



Protective Effects of Arginine Against 4 Nitrophenol Induced Liver Injury in Rats

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ABSTRACT

The aim of this study was to evaluate the possible protective effects of arginine against hepatotoxicity induced by 4-nitrophenol (PNP) in rats. Twenty-four rats were allocated to a 2×2 factorial arrangements with six rats each. The primary variations were dietary arginine (Arg) supplemental levels (0 g/kg Arg or 13 g/kg Arg) and PNP injection (0 or 100 mg/kg b.w.). Dietary Arg and subcutaneous injection PNP were performed simultaneously. Rats were sacrificed on d 8. Treatment of rats with 13 µg/ kg Arg resulted in a significant increase in mean daily weight gain (ADG), liver weight, and liver coefficient. However, Arg treatment in the PNP group showed an elevated trend relative to the rats treated with PNP alone, and the results were not significant. Moreover, in animals treated with 13 g/kg Arg plus PNP, hepatic morphological impairment was alleviated, including hepatic sinusoid damage and inflammatory infiltration by PNP challenge. Compared with the PNP group, Arg plus PNP-treated rats did not show any significant change in the serum malondialdehyde (MDA), hydrogen peroxide (H₂O₂), superoxide dismutase (T-SOD), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Meanwhile, the mRNA expression of HO-1 and NQO1 in liver tissue was also not significantly different. These results indicate that PNP may damage liver tissue through oxidative stress. Arg may improve growth performance and attenuate PNP-induced liver inflammation in healthy animals, but not by reducing oxidative levels.

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Authors' Contribution

WK performed conception, writing and review. XWF and DPY planned and conducted methodology, data curation, and investigation. LCM supervised the study.

Key words

Arginine, 4-nitrophenol (PNP), Antioxidant, Hepatoprotective effects, Rats

INTRODUCTION

4-nitrophenol (PNP) is a final primary product of organophosphorus insecticides and derivatives of diesel exhaust particles (Kwong, 2002). Due to the widespread and excessive use of pesticides and equipment in the diesel industry, which leads to PNP environment and bioaccumulation, studies have found it to be a persistent organic pollutant with poor degradation (Ahmed *et al.*, 2015; Yang *et al.*, 2016). Because of the wide exposure range, PNPs have been detected in animal, bird, and human urine samples (Ahmed *et al.*, 2015). Previous studies on PNP mainly believed that it could affect the secretion of reproductive hormones and cause damage to the reproductive system (Zhang *et al.*, 2017; Wei *et al.*, 2021). Given the finding in later studies that PNP can affect

biological enzyme activities such as CYP450, as well as cause toxicity to cells, and that PNP is mainly metabolized in the liver, it is crucial to investigate the toxic effects of PNP on the mammalian liver (Sechman *et al.*, 2020). In a previous study, PNP was found to induce parenchymal liver injury by severe vacuolization (Kwong, 2002). Our previous study found that PNP activated the AhR signaling pathway and regulated the related *CYP1A1* and *GST* gene expression in the liver, which induced liver injury, possibly by causing oxidative stress (Li *et al.*, 2017). However, studies on treating and alleviating the hepatotoxic effects of PNPs are minimal.

Thus far, many natural or artificial compounds have been found to relieve hepatic damage and liver function failure (Chen *et al.*, 2016). Some evidence suggests that Arg could contribute to the detoxification process from ROS (Dasgupta *et al.*, 2006) and may interact with superoxide anion and other radicals to produce less toxic species (Bergeron *et al.*, 2017). Arginine (Arg) was reported to be used as a cell protector because of its inhibitor effects, particularly on the anti-oxidant parameters (Kehinde *et al.*, 2015; Wang *et al.*, 2015). In addition, Arg shows positive health effects, including strongly exhibited function of hepatoprotective, anti-oxidant, anti-lipid peroxidative, anti-inflammatory, and anti-aging (Lubec *et al.*, 1997; Lass *et al.*, 2002; Hnia *et al.*, 2008; Masaki, 2010; Zheng *et al.*,

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2013). Therefore, Arg may be an effective alleviator for preventing liver injury caused by chemical detriments.

