# The Inhibition of Nrf2/NQO1 pathway was involved in Polymyxin B Exerts Nephrotoxicity Effects in vivo and in vitro

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**Research Article**

**Keywords:** Polymyxin B, Oxidative stress, Nrf2/NQO1 pathway, Apoptosis, Nephrotoxicity

**Posted Date:** May 10th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-1629876/v1

# Abstract

Polymyxin B (PMB) is a Polypeptide antibiotic widely applied in multidrug-resistant Gram-negative bacteria. However, nephrotoxicity is one of the serious adverse effects that limit its clinical use. Therefore, clarification of the molecular mechanism of PMB-induced renal injury appears to be particularly important. Our study aimed to explore the possible mechanism of PMB-induced nephrotoxicity *in vivo* and *in vitro*. Mice were treated with PMB to construct models of kidney injury. The antioxidant capacity was assessed by measuring the activity of SOD and CAT and the contents of GSH and MDA, and the Nrf2/NQO1 pathway was examined after PMB treatment in NRK-52E cells and mice. Finally, we assessed the expression of apoptosis-related genes and proteins (Bax, Bcl-2, Caspase-3, Caspase-9) through qPCR and WB assay. The study verified PMB-induced nephrotoxicity in mice and NRK-52E cells by a dose- and time-dependent manners. PMB treatment significantly down-regulated the expression of Nrf2 and its downstream target genes NQO1 and up-regulated the apoptosis-related protein expression. In summary, our results suggested PMB induced oxidative stress damage by inhibiting the Nrf2/NQO1 pathway and promoting apoptosis in kidney tissue.


# Introduction

With the increasing emergence of antibiotic-resistant bacteria in recent years [1], polymyxin has been the last-resort antibiotic for treating infections caused by multidrug-resistant Gram-negative bacteria[2, 3]. Polymyxins group consisted of five chemically different compounds, including polymyxin A, B, C, D, and E. Among five compounds, only polymyxin B (PMB) and polymyxin E (colistin) were clinically available[4]. Unlike colistin administered as an inactive prodrug (colistimethate), PMB is delivered as an active compound, which means PMB has superior PK characteristics in humans. Therefore, PMB is recommended as the preferred agent for routine systemic use in invasive infections [5]. However, it is now evidenced that nephrotoxicity is the major dose-limiting factor impacting the effective clinical use of PMB. Early clinical studies indicated the incidence of nephrotoxicity was 10–60% after intravenous administration of PMB [6–9]. Older adults, high daily dose, underlying diseases, the use of concomitant nephrotoxic drugs were among the independent predictors of nephrotoxicity [10].

The underlying mechanism of PMB-induced nephrotoxicity has not been fully established. Some studies [11, 12] found that PMB possessed noticeable accumulation in the renal cortex, especially in proximal tubular cells, which resulted in renal tubular epithelial cells apoptosis or necrosis. Recently study [13] has indicated that PMB-related nephrotoxicity was connected with reactive oxygen species (ROS), PMB damaged the capability of scavenging ROS in HK-2 and NRK-52E cells. Superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) are essential enzymes that participate in the removal of ROS from the cellular environment. SOD is the first line of defensing against ROS and is vital for the antioxidant enzymatic defensive system. MDA level reflects the extent of oxidative damage to cell membranes [14]. Normally, the production of ROS is usually in balance with the availability and cellular localization of antioxidant enzymes (SOD, CAT, GSH). Overproduction of ROS ultimately affects the activity of endogenous antioxidant enzymes and causes oxidative damage in renal cells [15].

The Kelch-like ECH-associated protein 1-NF-E2-related factor 2 (KEAP1-Nrf2) system possesses a cytoprotective role in maintaining redox homeostasis [16]. Nrf2 is a primary sensor of oxidative stress and a master regulator of the antioxidant defensive pathway [17]. Under physiological conditions, Keap1 binds to Nrf2 and mediates Nrf2 proteasomal degradation and ubiquitination. When under oxidative stress, Nrf2 dissociates from the Keap1/Nrf2 complex in the cytoplasm and translocates into the nucleus to activate the expression of downstream proteins involving antioxidant defensive systems[18, 19]. A previous study [20] showed that activating the Nrf2 signal pathway induced by Procyanidins inhibits apoptosis of delicate particulate matter-induced vascular smooth muscle cells. Bo Yoon Chang's study [21] has demonstrated that binge drinking patterns efficiently produce a state of oxidative stress that disturbs liver function. ETZL enhanced Nrf2 nuclear translocation and increased expression of the downstream target genes HO-1 and NQO1 as an antioxidant defensive factor, finally ameliorating binge ethanol-induced liver injury. Fei Gao's study [22] has indicated that activating the Nrf2/HO-1 pathway suppressed apoptosis and attenuated LPS-induced renal tubular epithelial cell injury.

Renal injury limits the efficacy and usage of PMB and degrades the quality of life, bringing severe psychological and economic burdens. Until now, study on the mechanism of PMB-related nephrotoxicity was scarce, the existing research[23, 24] just identified ROS connected with PMB-induced nephrotoxicity, further studies are warranted to understand the underlying molecular mechanisms. Our study was devoted to the identification of molecular mechanisms of PMB-related nephrotoxicity.

## Materials And Methods

## 2.1. Chemicals and reagents

Polymyxin B sulfate purchased from SPH NO.1 Biochemical& Pharmaceutical CO., LTD. (batch number A1420947). Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Gibco. Fetal bovine serum (FBS) was purchased from Procell. Cell Counting Kit-8 (CCK8) was purchased from Meilunbio®. Reactive Oxygen Species Assay Kit and Annexin V-FITC Apoptosis Detection Kit were purchased from Biyuntian Biotechnology (Shanghai, China).

## 2.1 Cell culture

The NRK-52E cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium supplemented with 5% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, in a humidified atmosphere containing 5% $CO_2$ at 37˚C.

## 2.2 Measurement of cell viability

Cell viability was determined by the CCK8 assay. NRK-52E cells were exposed to culture media containing PMB at different dosages for 12, 24, 36 and 48 h at 37°C. Then NRK-52E cells were handled by CCK8 reagent and continued to incubate for another 1–2 h. Absorption was measured at 450 nm using a microplate reader. Each experiment was repeated in triplicate, and the viability of each group was

expressed as a relative value compared to the control group. Cell viability (%) = (A450sample − A450blank)/ (A450control − A450blank) × 100%.

## 2.3 Flow Cytometry Analysis of Apoptosis

NRK-52E cells were incubated in 6-well plates by 1 × $10^6$ cells/well in medium with different concentrations of PMB (125, 500, 1000 μM) for 24 h. Following the manufacturer's instructions, cells were pelleted by centrifugation and incubated with anti-Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). Then, fluorescence was detected by flow cytometry (Beckman Coulter, USA) to assay the percentage of cell apoptosis.

## 2.4 Reactive oxygen species (ROS) detection

Total ROS Assay kits were used to quantify ROS in cells. After PMB treatment about 4 h, NRK-52E cells were centrifuged for 5 min at 1000 g and washed with serum-free DMEM medium. Washed cells (1x$10^6$ cells/ml) were stained with 10 μM of DCFH-DA at 37°C for 20 minutes. Then, labeled NRK-52E cells were trypsinized and analyzed by flow cytometry. Also, fluorescence microscope was applied to observe the level of ROS after PMB treatment 4 h.

