

Original Article

Effects of atrazine on the oxidative damage of kidney in Wister rats

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Abstract: The environmental persistence and bioaccumulation of herbicide atrazine may pose a significant threat to human health. In this experiment, 4 weeks old female Wister rats were treated by 0, 5, 25 and 125 mg/kg atrazine respectively for 28 days, and the oxidative stress responses as well as the activations of Nrf2 signaling pathway in kidney tissues induced by atrazine were observed. The results showed that after be treated by atrazine, the Blood urea nitrogen (BUN) and creatinine (CREA) levels in serum were increased, the contents of nitric oxide (NO) and malondialdehyde (MDA) in the kidney tissue homogenates were increased, the over-expressed Nrf2 transferred into the nuclei and played an antioxidant role by up-regulated the expression of II phase detoxifying enzymes such as heme oxygenase-1 (HO1) and NAD(P)H quinone oxidoreductase (NQO1) and the expression of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).

Keywords: Atrazine, oxidative injury, Nrf2, rat, kidney

Introduction

Chemical pollution in the environment with pesticides has been increasing due to their extensive usage in agriculture. Atrazine (2-chloro-4-ethylamine-6-isopropylamines -triazine, ATR) is a chlorotriazine herbicide that has been used worldwide for the control of broadleaf weeds and is applied on crops such as corn, sugarcane, and sorghum [1]. In the United States alone, ATR's annual use is approximately 65-80 million pounds per year [2], and in the European countries more than 1.2 million tons per year [3]. Atrazine and degradation products are the most common contaminants of groundwater and surface water, and this contamination has spread well beyond areas where it was once applied. Furthermore, ATR can persist for several years after its application [4]. In China, atrazine concentrations exceeding the standards for drinking-water (3 µg/l) have been reported in Guanting reservoir [5] and Taihu Lake [6]. In the surface water in East Liaoh River Basin of Jinlin province, the average atrazine content in waters in regions with glebe or without glebe were namely 9.7 µg/l and 8.854

µg/l, and up to the highest content of 18.93 µg/l in July [7]. Although the use of atrazine has recently been banned in the European Union, because of its high water solubility and stability, atrazine could be ubiquitous in waters for a long time and cause eco-toxicity.

Limited human data [8, 9] and extensive animal data indicate that exposure to high levels of ATR is detrimental to the nervous systems, reproductive, immune, and endocrine. For example, several studies reported that ATR is detrimental to the brain [10-12], by interfering with the vesicular storage and/or cellular uptake of DA [13, 14]. Previous studies have shown that atrazine could affect the reproductive system of wild animals [15]. Some researchers have demonstrated that excessive ATR exposure has a negative impact on the immune system [16, 17]. Atrazine can act as an endocrine disrupting compound (EDC) with effects on the endocrine system [18-21].

Recent studies have indicated that oxidative stress (OS) has been implicated in ATR toxicity through the evaluation of specific biomarkers in

Atrazine on oxidative damage

tissues such as liver, erythrocytes, testis, and epididymis in the rat [22-24]. Bhatti reported that after treated by atrazine, a significant increase in the malondialdehyde (MDA) levels and decrease in the GSH was observed, and significant increase in the activities of superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPx), and serum glutathione S epoxide transferase (GST) were observed in atrazine treated group compared to controls [25]. Adesiyun reported that atrazine could induce toxicity in the liver and reproductive system of rats, with the increase of MDA anabolism and the decrease of SOD catabolism in liver and Testis [23]. Abarikwu reported atrazine induced toxicity by increased Reactive Oxygen Species (ROS) and MDA levels and up-regulated the expression of glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione-S-transferase (GST) and down-regulated the expression of superoxide dismutase 1 (SOD-1) and superoxide dismutase 2 (SOD-2) [26].

While data pertaining to ATR's toxicity in mammals are increasing, studies of ATR effects on oxidative injury in the kidney are still scarce. In this study, we investigated the histopathological characteristics and antioxidant responses in the kidney of Wister rats that received orally administration of ATR. These results will enable us to further understand the kidney damage and antioxidation mechanisms in mammals.