Previously we showed that PNP might induce liver injury through oxidative stress (Li *et al.*, 2017). Given the anti-oxidant effect of Arg, this study aimed to evaluate the potential protective effect of Arg against hepatotoxicity resulting from PNP exposure by evaluating growth performance, histopathological changes, liver function enzyme indicators, oxidation product levels, and the expression of liver injury-related genes.

MATERIALS AND METHODS

Chemicals

PNP monomer dry crystals ($C_6H_5NO_3$, > 99.9% purity, CAS 100-02-7) were purchased from Chengdu Kelong Chemical Reagent Factory, China. Arg ($C_6H_{14}N_4O_2$, > 98.5% purity, CAS 1119-24-2) was purchased from Aladdin Chemistry Co. Ltd, Shanghai, China. The diagnostic kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AKP), superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT), hydrogen peroxide (H_2O_2), and protein were purchased from Nanjing Jiancheng Biological Institute Co. Ltd, China. All other chemicals were of analytical grade were obtained from standard commercial suppliers.

Animals

Twenty-four female Sprague-Dawley (SD) rats postnatal day 21 (weighting 50-60 g), obtained from Qinglongshan Laboratory Animal Centre (Nanjing, China), were selected. These animals were maintained under the standard laboratory conditions (temperature 25 ± 2 °C, relative humidity $50 \pm 10\%$) with dark and light cycles (12/12 h). They were free to access to a standard dry pellet diet and sterile distilled water available ad libitum. Rats were adapted to the new environment before the formal experiment. The experimental protocols were approved following the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

Experimental design

The experiment was arranged as a 2×2 factorial design, including diet type (0 g/kg Arg or 13 g/kg Arg) and PNP injection (100 mg/kg b.w. or the same dose of PBS). Twenty-four rats aged 3 weeks were randomly divided into 4 groups, each of 6 replicates, CT group (fed on control diet and PBS), PNP group (fed on control diet and 100 mg/kg b.w. PNP administrated subcutaneous), CTA group (fed on diet supplemented with 13 g/kg Arg and PBS), and

PNPA group (fed on diet supplemented with 13 g/kg Arg and PNP). Dietary Arg and subcutaneous PNP injection were performed simultaneously. PNP was dissolved in PBS and stored under refrigeration. The experiment lasted for 1 week.

Sample collection

Twenty-four hours after the final injection, the rats were weighed and exposed to mild anaesthesia. The blood was collected from the cervical vessels at room temperature and centrifuged at 3500 rpm for 15 min at 4 °C. Separated serum was then kept at -20 °C until biochemical analysis. Rats' livers were removed and immediately weighed after killing. A portion of the livers was fixed in 10% paraformaldehyde for histological examination. A second part was assayed for superoxide dismutase (T-SOD), CAT, MDA and H_2O_2 concentrations. The remaining liver was stored in liquid nitrogen for RT-PCR analysis.

Hepatic function biomarker detection

After blood collection, serum was separated by centrifugation at 3500 rpm at 4 °C for 15 min. The serum ALT, AST, and AKP activities were measured with commercially available diagnostic kits obtained from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China), according to the manufacturer's protocols.

Determination of hepatic oxidative stress parameters

The liver was excised immediately after the animals were euthanized and sacrificed. Before determinations, thawed tissue samples were homogenized with standard saline buffer and centrifuged at 3500 rpm at 4 °C for 15 min. Using commercially available diagnostic kits, the supernatant was used to determine MDA, H_2O_2 levels, T-SOD, CAT activities, and protein concentration. The absorbance of the supernatants was measured by spectrophotometric assay at 532 nm for MDA, 405 nm for H_2O_2 , 560 nm for T-SOD, and 405 nm for CAT, and the values were expressed as nmol mg^{-1} protein for MDA, mmol g^{-1} protein for H_2O_2 and U mg^{-1} protein for T-SOD and CAT.