## 2.5 Mice model of polymyxin-induced nephrotoxicity

Animal studies were approved by the Institutional Animal Care and Use Committee at the Hunan Provincial Institute of Traditional Chinese Medicine. 80 ICR mice (male, 6–8 weeks, 18–22 g) were purchased from Hunan SJA laboratory animal Co., Ltd (Hunan, China). The mice model of nephrotoxicity was established through intraperitoneal PMB injection. After the nephrotoxicity model was successfully constructed, mice were divided into four groups randomly. 20 mice per group, as described below. Control (intraperitoneal injection with 0.9% saline), PMB-L (intraperitoneal injection with 9.3mg/kg PMB), PMB-M (intraperitoneal injection with 14.0 mg/kg PMB) and PMB-H (intraperitoneal injection with 18.7 mg/kg PMB). The injection was given twice daily at 8:00 a.m. and 8:00 p.m. for 3 or 7 days. Mice were euthanized by intraperitoneal sodium pentobarbital (80 mg/kg) at 3- or 7-days post-administration and groups of 10 mice were used for each comparison (Fig. 1).

All blood samples were centrifuged (3000g, 10min) and stored at − 80°C until analysis. Renal and liver injury was evaluated through serum creatinine (Cre), blood urea nitrogen (BUN), glutamic pyruvic transaminase (ALT), glutamic oxaloacetic transaminase (AST), alkaline phosphatase (ALP), and total bile acids (TBA) levels using the corresponding ELISA assay kit according to the manufacturer's protocol. The liver and kidney of mice were weighed to assess the liver and kidney function index: liver (or kidney) function index = liver (or kidney) weight/body weight × 100. The left ones were stored in 10% buffered formalin for immunohistochemistry (hematoxylin-eosin (HE) staining) study [25], the right ones were used for biochemical and molecular studies.

## 2.6 Determination of CAT, GSH, SOD, and MDA levels in NRK-52E cells and mice kidney

CAT, GSH, SOD, and MDA were key parameters usually applied to evaluate the antioxidant abilities of cells. The activity of CAT and SOD and the contents of GSH and MDA were measured as markers of oxidative stress using the instructions of their corresponding kits (Jiancheng Biotech Inc., Nanjing, China).

# 2.7 The quantitative real-time PCR (qPCR) analysis

According to the manufacturer's instructions, total RNA was isolated from frozen kidney tissues stored at − 80 °C. qPCR analysis of mice *Nrf2*, *NQO1*, *Caspase-3*, *Caspase-9*, *Bax*, *Bcl-2* mRNA was performed using the ABI7500 Real-time PCR detection system. The corresponding primer sequences were listed in Table 1. *GAPDH* gene was used as an internal reference to normalize target gene expressions, and the $2^{-\Delta\Delta CT}$ method was used for quantitative analysis [26].

Table 1
Primer sequences used for quantitative real-time PCR

| Gene | Direction | Primer sequence (5' to 3') |
|---|---|---|
| Nrf2 | Forward | 5'-AGC CAG CTG ACC TCC TTA GA -3' |
| | Reverse | 5'-AGT GAC TGA CTG ATG GCA GC-3' |
| NQO1 | Forward | 5'-GCA TTG GCC ACA CTC CAC CAG − 3' |
| | Reverse | 5'-ATG GCC CAC AGA GAG GCC AAA − 3' |
| Caspase-3 | Forward | 5'-CTG ACT GGA AAG CCG AAA CTC-3' |
| | Reverse | 5'-GAC TGG ATG AAC CAC GAC CC-3' |
| Caspase-9 | Forward | 5'-GCG GTG GTG AGC AGA AAG A-3' |
| | Reverse | 5'-CCT GGG AAG GTG GAG TAG GA-3' |
| Bax | Forward | 5'-GGA TGC GTC CAC CAA GAA G-3' |
| | Reverse | 5'-CAA AGT AGA AGA GGG CAA CCA C-3' |
| Bcl-2 | Forward | 5'-TGT GGT CCA TCT GAC CCT CC-3' |
| | Reverse | 5'-ACA TCT CCC TGT TGA CGC TCT-3' |
| GAPDH | Forward | 5'-AGG TCG GTG TGA ACG GAT TTG − 3' |
| | Reverse | 5'-GGG GTC GTT GAT GGC AAC A-3' |

# 2.8 Western blot analysis

Kidney tissue was processed to obtain cell suspension, cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer with 1% PMSF for 30 minutes on ice and then centrifuged for 10 minutes at 12,000g and 4°C. Bicinchoninic acid (BCA) protein assay kits (Boster Biotechnology, Wuhan, China) were used to measure protein supernatant protein concentrations. Protein samples were separated using SDS-PAGE

(polyacrylamide gel electrophoresis) and then transferred onto PVDF membrane (Immobilon) hydrated with methanol. The membranes were incubated with primary antibodies overnight at 4°C and secondary antibodies for 2 h at room temperature. The ECL Chemiluminescence Detection Kit (Boster Biotechnology, Wuhan, China) was used for signal detection. The protein bands were quantified using ImageJ software.

As primary antibodies, mice monoclonals anti-Nrf2 antibody (1:1000, ab89443), NQO1 (1:10000, ab80588), Caspase-3 (1:2000, ab184787), Caspase-9 (1:2000, ab184786), Bcl-2 (1:1000, ab186495), Bax (1:2000, ab8182733) were from Abcom Plc, β-actin (1:3000, T0022) and GAPDH (1:3000, AF7021) were from Affinity Biosciences. Goat anti-rabbit IgG and goat anti-mice as secondary antibodies (1:5000) were from Boster Biological Technology co.ltd.

## 2.9 Statistical analysis

The *in vitro* experiments were repeated at least three times unless stated otherwise. As indicated in the figure legends, all quantitative data were presented as the mean ± SEM of biologically independent experiments or samples. Statistical analyses were carried out by GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA) and statistical comparisons were by one-way analysis of variance (ANOVA) for multiple comparisons. A $p$ value $< 0.05$ was considered statistically significant.

# Results

### 3.1 PMB decreased cells viability and promoted apoptosis in NRK-52E cells in a dose- and time-dependent way.

We initially tested the toxicity of PMB *in vitro*, NRK-52E cells were treated with different dosages of PMB about 12, 24, 36, 48 h. CCK8 assay showed that PMB treatment decreased viability of NRK-52E cells in a dose- and time-dependent manners, the IC50 value of PMB in 12, 24, 36, 48 h was 653.0 μM, 450.5 μM, 338.1 μM and 311.4 μM, respectively (Fig. 2A). Flow cytometry analysis was conducted to detect the levels of cell apoptosis. A significant time- and dose-dependently increase in apoptosis rate was observed after PMB treatment about 12 and 24 h, the percentage of early-apoptotic and late-apoptotic cells in PMB-125 μM, PMB-500 μM, PMB-1000 μM treatment about 12h groups were 3.6%±0.6%, 9.6%±2.6% and 24.3%±10.0%, respectively, and 24h were 7.7%±1.9%, 16.9%±2.5% and 36.9%±10.6%, respectively (Fig. 2B). Western blot experiment was performed to assess the expression of apoptotic pathway proteins in NRK-52E after PMB treatment. As shown in Fig. 2C-D, the expression of caspase-9 and caspase-3 proteins were increased after PMB treatment about 24 h, and the Bax/Bcl-2 ratio was also increased.