Materials and methods

Experimental animals

Four-week-old pathogen-free female Wister rat were purchased from the Experimental Animals Center of Norman Bethune Medical College, Jilin University (Changchun, China). The rats were housed at constant room temperature ($23\pm 1^\circ\text{C}$) and relative humidity (50%) under a regular light/dark schedule (light on from 7:00 A.M. to 7:00 P.M.) with free accessing food and water. The animal study was conducted following internationally recognized guidelines and was approved by the Animal Research Committee of Norman Bethune College of Medicine, Jilin University.

Reagents

Atrazine (99% purity) and SDS, TEMED, Acrylamide, N, N- Dimethyl-bis-acrylamide, DTT and

PMSF were obtained from Sigma Chemical Company (USA). ATR solutions (0.5 mg/ml, 2.5 mg/ml and 12.5 mg/ml) were prepared by dissolving ATR in corn oil. All the solutions were kept at 4°C for a maximum of 1 week. NO, MDA, SOD, CAT and GSH-Px detection kits were purchased from Nanjing Jiancheng Company. Rabbit anti-Nrf2, Keap1, HO1 and NOQ1 Monoclonal antibody, HRP-labeled anti-rabbit IgG secondary antibodies were acquired from Protentech Group (USA). Pierce ECL Plus Kit were acquired from Thermo Fisher Scientific Inc (USA).

Treatment of animals

After 1 week adaption in laboratory, the rats were randomly divided into four groups (8 animals per group) and were treated by a daily gavage of 0, 5, 25 and 125 mg/kg atrazine for 28 consecutive days. The animals were sacrificed by bleed from abdominal aorta under anesthesia on day 29. The blood was centrifuge with 3000 r/min for 10 min, and serum were collected and stored at -20°C until assayed. The kidneys were removed, partly fixed in 10% formalin and partly stored at -80°C .

Assessments of renal function injury

Blood urea nitrogen (BUN) and creatinine (CREA) levels were measured using an automated biochemical analyzer according to the manufacturer guides (Mairui, Shenzhen, China).

Detection of contents of NO, MDA and the activity of SOD, CAT, GSH-PX

A 10% homogenate of the kidney tissue was prepared in 1 ml PBS buffer containing 100 mg tissue, and kept in -20°C . Protein content was estimated by the method of Bradford [27]. The contents of NO, MDA and the activities of SOD, CAT, GSH-Px were determined as described in the detection kits instruction.

Western blot analysis

Kidney tissues stored at -80°C were homogenized in ice-cold buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris base, pH 7.4). The tissue lysates were then centrifuged for 30 min at $12\ 000\times g$ and the protein concentrations were determined using the Protein Assay Kit (Bio-Rad, Hercules, CA). Forty micrograms of protein were separated by 12% SDS-PAGE gel and transferred onto a PVDF membrane (Millipore,