RNA extraction, reverse transcription (RT), PCR, and quantitative PCR

Total RNA was isolated from frozen livers using TRIzol reagent according to the manufacturer's instructions. The total RNA concentration was determined by detecting the absorption value at 260 and 280 nm. The total RNA (1 μg) was reversely transcribed into cDNA with an Omniscript® Reverse Transcription kit (Takara) and Oligo-dT primer (Takara) according to the manufacturer's protocol. RT-PCR quantified the target

Table I. Primer sequences used for real-time PCR analysis.

Gene name	Accession No.	Primer sequence (5'→3')	Product size (bp)
<i>β-actin</i>	NM_031144	F: CGTTGACATCCGTAAAGACC R: GGAGCCAGGGCAGTAATCT	108
<i>Nrf2</i>	NM_031789	F: TTTGGAGGCAAGACATAG R: TGGGCAACCTGGGAGTA	253
<i>NQO1</i>	NM_017000	F: CCATTCCAGCCGACAAC R: AGCCGTGGCAGAACTATC	199
<i>HO-1</i>	NM_012580	F: TTCACCTTCCCGAGCAT R: GCCTCTTCTGTCAACCTGT	110

Nrf2, Nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H: quinone oxidoreductase 1; HO-1, Heme Oxygenase-1.

Table II. Effects of PNP (100 mg/kg PNP administrated subcutaneously) and arginine exposure on body weight, growth, liver weight and coefficient.

Items	Arg (0 g/kg)		Arg (13 g/kg)		p-value			
	PBS	PNP	PBS	PNP	SEM	PNP	Arg	PNP × Arg
Initial weight (g)	39.15	42.00	40.06	38.31	0.889	0.760	0.443	0.211
Final weight (g)	66.01	66.33	67.44	66.82	1.548	0.962	0.760	0.882
Average daily gain (g)	4.48	4.13	4.56	4.61	0.182	0.040	0.052	0.220
Liver weight (g)	3.20	2.75	3.90	2.96	0.097	0.002	0.029	0.222
Final weight (g)	66.01	66.33	67.44	66.82	1.548	0.962	0.760	0.882
Liver coefficient (%)	4.86	4.16	5.79	4.44	0.119	0.052	0.020	0.187

The values shown are the mean ± SEM of 6 animals per group (Two-way ANOVA). In the same row, differences were considered significant at $p < 0.05$. Average daily gain (g) = [(final body weight – initial body weight) / days] × 100%. Liver coefficient (%) = [liver weight / final weight] × 100

fragments with a QuantiTect™ SYBR Green® PCR Kit (Roche) with 300 ng of the cDNA template. The gene expression data were normalized to β -actin expression. The primers were designed using the Primer 5 Plus Program (Table I). For the quantification of RT-PCR results, the threshold value for fluorescence intensity for all samples was set manually. Ct values for each gene were normalized to β -actin. PCR amplification efficiencies were considered by amplifying various amounts of target cDNA for each reaction.

Statistical analysis

All the data were analyzed by two-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test. The statistical analysis was performed using the SPSS package version 20.0 (SPSS Inc., USA). Quantitative data were presented as mean and SEM. Differences between experimental groups were considered significant at $p < 0.05$.

RESULTS

Body weight and growth

The body weights before and after the experiment

were weighed and compared. There were no significant differences between the initial and final weights among the different groups. Rats injected subcutaneously with PNP showed a significantly reduced ADG ($p < 0.05$) when compared with rats injected with PBS, indicating that PNP adversely impacts body growth at the current dosage. However, rats supplemented with Arg showed an increase in ADG ($p = 0.052$) when compared with rats fed with a control diet (Table II), which implied ameliorative effect, possessing the ability to promote growth in rats.

Liver weights and coefficients

The values of liver weights and coefficients in all experimental groups were measured. As shown in Table II, compared with rats injected with PBS, liver weights were significantly decreased ($p < 0.05$), and liver coefficients were subtly reduced ($p < 0.05$) in rats injected with PNP. On the other hand, Arg diet-treatment rats increased liver weights ($p < 0.05$) and liver coefficients ($p < 0.05$) more than those of control diet-treated rats. However, liver weight and coefficient were not significantly different in PNP-treated rats treated with Arg.