## 3.2 PMB induced kidney injury in mice in a dose- and time-dependent manner

To detect the toxicity of PMB *in vivo*, the following experiment was done. All mice were randomly divided into 4 groups with different treatments including control (0.9% saline), PMB-L (9.3mg/kg), PMB-M (14.0 mg/kg), PMB-H (18.7 mg/kg) for 3 days or 7 days. The injury markers of the liver and kidney were investigated to assess the potential toxic effect of PMB. Serum levels of BUN and Cre were estimated to

assess kidney injury. Compared with no change after PMB treatment 3 days, increased serum BUN and Cre levels were found after PMB treatment 7 days. A similar tendency was also observed in the kidney function index (Fig. 3A). For liver function, PMB didn't induce any significant effect on mice serum level of AST, ALT and TBA in 3 days or 7 days, while the serum level of ALP was increased in 7 days ($p < 0.05$). The liver function index was also detected and unchanged (Fig. 3B). These results indicated that PMB induced kidney injury in a dose- and time-dependent manner but had no impact on the liver. Additionally, HE stained was applied to detect injury in the liver and kidney after PMB treatment. As shown in Fig. 4, marked histopathological alterations such as tubular dilation and vacuole formation were manifested in the kidneys of mice in the group treated with 18.7 mg/kg polymyxin B (PMB-H), though only slightly tubular and glomerular injury were observed in 9.3 mg/kg PMB group (PMB-M) and 14.0 mg/kg PMB group (PMB-H).

Based on the above results, considering that PMB treatment for 3 days did not cause significant damage to kidney tissue, we mainly studied the kidney damage of mice after PMB treatment for 7 days in the follow-up experiments.

# 3.3 PMB promoted apoptosis in kidney tissue

Next, the apoptosis level in mice kidney tissue was explored after PMB treatment. Results from qPCR assay showed that compared with control group, the ratio of *Bax/Bcl-2* mRNA expression was increased in PMB-M ($p < 0.05$), PMB-H ($p < 0.01$) in a dose-dependent manner, *Caspase-9* mRNA expression was dose-dependently increased in PMB-L ($p < 0.01$), PMB-M ($p < 0.01$), PMB-H ($p < 0.01$), and *Caspase-3* mRNA expression was dose-independently increased in PMB-M ($p < 0.01$) and PMB-H ($p < 0.01$) (Fig. 5A). Then, the expression of apoptosis-related proteins was measured by Western Blot analysis. Consistence with the results from the qPCR assay, PMB increased the ratio of Bax/Bcl-2 protein in a dose-dependent manner, the expression of Caspase-9 protein was dose-dependently increased in PMB-L ($p < 0.05$), PMB-M ($p < 0.01$), PMB-H ($p < 0.01$), the expression of Caspase-3 protein was increased after PMB treatment and arrived at highest in PMB-M group ($p < 0.01$) (Fig. 5B-C). The above results indicated that PMB promoted apoptosis in kidney tissue.

### 3.4 The decline of antioxidant ability and the generation of Reaction Oxidative stress was involved in PMB-induced kidney injury in vivo and in vitro

To investigate the effect of PMB on the antioxidant level *in vivo* and *in vitro*, we firstly measured the activities or levels of CAT, GSH, SOD and MDA in NRK-52E cells. NRK-52E cells were treated with PMB at three different dosages (125 μM, 500 μM, 1000 μM), the results indicated that PMB decreased activity of CAT, GSH, and SOD in a dose-dependent manner, while the content of MDA was dose-dependently increased in the PMB-125 μM group ($p < 0.05$), PMB-250 μM group ($p < 0.01$) and PMB-1000 μM group ($p < 0.05$) (Fig. 6A). *In vivo* study, after 7 days of drug exposure, decreased activity or content of CAT, GSH, and SOD were observed in PMB-L (no significant), PMB-M ($p < 0.05$), and PMB-H group ($p < 0.05$) by a dose-dependent manner, while the content of MDA was dose-independently increased in PMB treatment ($p < 0.05$) (Fig. 6B). Then, intracellular ROS was evaluated after PMB treatment, the results from flow

cytometry analysis and fluorescence microscope showed that PMB induced the production of ROS in a dose-dependent manner (Fig. 6C-D). These results indicated that PMB declined antioxidant ability and induced the generation of ROS *in vivo* and *in vitro*, which resulted in kidney injury.

## 3.5 The inhibition of the Nrf2/NQO1 pathway was involved in PMB-induced toxicity

The Nrf2/NQO1 signaling pathway is an important regulator of oxidative stress. We next evaluated the Nrf2/NQO1 pathway in NRK-52E cells, western blot experiment was applied to detect the expression of Nrf2 and NQO1 protein, PMB induced dose-dependently decrease in the expression of Nrf2 and NQO1 protein (Fig. 7A). In mice study, the expression of *Nrf2* and *NQO1* mRNA was measured through qPCR experiments. Compared with the control group, the expression of *Nrf2* mRNA was decreased after PMB-H treatment ($p < 0.05$), but there was no significant change in PMB-L and PMB-M groups. Besides, PMB declined the level of *NQO1* mRNA in the PMB-L group ($p < 0.05$), PMB-M group ($p < 0.01$), PMB-H group ($p < 0.01$) with a dose-dependent manner (Fig. 7B). Western Blot experiment showed that PMB down-regulated the expressions of Nrf2 and NQO1 proteins in kidney tissues, the expression of Nrf2 protein was decreased in PMB-L (no significant), PMB-M ($p < 0.05$), PMB-H ($p < 0.01$) in a dose-dependent manner, while NQO1 protein expression was lowest in PMB-M group ($p < 0.01$) (Fig. 7C). These results demonstrated that the inhibition Nrf2/NQO1 pathway was involved in PMB-induced nephrotoxicity.

## Discussion

Although PMB has potent antibacterial activity, nephrotoxicity limits its systemic use in patients. In this study, we first demonstrated that PMB decreased viability and increased apoptosis in NRK-52E cells, and *in vivo* analyses were carried out to explore PMB-induced nephrotoxicity in mice. According to “International Consensus Guidelines for the Optimal Use of the Polymyxins,” [5] recommend a loading dose of 2.0-2.5 mg/kg for PMB based on total body weight (TBW) (equivalent to 20,000–25,000 IU/kg) over 1 hour. For patients with severe infections was suggested that a PMB dose of 1.25–1.5 mg/kg (equivalent to 12,500 − 15,000 IU/kg TBW) every 12 hours is infused over 1 hour. PMB dose in the mice model was calculated by normalization of body surface area. Histological examination showed PMB induced some damage in the kidney in a dose-dependent manner, and no apparent damage was observed in the liver. Previous studies[27, 28] revealed a substantial accumulation of PMB in the renal cortex induced nephrotoxicity. However, no central accumulation was detected in the other organs in mice, such as lungs, liver, or heart. Pooja Manchandani’s study[29] indicated that PMB-induced nephrotoxicity was correlated with renal drug exposure. Vital clinical characteristics of polymyxin-associated nephrotoxicity include increased serum BUN and Cre[30]; the same results were also observed in our study. Our results found that PMB increased serum BUN and Cre levels in a dose- and time-dependent manner, which was consistent with some clinical studies [10, 31]. A previous study [12] indicated PMB-induced apoptosis in rat and human kidney proximal tubular cells (NRK-52E cells and HK-2 cells) in a concentration- and time-dependent manner. Similarly, our study found that PMB increased

the expression of Caspase-9 and Caspase-3 proteins, the ratio of Bax/Bcl-2 was also increased in mice and NRK-52E cells.