Atrazine on oxidative damage

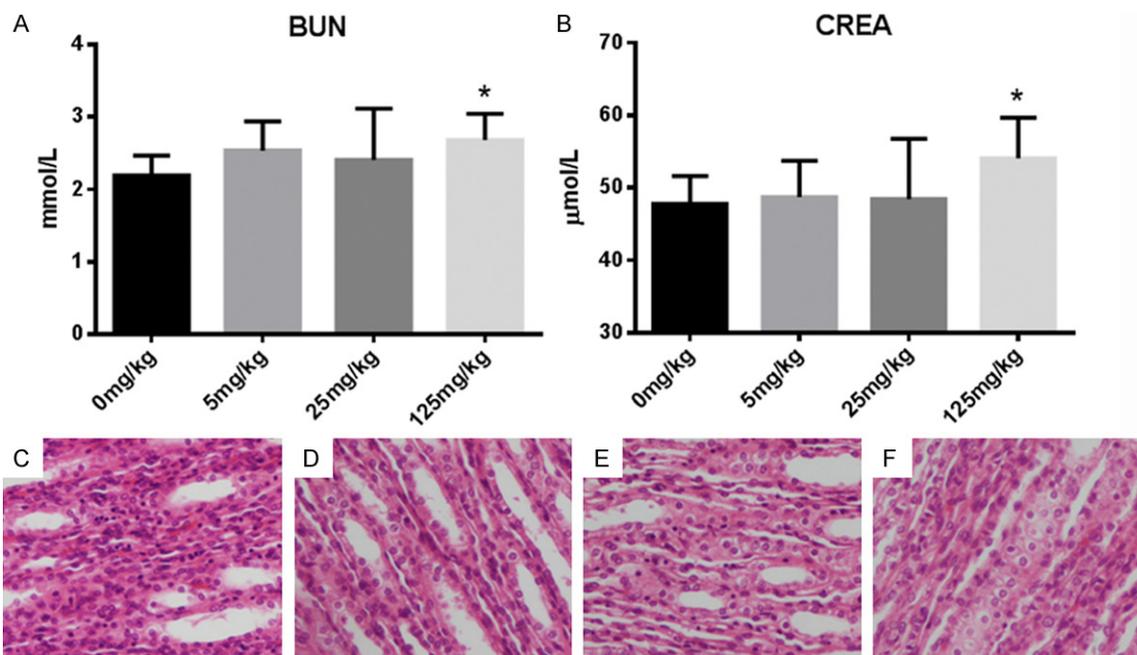


Figure 1. Subacute exposure of atrazine induce kidney injury of rats. (A) The serum BUN levels in rats treated by different dosage of ATR; (B) The serum creatinine (CREA) levels in rats treated by different dosage of ATR; (C-F) The renal histological results of the 0 mg/kg (C), 5 mg/kg (D), 25 mg/kg (E) and 125 mg/kg (F) ATR treated groups separately. (* $p < 0.05$, compared with the control group).

Bedford, MA). The membranes were blocked with 5% nonfat milk diluted in buffer (10 mM Tris-HCl, 100 mM NaCl and 0.1% Tween 20) for 1 h at room temperature and probed with antibodies of anti-Nrf2, anti-Keap1, anti-HO1, anti-NQO1, and anti- β -actin (proteintech group, USA) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies (Thermo, Waltham, MA) at a dilution of 1:2000 were then applied for 1 h at room temperature. The protein bands were then detected using an Enhanced Chemiluminescence kit (Pierce Biotechnology Ltd., Rockford, IL). The protein levels were quantified by densitometry using Quantity One software (Bio-Rad).

HE staining and immunohistochemistry assay

Kidney specimens were fixed in 10% formalin, embedded in paraffin, and cut into 4 μ m-thick slides. For HE staining, the slides were dewaxed, and then stained routinely with hematoxylin-eosin for histological assessment.

For immunohistochemistry assays, the endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide solution in methanol for 20 min. Epitope retrieval was performed by treating the slides with 10 mM sodi-

um citrate buffer (pH 6.0) and heating in a microwave oven for two times at the high power for 6 min each. Non-specific binding was prevented by blocking with normal goat serum (1:10) for 10 min. Immunostaining of Nrf2 was performed using a rabbit anti-Nrf2 monoclonal antibody. Goat anti-rabbit IgG conjugated with horseradish peroxidase was used as the secondary antibody. The staining procedure was carried out manually at room temperature, using an avidin-biotin-peroxidase complex method. After incubation with the primary antibody for 60 min, the slides were incubated with the biotinylated goat anti-rabbit IgG (H+L) (DAKO, Carpinteria, CA) at 37°C for 30 min, followed by incubation with a 1:200 streptavidin-biotin-peroxidase complex (Sigma, St. Louis) for 30 min. Reactive products were visualized with 3, 3'-diaminobenzidine (DAB) as the chromogen, and the slides were counterstained with hematoxylin and coverslipped. The stained slides were analyzed with a microscope, and cellular brownish staining was scored as positive.