Histopathology analysis of livers

The protective effects exerted by Arg against

PNP-induced hepatotoxicity were further confirmed by conventional histological assessment (as shown in Fig. 1). The liver histology in rats treated with PBS showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus, visible central veins, and thin sinusoids (Fig. 1A and B). The stained sections of rats injected with PNP alone revealed extensive liver injuries characterized by inflammatory cell infiltration around the central vein, hepatic sinusoid damage, and hepatic cell irregular arrangement (Fig. 1C). However, compared with the PNP group, apparent hepatic amelioration was found in the Arg + PNP group, including hepatic sinusoid damage and reducing inflammatory cells, while hepatic cells arranged in disorder still presented (Fig. 1D).

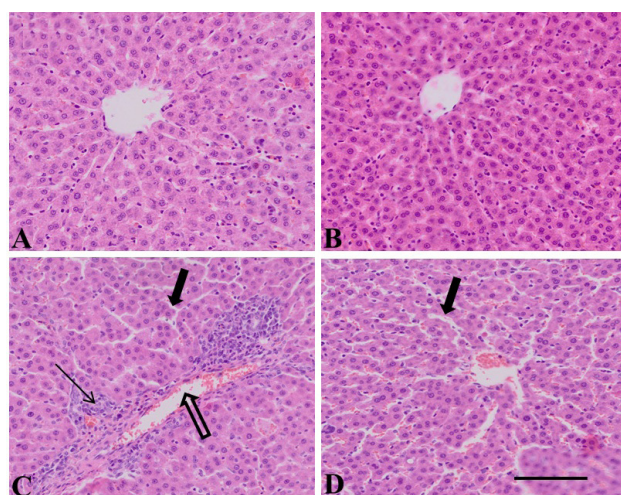


Fig. 1. Effects of PNP and Arg exposure on histological observation of liver section. (A) Control+PBS group (CT) (B) Arg+PBS group (CTA) (C) Control+PNP group (PNP) (D) Arg+PNP group (PNPA). Liver sections of rats showed single arrow inflammatory infiltration, open arrow hepatic sinusoid damage, and filled arrow hepatic cells irregular arrangement scale bar=100 μ m.

Serum enzyme activity

The results of the hepatoprotective effect of Arg on the serum ALT, AST, and AKP activities are shown in Figure 2. Compared with rats injected with PBS, a significant increase in the activities of serum ALT and AST ($p < 0.01$) was observed in the exact dosage of PNP-treated animals (Fig. 2A and B). There was no significant differences in the serum ALT and AST activities in rats supplemented with an Arg diet compared with those treated with the control diet. As for the AKP activity, no significant differences were observed among all experimental groups (Fig. 2C).