In general, the dysregulation of ROS signal or excessive production of nonspecific ROS impairs innate defense and antioxidant capacity, disrupts normal organismal function, which is the pathophysiological basis of many diseases. ROS generation is one of the effective mechanisms of cells apoptosis, and drug-induced nephrotoxicity was manifested in previous studies [32, 33]. Our study evaluated the level of antioxidant enzymes after PMB treatment, such as MDA, GSH, SOD, and CAT [34]. We found PMB increased the level of GSH, SOD, and CAT and decreased the level of MDA, demonstrating PMB inhibited antioxidant abilities of cells in kidney tissue. Additionally, our study showed PMB dose-dependently increased intracellular ROS levels in NRK-52E cells. Our experimental results were consistent with the study of Azad [35], their study proclaimed that PMB nephrotoxicity was correlated with an increased level of MitoSOX, methionine was protective against polymyxin-induced nephrotoxicity through alleviating mitochondrial superoxide production.

As key endogenous antioxidant factors, Nrf2 and downstream target genes play essential roles in counterbalancing ROS production and protecting cells against oxidative stress [36]. Activation of Nrf2 Signaling can alleviate the toxicity related to reactive oxygen species and is an important molecular target for renal injury prevention [37–40]. In contrast, Nrf2 impairment causes accumulation of peroxide radicals inflicting renal tissues injuries[41]. The study explored PMB-induced ROS accumulation in the kidney whether involved in the Nrf2 pathway. Our study found that Nrf2 and NQO1 protein expression decreased in NRK-52E and mice. Unlike our result, one study [23] showed that reduced levels of HO-1 protein were involved in PMB-induced nephrotoxicity. We guessed that PMB might influence Nrf2 other downstream targets such as HO-1 but not only NQO1 protein. However, we failed to explore the further mechanism of PMB-induced kidney injury due to limitations in the study.

Together, our results demonstrated that the inhibition of the Nrf2 pathway increased ROS accumulation, which resulted in cell apoptosis and kidney injury (Fig. 8). Inevitably, there were still some limitations in this study. The specific molecular mechanisms of PMB nephrotoxicity need to be further profoundly studied. And some aggressive strategies were required to address the issue of nephrotoxicity.

# Conclusion

In summary, this was the first study demonstrating that the Nrf2/NQO1 pathway inhibition and ROS accumulation were involved in PMB-induced nephrotoxicity. The findings of our study highlighted that the profile of the Nrf2/NQO1 pathway and oxidative damage play essential roles in PMB-induced nephrotoxicity. However, future research was needed to focus on translating the reno-protective properties of the Nrf2/NQO1 pathway into clinical practice.

# Declarations

**Acknowledgments**

This work was supported by the Department of Science and Technology of Hunan Province (Grant numbers NO.2016SK4008); Changsha Science and Technology Project (Grant numbers kzd2001096).

**Authors' contributions** All the authors contributed substantially to the manuscript.

**Compliance with ethical standards**

**Conflict of interest** All authors have declared that no competing interests exist.

**Ethical approval** All animal experiments were conducted according to the National Institutes of Health's Guide for the Use and Care of Laboratory Animals and were approved by the Hunan Provincial Institute of Traditional Chinese Medicine (Changsha, China).

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# Figures

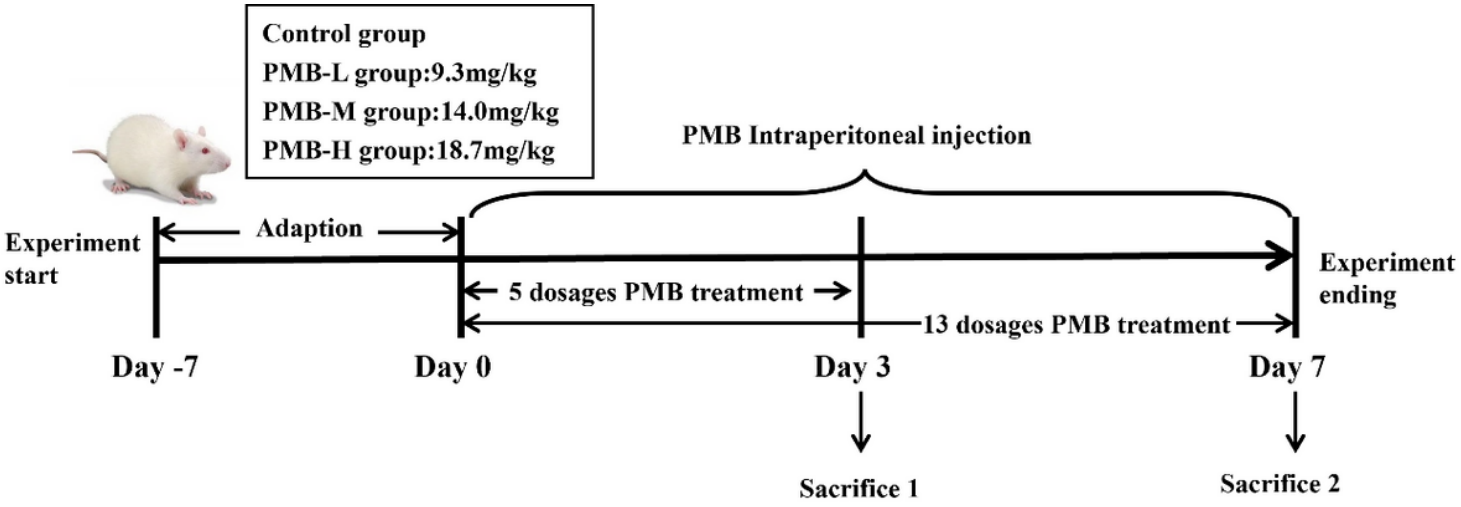
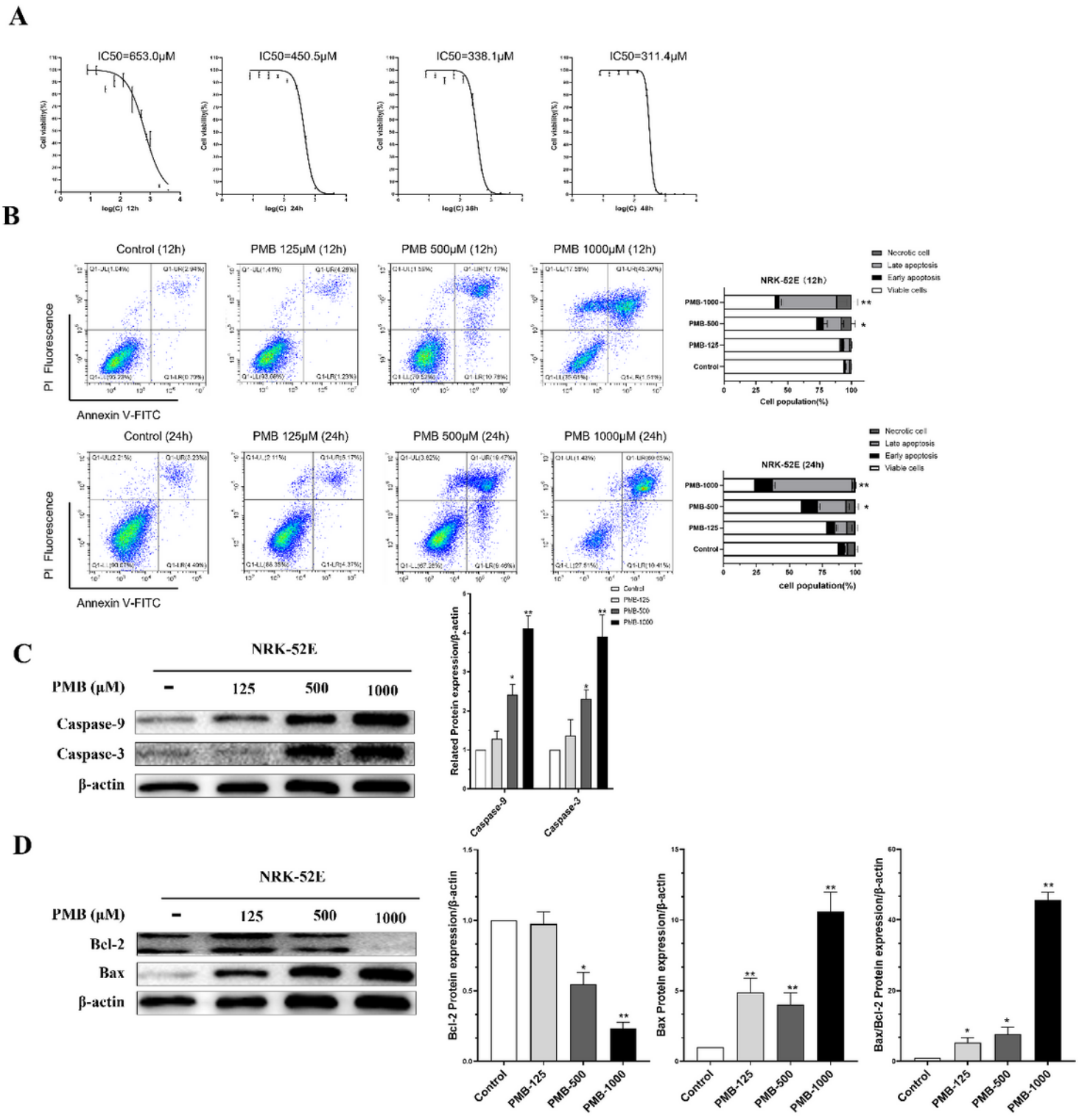