Statistical analysis

Data are presented as the mean \pm SE and represent three independent experiments. Statistical

Atrazine on oxidative damage

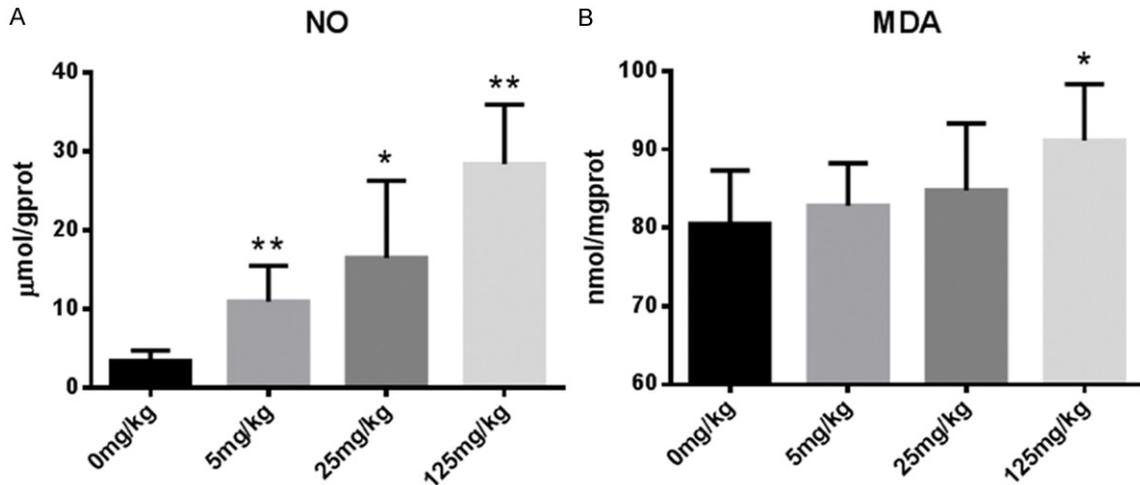


Figure 2. Subacute exposure of atrazine induce oxidative damage in kidney of rats. A. The NO levels in kidney tissue homogenates treated by different dosage of ATR; B. The MDA content in kidney tissue homogenates treated by different dosage of ATR. (* $p < 0.05$, ** $p < 0.01$, versus control group).

analyses were performed with SPSS17.0, difference among the groups were compared with one-factor analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant.

Results

Changes of renal function in rats treated by atrazine

To evaluate the renal function of rats treated by ATR, Blood urea nitrogen (BUN) and creatinine (CREA) levels were measured using an automated biochemical analyzer. As showed in **Figure 1**, the levels of BUN and CREA were significantly increased in serum in the 125 mg/kg group compared with the control group ($p < 0.05$). These changes suggest that the kidney was less able to eliminate BUN and CREA from serum.

Histopathology changes of kidney

To evaluate whether exposure to atrazine would elicit changes in the kidney tissue, the sections of kidney tissue were stained with hematoxylin-eosin for histological assessment. As shown in **Figure 2**, the kidney of rats displayed different degrees of swelling of epithelial cells of juxtamedullary renal tubules.

Contents of NO and MDA

In order to clarify whether renal function associated with oxidative stress, we examined con-

tent of MDA and NO in kidney tissue homogenates. The Data presented in **Figure 3** pointed that the contents of NO and MDA were significantly increased in kidney homogenate in 125 mg/kg atrazine treated group, which indicated that oxidative stress was occurred in the kidney tissues ($p < 0.05$).

