., Žunec, S., Micek, V., Kašuba, V., Mikolić, A., Lovaković, B. T., … Želježić, D. (2018). Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28-day exposure to glyphosate. *Archives of Industrial Hygiene and Toxicology*, *69*, 154–168. https://doi.org/10.2478/aiht-2018-69-3114

Myers, J. P., Antoniou, M. N., Blumberg, B., Carroll, L., Colborn, T., Everett, L. G., … Benbrook, C. M. (2016). Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. *Environmental Health*, *15*, 19. https://doi.org/10.1186/s12940-016-0117-0

NAWQA (National Water-Quality Assessment) (2012). Estimated Agricultural Use for Glyphosate. Retrieved from https://water.usgs.gov/nawqa/pnsp/usage/maps/show_map.php?year=2012&map=GLYPHOSATE&hilo=L&disp=Glyphosate

Niemann, L., Sieke, C., Pfeil, R., & Solecki, R. (2015). A critical review of glyphosate findings in human urine samples and comparison with the exposure of operators and consumers. *Journal of Consumer Protection Food Safety*, *10*, 3–12. https://doi.org/10.1007/s00003-014-0927-3

Peraza, S., Wesseling, C., Aragon, A., Leiva, R., García-Trabanino, R. A., Torres, C., … Hogstedt, C. (2012). Decreased kidney function among agricultural workers in El Salvador. *American Journal of Kidney Diseases*, *59*, 531–540. https://doi.org/10.1053/j.ajkd.2011.11.039

Ryan, M. J., Johnson, G., Kirk, J., Fuerstenberg, S. M., Zager, R. A., & Torok-Storb, B. (1994). HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney International*, *45*, 48–57. https://doi.org/10.1038/ki.1994.6

Sprankle, P., Meggitt, W. F., & Penner, D. (1975). Adsorption, mobility, and microbial degradation of glyphosate in soil. *Weed Science*, *23*, 229–234.

Struger, J., Thompson, D., Staznik, B., Martin, P., McDaniel, T., & Marvin, C. (2008). Occurrence of glyphosate in surface waters of southern Ontario. *Bulletin of Environmental Contamination and Toxicology*, *80*, 378–384. https://doi.org/10.1007/s00128-008-9373-1

Swanson, N. L., Leu, A., Abrahamson, J., & Wallet, B. (2014). Genetically engineered crops, glyphosate and the deterioration of health in the United States of America. *Journal of Organic Systems*, *9*(2), 6–37.

Tang, J., Hu, P., Li, Y., Win-Shwe, T. T., & Li, C. (2017). Ion imbalance is involved in the mechanisms of liver oxidative damage in rats exposed to glyphosate. *Frontiers in Physiology*, *8*, 1083. https://doi.org/10.3389/fphys.2017.01083

Tizhe, E. V., Ibrahim, N. D., Fatihu, M. Y., Igbokwe, I. O., George, B. D., Ambali, S. F., & Shallangwa, J. M. (2014). Serum biochemical assessment of hepatic and renal functions of rats during oral exposure to glyphosate with zinc. *Comparative Clinical Pathology*, *23*, 1043–1050. https://doi.org/10.1007/s00580-013-1740-6

WHO (World Health Organization) (2017). Some organophosphate insecticides and herbicides. Retrieved from http://apps.who.int/bookorders/anglais/detart1.jsp?codlan=1&codcol=72&codcch=112

Willard, S. S., & Koochekpour, S. (2013). Glutamate, glutamate receptors, and downstream signaling pathways. *International Journal of Biological Sciences*, *9*, 948–959. https://doi.org/10.7150/ijbs.6426

Williams, A. L., Watson, R. E., & DeSesso, J. M. (2012). Developmental and reproductive outcomes in humans and animals after glyphosate exposure: a critical analysis. *Journal of Toxicology Environmental Health-Part B-Critical Reviews*, *15*, 39–96. https://doi.org/10.1080/10937404.2012.632361

Williams, G. M., Kroes, R., & Munro, I. C. (2000). Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. *Regulatory Toxicology and Pharmacology*, *31*, 117–165. https://doi.org/10.1006/rtph.1999.1371

Wunnapuk, K., Gobe, G., Endre, Z., Peake, P., Grice, J. E., Roberts, M. S., … Liu, X. (2014). Use of a glyphosate-based herbicide-induced nephrotoxicity model to investigate a panel of kidney injury biomarkers. *Toxicology Letters*, *225*, 192–200. https://doi.org/10.1016/j.toxlet.2013.12.009

Yu, Y., Jin, H., Holder, D., Ozer, J. S., Villarreal, S., Shughrue, P., … Gerhold, D. L. (2010). Urinary biomarkers trefoil factor 3 and albumin enable early detection of kidney tubular injury. *Nature Biotechnology*, *28*, 470–477. https://doi.org/10.1038/nbt.1624

Zhang, B., & Shim, V. P. W. (2010). Effect of strain rate on microstructure of polycrystalline oxygen-free high conductivity copper severely deformed at liquid nitrogen temperature. *Acta Materialia*, *58*, 6810–6827. https://doi.org/10.1016/j.actamat.2010.09.009

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