**Figure 1**

The study flow diagram for animal experiments.




## Figure 2

PMB decreased viability and promoted apoptosis in NER-52E cells.

(A) NRK-52E cells were treated with PMB at different concentrations (7.8, 15.6, 31.25, 62.5, 125, 250, 500, 750, 1000, 2000, 4000μM) about 12, 24, 36, 48 h. Cell viability was measured by CCK8 assay. (B) NRK-52E cells were treated with PMB for about 12 and 24 h, and the apoptosis level was tested by Annexin

V/PI staining assays. The statistical result of the graphs was shown on the right. * p < 0.05 compared with control for total apoptosis (early and late apoptosis). ** p < 0.01 compared with control for total apoptosis (early and late apoptosis). (C-D) The expression of apoptotic-related proteins (Caspase-3, Caspase-9, Bcl-2, Bax) were measured using the western Blot experiment after PMB-125 (125μM), PMB-500 (500μM), PMB-1000 (1000μM) treatment, respectively. Line chart of western blot after the gray scale analysis was shown in right. Data were represented as mean ± SEM, * *p < 0.05* compared with control. ** *p < 0.01* compared with control.

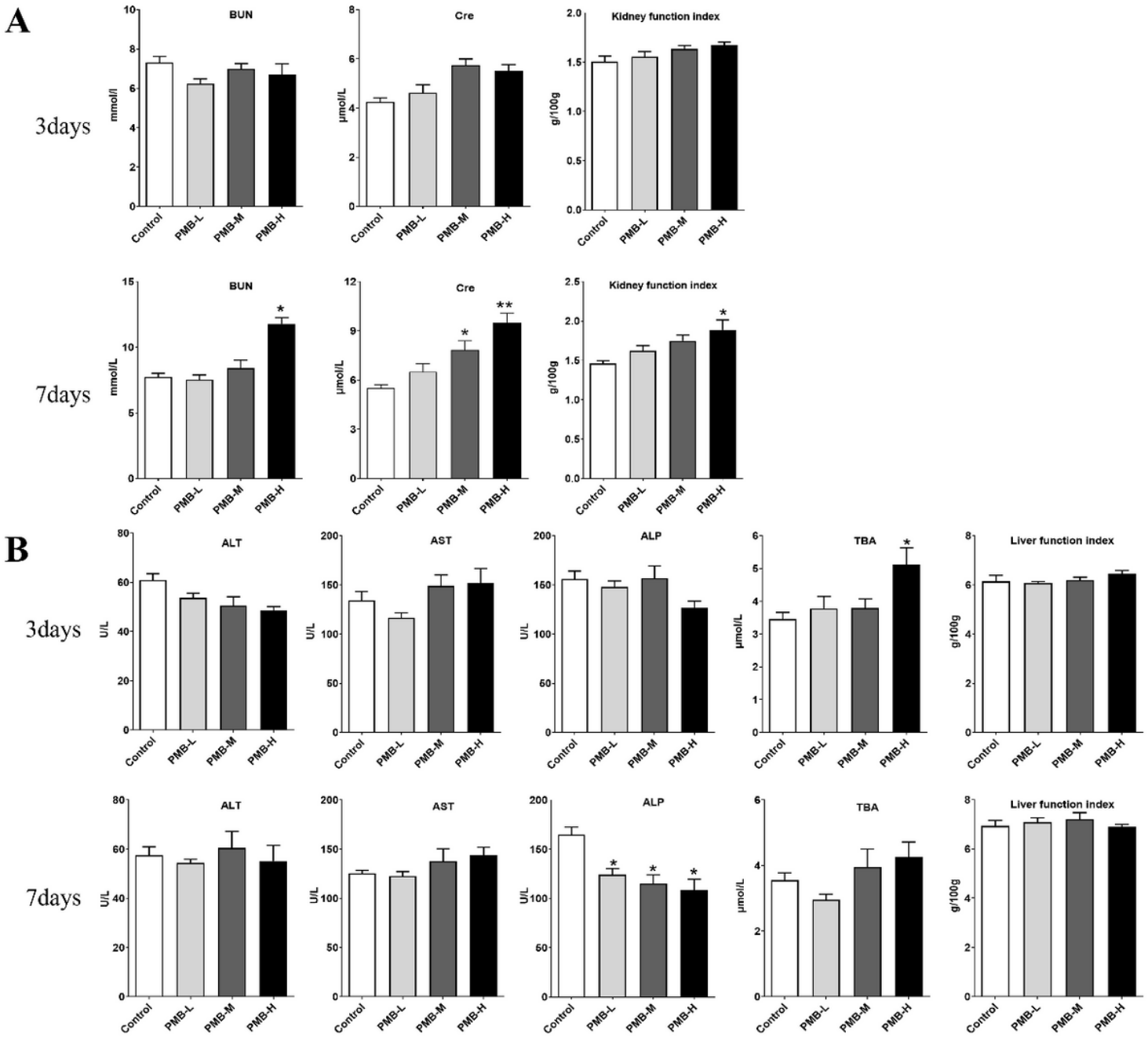
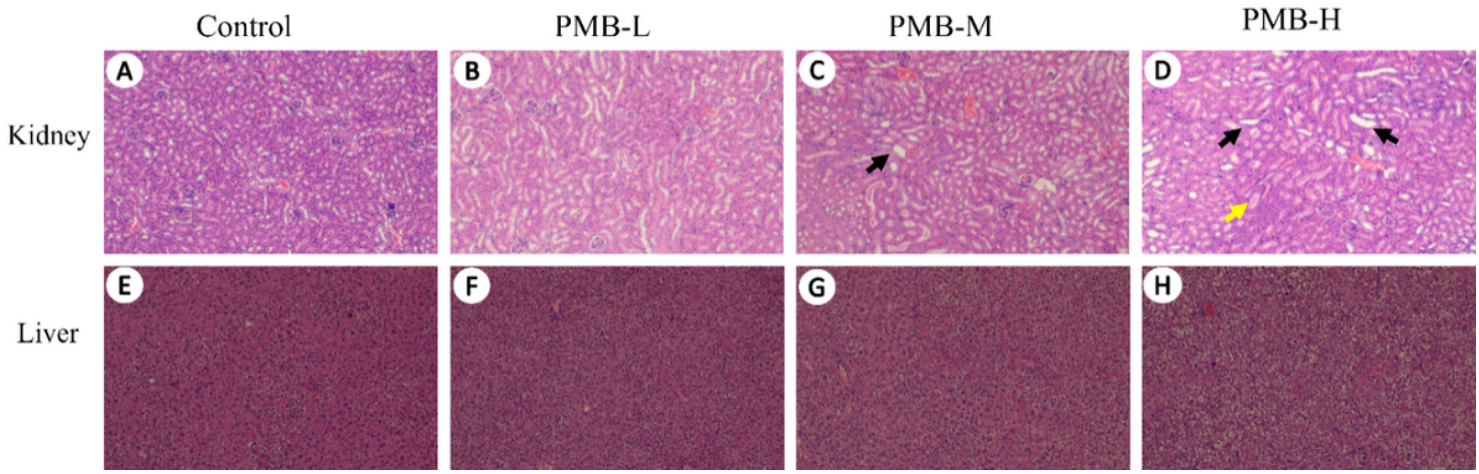