Expression of Nrf2 and Keap1

To further explore the effects of oxidant stress on Nrf2 pathway in atrazine treated rats, we estimated the expression of Nrf2 and Keap1 in kidney tissue by western blot. The results showed that the expression of Nrf2 was significantly increased in a dose-dependent manner in 25 mg/kg and 125 mg/kg atrazine treated groups ($p < 0.05$, $p < 0.01$). The expression of Keap1 was increased in 5 mg/kg atrazine treated rats, while a dose-related decrease of Keap1 content was presented with the increase of atrazine dose. The contents of Keap1 in 125 mg/kg atrazine treated rats were significantly decreased compared with those of the control group ($p < 0.05$) (**Figure 4**).

Translocation of Nrf2 to the nucleus

To investigate whether the up-regulated Nrf2 can translocate to the nucleus and thus exerts its biological function, we used Immunohistochemistry to determine the effect of atrazine on the intercellular localization of Nrf2 in rat. As was shown in **Figure 4**, there was a significant increase of positive expression of Nrf2 in atrazine treated kidney cell nucleus, which

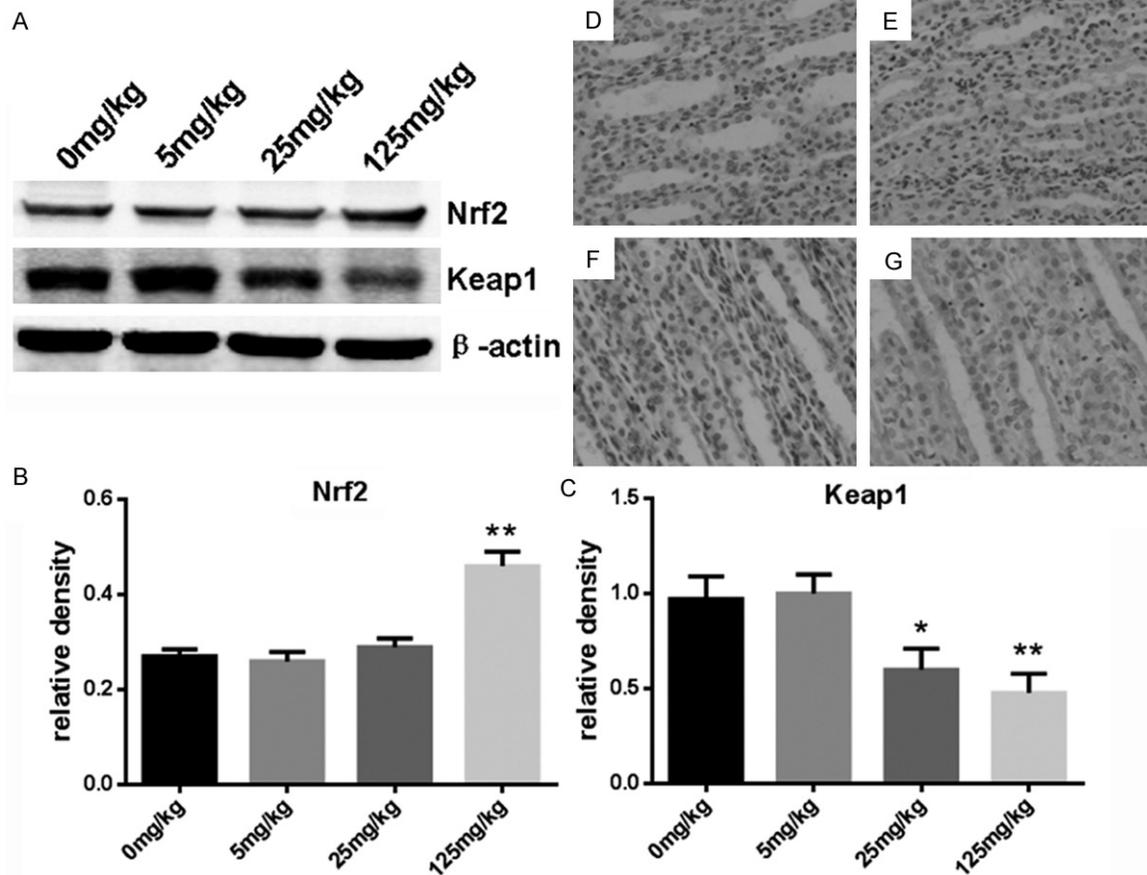


Figure 3. Subacute exposure of atrazine induce activation of Nrf2. (A) Western blot results of Nrf2 and Keap1; (B) Quantified Nrf2 protein level from three rats; (C) Quantified Keap1 protein level from three rats; D-G. Immunohistochemical analysis of Nrf2 location in kidney of 0 mg/kg (D), 5 mg/kg (E), 25 mg/kg (F) and 125 mg/kg (G). (* $p < 0.05$, ** $p < 0.01$, versus control group).

indicated that Nrf2 was activated and transferred into nucleus.

Expression of phase II detoxification enzymes