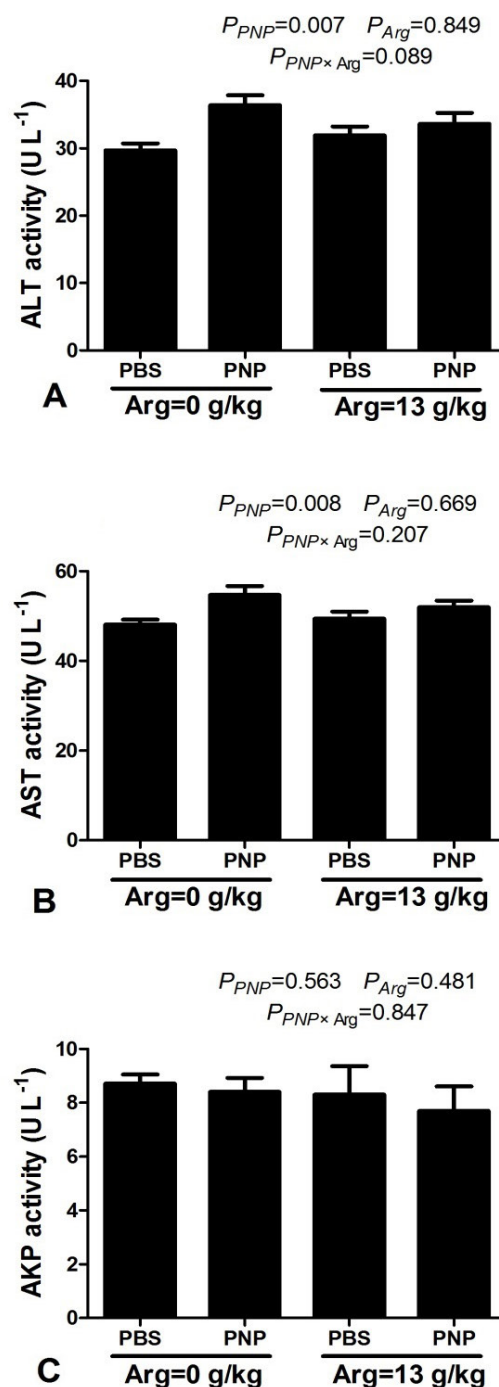


Fig. 2. Effects of PNP and Arg exposure on serum. ALT activity (A), AST activity (B), and AKP activity (C). The statistical differences among groups were analyzed using a two-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test. Bars represent the mean $\pm$ SEM. Differences between experimental groups were considered significant at $p < 0.05$. (n=6).

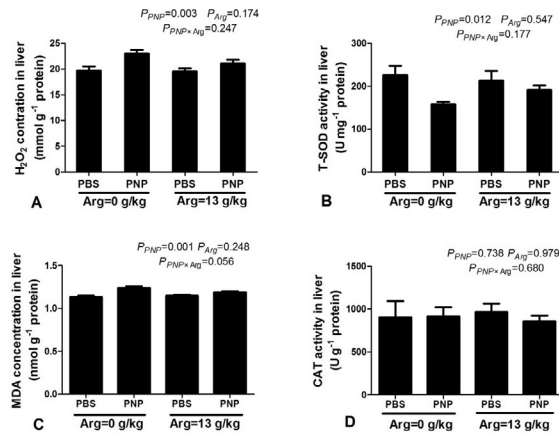


Fig. 3. Effects of PNP and Arg exposure on lipid peroxidation and antioxidative status in the liver. (A) H_2O_2 concentration, (B) T-SOD activity, (C) MDA concentration (D) CAT activity. For statistical details see Fig. 2.

Liver antioxidant enzyme activities and lipid peroxidation

To examine the effects of Arg on anti-oxidant enzymes in PNP-induced hepatic injury, we investigated the activity of T-SOD, CAT, and the concentration of H_2O_2 and MDA. As shown in Figure 3, there was a significant increase in the production of H_2O_2 and MDA ($p < 0.01$); a significantly lower activity of T-SOD was also observed in PNP treatment group rats' livers, compared with those treated with PBS rats (Fig. 3A, B, C). However, compared with control diet treatment animals, the concentration of H_2O_2 and MDA in rats supplemented with Arg showed no significant difference (Fig. 3A and C), along with the T-SOD activity (Fig. 3B). There was no significant difference in CAT activity among all experimental groups (Fig. 3D).

Expression of hepatic Nrf2-regulated genes

To examine the effects of Arg on anti-oxidant pass way genes in PNP-induced hepatic injury, we investigated the mRNA expression levels of Nrf2, HO-1, and NQO1. As shown in Figure 4, the mRNA expression of HO-1 ($p = 0.008$) and NQO1 ($p = 0.043$) in the PNP-injected group was significantly higher than the corresponding values in the rats treated with PBS. Meanwhile, the mRNA expression level of HO1 ($p = 0.184$) and NQO1 ($p = 0.374$) exhibited no significant differences in animals treated with an Arg diet compared with those fed with a control diet (Fig. 4B, C). The mRNA expressions of the Nrf2 gene showed no significant differences among all groups (Fig. 4A).