Figure 3

The kidney and liver injuries were assessed after PMB treatment for 3 or 7 days.

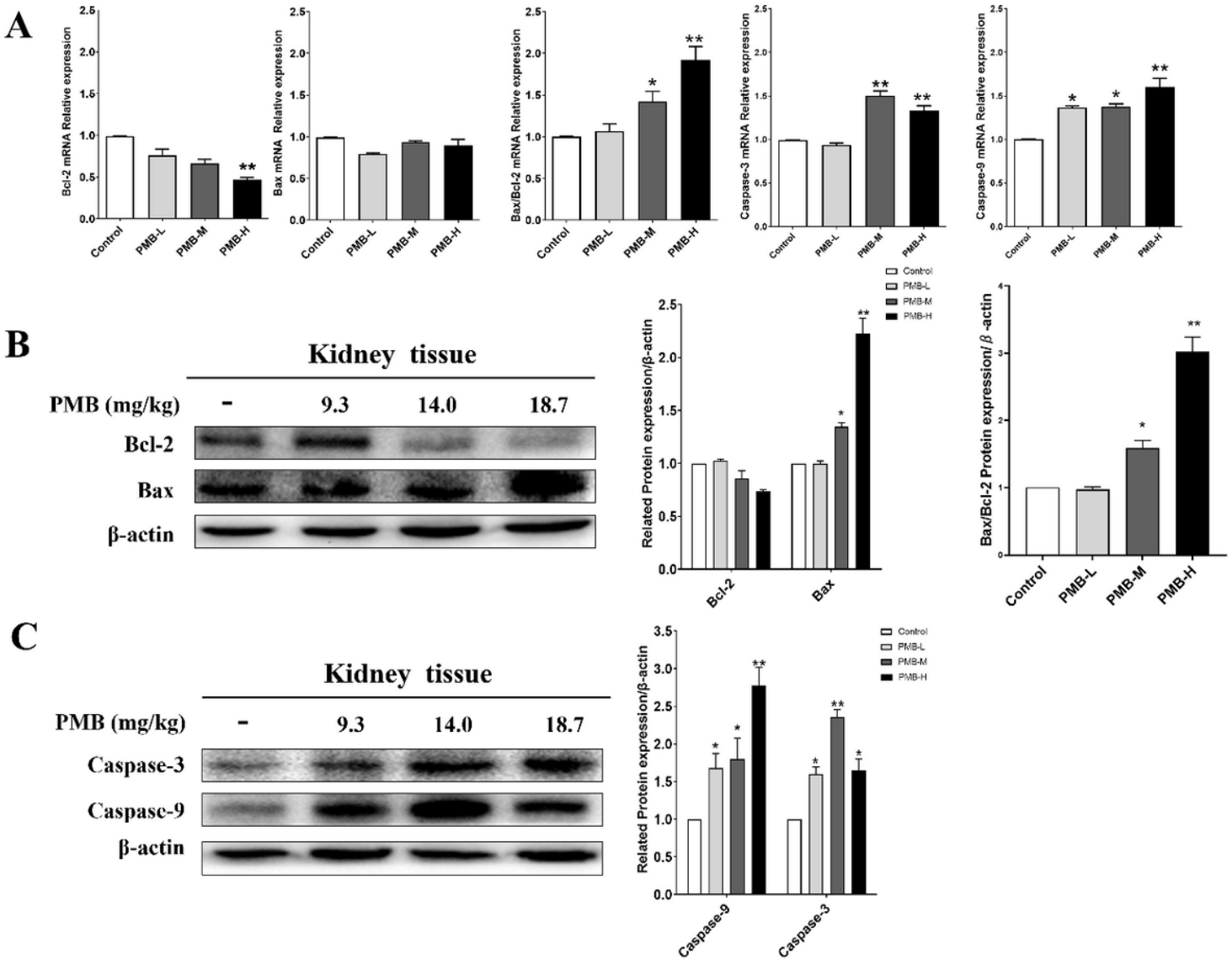
(A) Levels of Cre, BUN and kidney function index in mice was measured after PMB treatment 3 or 7 days. Control (Intraperitoneal injection 0.9% NaCl), PMB-L (9.0mg/kg), PMB-L (14.0mg/kg), measured PMB-L (18.7mg/kg). (B) Levels of ALT, AST, ALP, TBA and liver function index in mice was after PMB treatment 3 or 7 days. Control (Intraperitoneal injection 0.9% NaCl), PMB-L (9.0mg/kg), PMB-M (14.0mg/kg), PMB-H (18.7mg/kg). Data were represented as mean ± SEM, * $p < 0.05$ compare with control, * *$p < 0.01$ compared with control.




## Figure 4

Representative histological images of HE stained kidney and liver sections from mice in the control and PMB treatment groups.

(A-D) The kidney tissue of four groups of mice was HE stained to detect kidney damage after drug treatment. the black arrow represents dilated tubules, the yellow arrow represents the degeneration and necrosis of tubular epithelial cells and tubular casts. (E-H) Liver tissue of four groups of mice was HE stained to detect liver damage after drug treatment. Control (Intraperitoneal injection 0.9% NaCl), PMB-L (9.0mg/kg), PMB-M (14.0mg/kg), PMB-H (18.7mg/kg).