Western blot assays were performed to determine the effect of Nrf2 activation on the expression of Phase II Detoxification enzyme, which includes HO1 and NQO1. The expression of HO1 and NQO1 were significantly up-regulated in 5 mg/kg atrazine treated group compared with the control group, and then decreased with the increase of atrazine dose. The expression of HO1 and NQO1 was significantly decreased in 125 mg/kg atrazine treated rats compared with that of the control group (Figure 5).

Activities of CAT and GSH-PX

To further explore the effect of Nrf2 activation on Antioxidant enzymes, the activities of SOD,

CAT, GSH-PX was determined. As is shown in Figure 5, the activities of CAT and GSH-Px of 125 mg/kg groups were significantly decreased compared with those of control group ($p < 0.05$) (Figure 5).

Discussion

The biological toxicity of ATR has been widely reported, but studies concerning its effects on the kidney peroxidation are scarce. The aim of this study was to assess the adverse effects of a subacute exposure of ATR on rat by renal function and histological detection and to investigate the possible mechanism by using selected oxidative stress parameters. In this study, the BUN and Creatinine in serum were increased in the ATR treated group compared with the control group, indicated that renal injury occurred in rats. Pathological changes in rat are powerful indicators of exposure to environmental stressors. Our histological results

Atrazine on oxidative damage

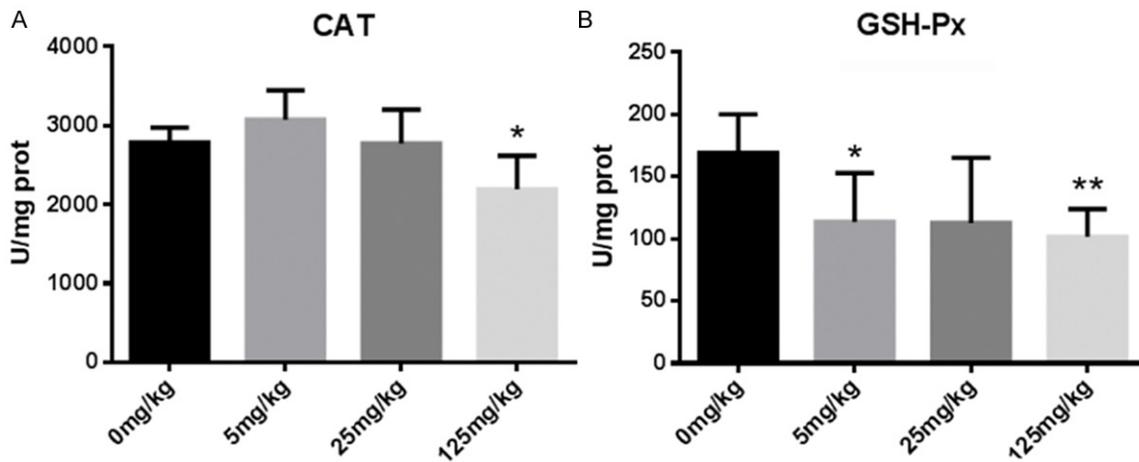


Figure 4. Subacute exposure of atrazine inhibit the activation of antioxidant enzymes. A. The activation of CAT in kidney tissue treated by different dosage of ATR; B. The activation of GSH-Px in kidney tissue treated by different dosage of ATR. (* $p < 0.05$, ** $p < 0.01$, versus control group).