DISCUSSION

In recent years, the wide use of PNPs and their low degradation rates have caused different toxicological

effects on animals and humans. Several research attempts to reveal the mechanisms underlying the effects of PNP in inducing liver injury. Our previous study elucidates how

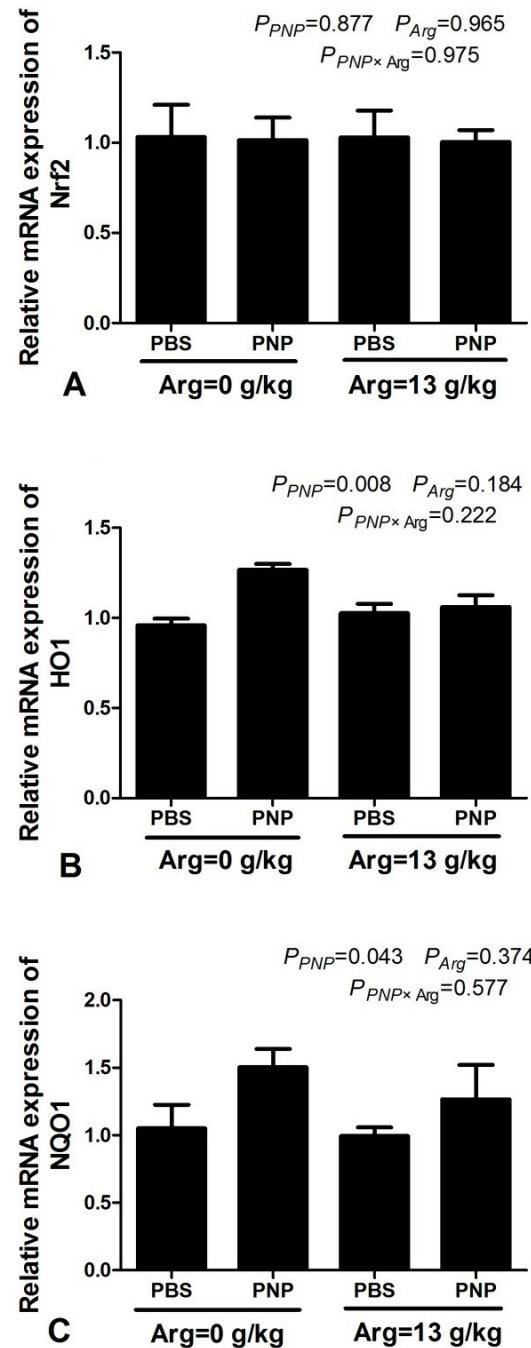


Fig. 4. Effects of PNP and Arg exposure on the expression of Nrf2 and regulated genes in the liver. (A) Nrf2 mRNA (B) HO-1 mRNA (C) NQO1 mRNA. All data were represented as the mean $\pm$ SEM from 6 rats per group. For statistical details see Fig. 2.

PNP may induce ovary injury by causing oxidative stress (Xu *et al.*, 2016). Nevertheless, research on how treatment alleviates the hepatotoxic effects caused by PNP is minimal. This prompted us to treat and alleviate PNP's effects on hepatotoxicity by searching for suitable anti-oxidants. We used 13g/kg of Arg in the present study to treat PNP-induced liver injury with 8d exposure times. After 8 days, it was found that the rats treated with 13 g/ kg Arg had no significant differences in mean daily weight gain, liver weight, and liver coefficient than the PNP-treated rats. This indicates that Arg cannot ameliorate rats' decreased body weight and organ-specific gravity caused by PNP toxicity. Meanwhile, further histological examination of rat livers was detected after PNP exposure. we noted that PNP produces typical toxicity characterization in liver tissues, including inflammatory infiltration, hepatic sinusoid damage, and hepatic cells' irregular arrangement. However, most of these typical hepatotoxicities were mitigated in the PNPA group, suggesting that Arg has potential protective effects against PNP-induced liver injury. Many other studies have also indicated similar results. For example, Arg-supplemented rats displayed reduced hepatic cell macrovesicular steatosis, portal inflammation, and fibrosis diet-induced obesity in rats (Alam *et al.*, 2013). Arg administration reduces liver tissue damage after ischemia injury (Calabrese, *et al.*, 1997). In addition, it has been reported that Arg could protect rats with liver cirrhosis from acute ammonia intoxication (Kim *et al.*, 2001). Supplementation of 0.5% Arg could alleviate liver morphological impairment, such as hepatocyte karyolysis, karyopycnosis, and fibroblast proliferation induced by LPS challenge (Li *et al.*, 2012). These observations were consistent with the results of our study. Another important indicator of hepatotoxicity is liver enzymes in circulating plasma. In this study, serum aminotransferase (ALT and AST) activities of PNP-induced rats increased significantly, indicating serious hepatocellular injury. At the same time, H_2O_2 and the product of lipid peroxidation (MDA) accumulated in the PNP-damaged liver. The increased MDA level in the liver showed enhanced peroxidation, resulting in organ injury and failure of the antioxidant-defence mechanisms to prevent the formation of excessive free radicals (Valko *et al.*, 2007). Supplementation with Arg could promote the ability to resist oxidation, eliminate free radicals and protect plasma membranes from damage. However, it cannot inhibit the formation of MDA and H_2O_2 in the liver and depressed the activities of serum ALT and AST induced by the PNP in this experiment. The above results showed that the liver underwent drastic changes during PNP treatment in rats and the body tried to overcome this stressful condition. Although Arg attenuates the development of hepatic inflammation at