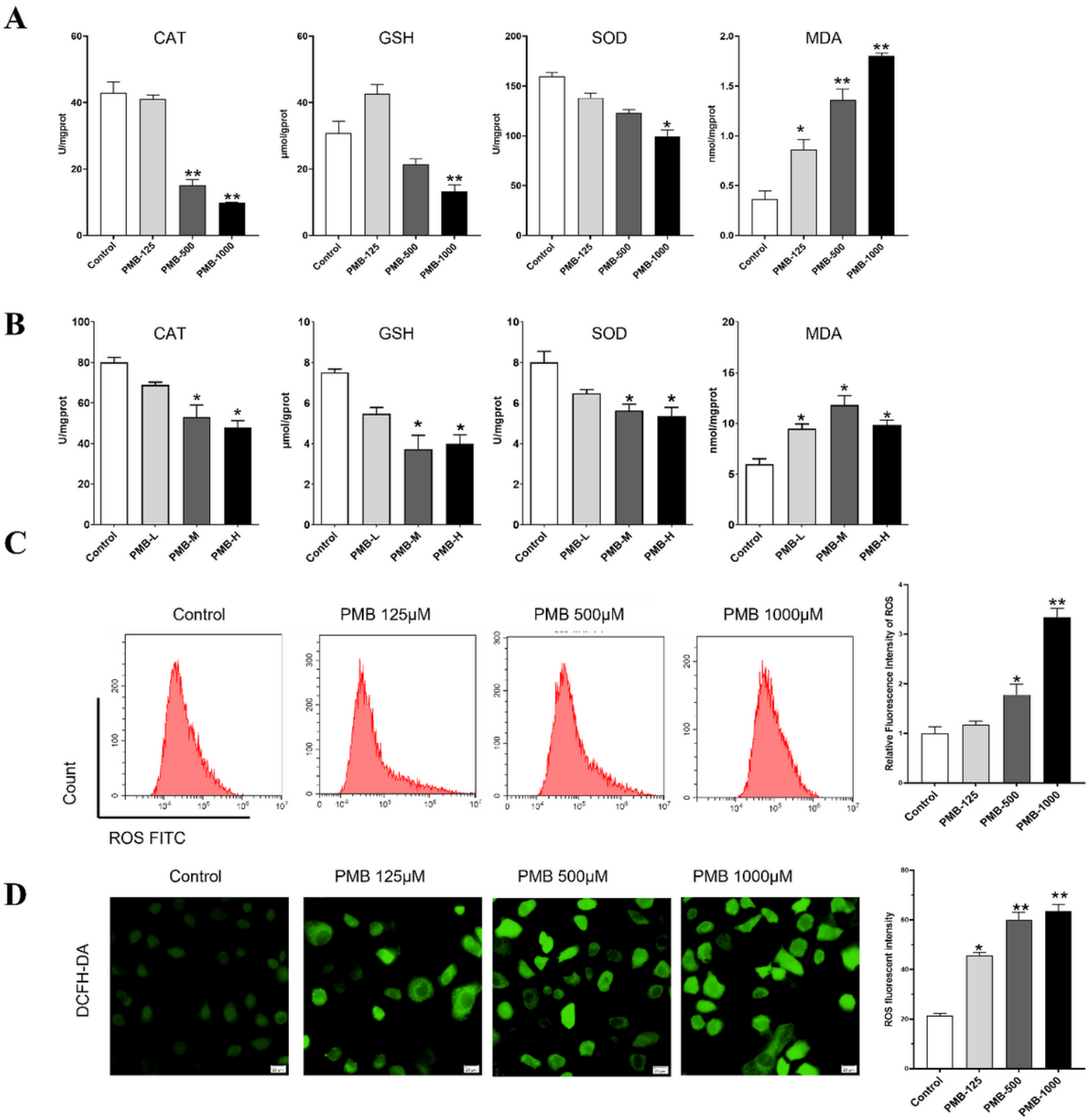



## Figure 5

Expression of apoptotic-related gene and protein in the kidney tissues after PMB treatment.

(A) Control (Intraperitoneal injection 0.9% NaCl), PMB-L (9.0mg/kg), PMB-M (14.0mg/kg), PMB-H (18.7mg/kg) four different treatments were used to treat mice, the apoptosis genes *Bax, Bcl-2, Caspase-9, Caspase-3* were examined by qPCR assay.

(B-C) Control (Intraperitoneal injection 0.9% NaCl), PMB-L (9.0mg/kg), PMB-M (14.0mg/kg), PMB-H (18.7mg/kg) four different treatments were used to treat mice, the apoptosis protein Bax, Bcl-2, Caspase-9, Caspase-3 were examined by western blot (left). Line chart of western blot after the gray scale analysis was shown in right. Data were represented as mean ± SEM, * $p < 0.05$ compared with control, * *$p < 0.01$ compared with control.




## Figure 6

PMB reduced the cell's antioxidant capacity in NRK-52E cells and mice.

(A) The oxidation indices of CAT, GSH, SOD, and MDA in NRK-52E cells were measured after PMB-125μM, PMB-500μM, PMB-1000μM treatment about 24 h. (B) The oxidation indices of CAT, GSH, SOD, and MDA was measured in mice after PMB treatment at different dosages: Control (Intraperitoneal injection 0.9% NaCl), PMB-L (9.0mg/kg), PMB-M (14.0mg/kg), PMB-H (18.7mg/kg), respectively. (C) The effects of PMB

concentrations on ROS production were analyzed by flow cytometry in NRK-52E cells and the relative fluorescent intensity of NRK-52E cells after PMB treatment. (D) NRK-52E cells were treated with different concentrations of PMB for 4h, and DCFH-DA probe was used to detect intracellular ROS levels. Scale bar = 20 μm. Data were represented as mean ± SEM, *$p < 0.05$* compared with control, * *$p < 0.01$* compared with control.