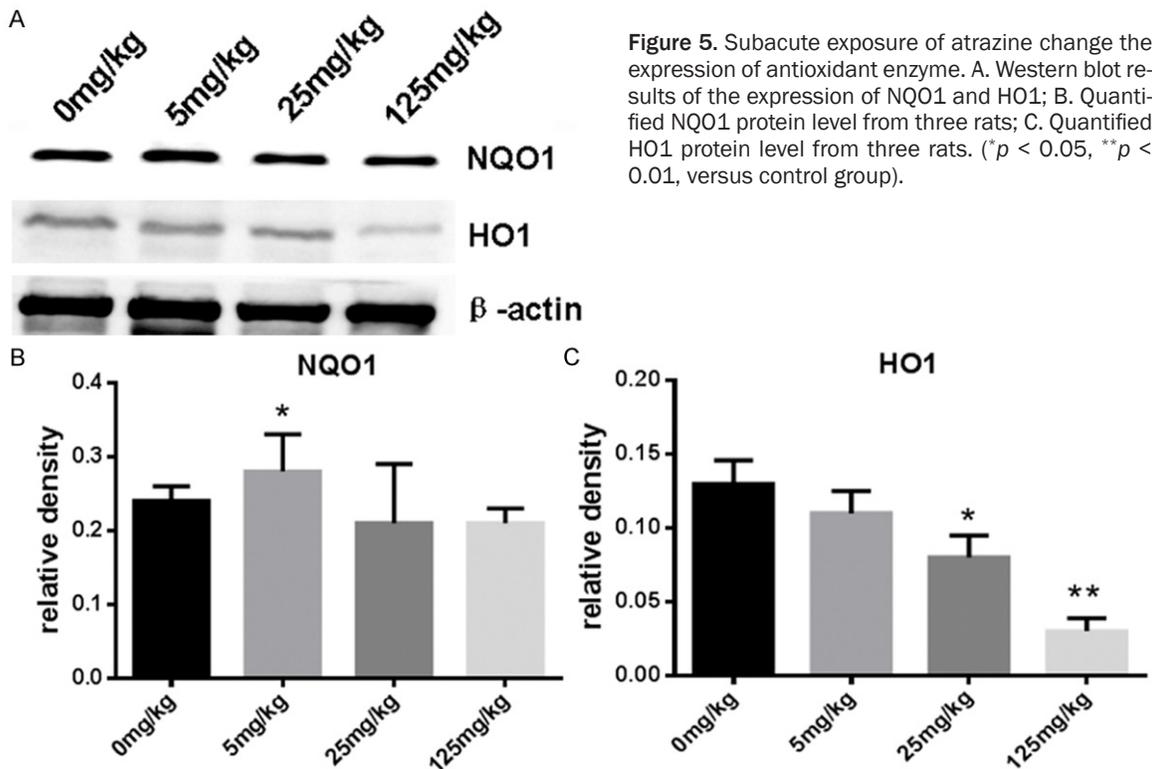


Figure 5. Subacute exposure of atrazine change the expression of antioxidant enzyme. A. Western blot results of the expression of NQO1 and HO1; B. Quantified NQO1 protein level from three rats; C. Quantified HO1 protein level from three rats. (* $p < 0.05$, ** $p < 0.01$, versus control group).

showed that exposed to ATR caused alterations to the kidney structure of the rat, as evidenced by the hydropic degeneration and formation of vacuoles in the epithelial cells of juxtamedullary renal tubules.