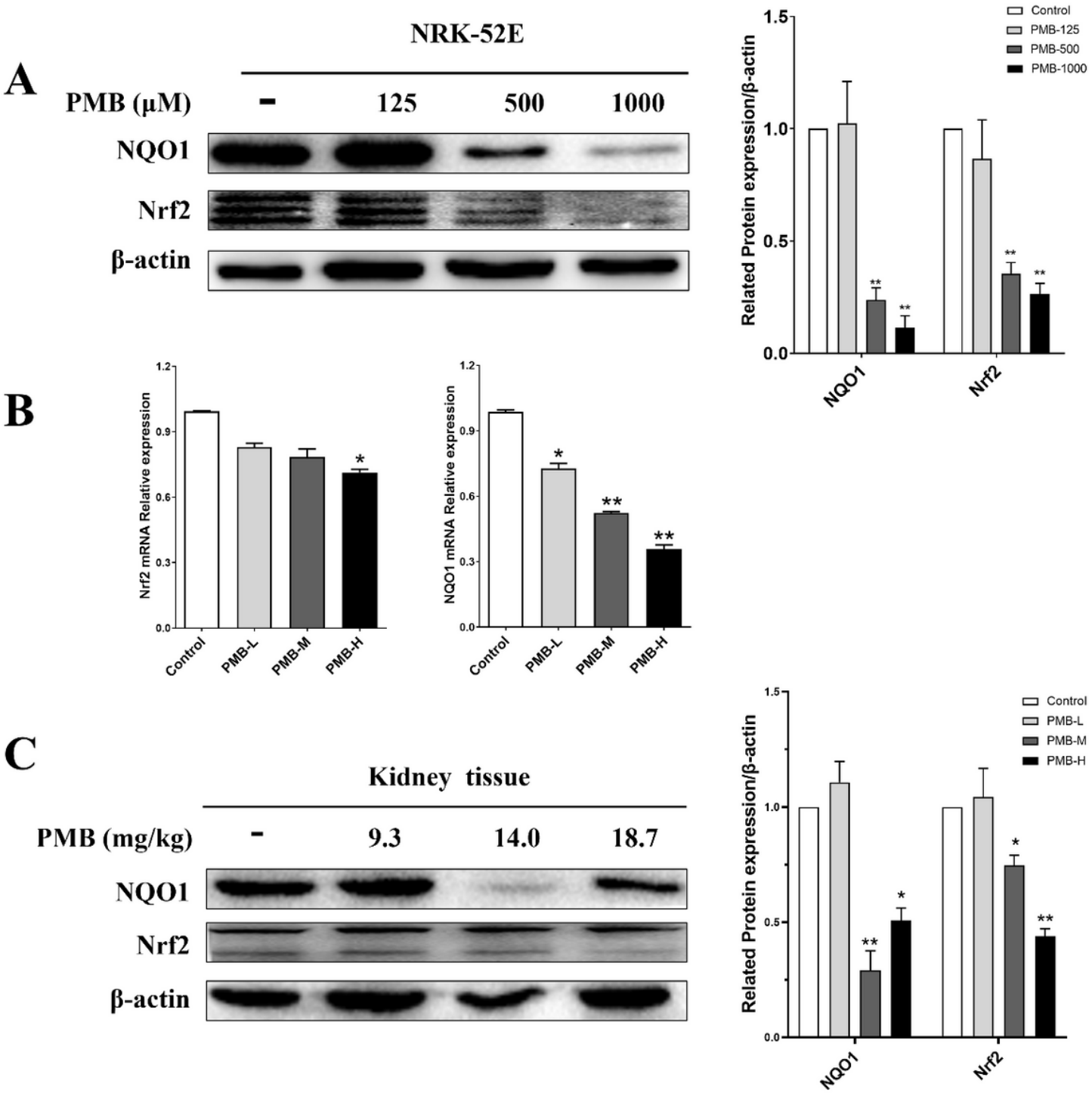



Figure 7

The change of the Nrf2/NQO1 pathway was measured after PMB treatment in NRK-52E and mice.

The effect of PMB treatment on the Nrf2 pathway was measured using Western Blot experiment. (A) The expression of Nrf2 and NQO1 protein in NRK-52E was measured after PMB-125μM, PMB-500μM, PMB-1000μM treatment 24 h, separately. (B) Control (Intraperitoneal injection 0.9% NaCl), PMB-L (9.0mg/kg), PMB-M (14.0mg/kg), PMB-H (18.7mg/kg) four different treatments were used to treat mice, the mRNA expression of *Nrf2* and *NQO1* were examined by qPCR assay. (C) Control (Intraperitoneal injection 0.9% NaCl), PMB-L (9.0mg/kg), PMB-M (14.0mg/kg), PMB-H (18.7mg/kg) four different treatments were used to treat mice, the protein expression of Nrf2 and NQO1were examined by western blot (left). Line chart of western blot after the gray scale analysis was shown in right. Data were represented as mean ± SEM, *$p < 0.05$ compared with control, **$p < 0.01$ compared with control.

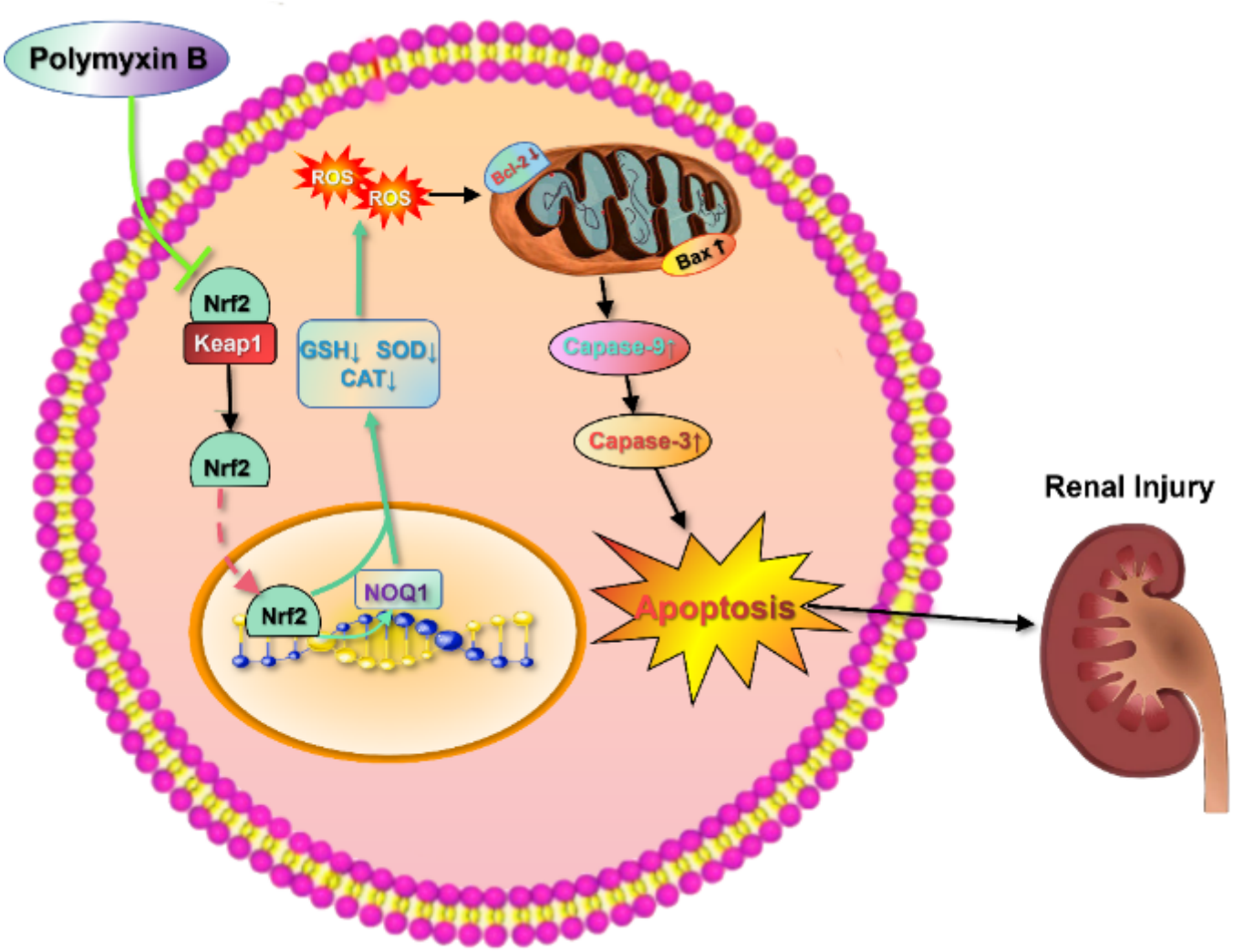



**Figure 8**

The inhibition of the Nrf2/NQO1 pathway and ROS accumulation involved in PMB induced apoptosis in the kidney.

PMB treatment suppressed Nrf2, NQO1 protein expression and decreased antioxidant capacity, which resulted in ROS accumulation and the activation of apoptosis-related proteins, finally indueced cells apoptosis in the kidney.