Xenobiotics can generate reactive oxygen species which are responsible for cell and tissue damage. Lipid peroxidation is the initial step to

cellular membrane damage caused by pesticides and is considered as a valuable indicator of oxidative damage in cellular components [28]. The increase in lipid peroxidation following ATR exposure may be attributed to the induction of ROSs, which enhances the oxidation of polyunsaturated fatty acids that lead to lipid peroxidation [29]. To further investigate the mechanism of renal injury induced by ATR, per-

oxidation product level in renal were detected in this study. In the high ATR concentration treatment group, MDA content, which is a secondary lipid peroxidation product, and NO, which is one of ROS, increased compared to the control group, suggesting that a large amount of lipid peroxidation has occurred in the treated rats. Similar results have also been reported in other species [30-32].

Many organisms have unique systems for protecting themselves against the damaging effects of activated ROSs. Oxidative stress as intracellular signaling molecules also activates several signaling pathways to regulate cell responses. Nrf2 is a redox-sensitive transcription factor that regulates the expression of phase II anti oxidant genes and confers cytoprotection against oxidative stress [33]. In unstressed cells Nrf2 is sequestered by its inhibitor, Keap1, which promotes rapid proteasome-mediated degradation [34-37]. However, in response to oxidative stress, Nrf2 is stabilized by dissociating from Keap1, and binds to cis-elements called antioxidant response elements (ARE) as a heterodimer with other members of the basic leucine zipper protein family, such as Maf or Jun [38]. Our study estimated the contents of Nrf2 and Keap1 in kidney tissues by western blot, and found that the expression of Nrf2 was significantly increased in atrazine treated groups. While the decrease of Keap1 content was presented with the increase of atrazine dose. Also, 25 mg/kg and 125 mg/kg atrazine could promote the translocation of Nrf2 into nucleus. These results indicated that Nrf2 might play an important role in the cytoprotective mechanism against oxidative damage induced by atrazine.

During oxidative stress, Nrf2 undergoes nuclear translocation, binds in heterodimeric combinations with members of the small Maf family of nuclear factors, to the 5'-upstream AREs [39, 40], and detoxify genes, such as Glutathione S-transferase (GST), NAD(P)H: quinone oxidoreductase I (NQO1), heme oxygenase 1 (HO1), Catalase (CAT), Superoxide Dismutase(SOD), Sulfiredoxin (SRX), Glutathione peroxidase (GSH-Px) and γ -glutamylcysteine synthetase (γ GCS), and thus modulates their expressions [41, 42].

HO1, formerly known as phase II detoxifying antioxidant enzyme, is the rate-limiting enzyme that catalyzes the degradation of heme to pro-

duce biliverdin, iron, and carbon monoxide [43]. NQO1 is a flavin protease which catalyzes quinone two-electron reduction reaction, thereby preventing the oxidation-reduction reaction and the generation of ROS [35, 44]. In this study, the upregulation of HO1 and NQO1 in 5 mg/kg atrazine treated group indicated that after translocation to the nucleus, Nrf2 combined with ARE and triggered the expression of phase II detoxifying enzymes. While with the increase of atrazine administration, the accumulation of Pro-oxidant substances consumed more phase II detoxifying enzymes simultaneously, thus the detectable contents of HO1 and NQO1 decreased on the contrary.

Rat can combat the elevated levels of ROSs in their systems with protective ROS-scavenging enzymes such as SOD, CAT and GSH-Px. In this study, GSH-Px and CAT activities in the kidney decreased after ATR exposure. Thus, it is possible that a decrease in the activity of these enzymes induced by ATR exposure contributes to the elimination of ROSs from the cells.

In summary, atrazine could induce oxidative stress response in rat kidney. Nrf2 has an important role in the defense against oxidative stress by regulating the expression of phase II detoxifying and antioxidative enzymes. Continued advances on the effect of atrazine exposure to Nrf2 signaling pathway will contribute to understanding the mechanism of atrazine-induced kidney damage.

In conclusion, the present study shows that atrazine could induce oxidative stress response in rats' kidney after 28 days administration. Nrf2 protects cells from oxidative stress by a mechanism that regulates ARE related genes including phase II detoxifying and antioxidant enzymes.

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Disclosure of conflict of interest

None.

Atrazine on oxidative damage

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Atrazine on oxidative damage

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