the tissue level, it does not significantly affect the level of hepatic oxidation and liver function. To further verify its anti-oxidant property, we also studied and observed the activities of T-SOD and CAT in the liver tissues to better understand the hepatoprotective effects of Arg in rats. Some enzymatic anti-oxidants have been recognized to play an important role in the anti-oxidant mechanism. Specifically, T-SOD converts $O_2^{\cdot-}$ into H_2O_2 and O_2 , and CAT reduces H_2O_2 into H_2O and O_2 (Reiter *et al.*, 2000). Lipid peroxides or reactive oxygen species could easily inactivate these anti-oxidant enzymes (Yang *et al.*, 2008). If their activities decreased, the oxygen-free radicals would cause progressive oxidative damage and cell death (Liochev, 2013). In the present study, the activity of the anti-oxidant enzyme T-SOD was significantly decreased in response to PNP treatment in rats' livers compared with the control group, indicating increased oxidative damage to the liver. Similar results were found in other authors' research. For instance, PNP induced oxidative damage, produced hepatotoxicity in zebrafish (Lam *et al.*, 2013), and altered the anti-oxidant system in rat testes (Zhang *et al.*, 2015). In addition, the decreased activity can lead to excessive availability of superoxide and hydrogen peroxide in biological systems, which in turn will generate hydroxyl radicals involved in the initiation and propagation of lipid peroxidation (Naziroglu, 2012). However, the activity of T-SOD was not significantly different by the administration of Arg to PNP-intoxicated rats, which suggests that it cannot restore and maintain T-SOD activity in PNP-damaged livers. Thus, dietary supplementation of Arg cannot protect against the hepatic lipid peroxidation induced by PNP.

The oxidative damage genes *Nrf2*, *HO-1*, and *NQO1* mRNA levels were detected. It was widely held that *Nrf2* and related genes play an essential role in protecting cells against internal and external stress (Taguchi *et al.*, 2011), also regulating anti-oxidant protective genes, the expression of phase II detoxification enzymes, and anti-oxidant enzymes (Ohta *et al.*, 2008). A recent study has found that the gene expression of *Nrf2* and *HO-1* could be activated under different stress conditions (Loboda *et al.*, 2016). Studies have shown that *Nrf2*- knockout mice exhibit more oxidative damage in the expression of *Nrf2* target genes compared to *Nrf2*- wild-type during antibody-induced arthritis (Wruck *et al.*, 2011) and that *Nrf2*-knockout mice are highly susceptible to hepatotoxicity due to decreased expressions of ARE-regulated drug-metabolizing enzymes and anti-oxidant genes (Enomoto *et al.*, 2001). In this study, PNP administration significantly increased *HO-1* and *NQO1* mRNA expression compared to the control group. The increased *HO-1* and *NQO1* mRNA expression in livers could be associated with a

possible response against the xenobiotic injury determined by PNP. However, Arg supplementation did not affect the expression of oxidative damage genes.

In conclusion, the present study demonstrated that the liver damage ability of PNP was significant, mainly characterized by the inflammatory invasion of cells and increased oxidation levels in serum and intrahepatic. In addition to this, the addition of Arg significantly improved the growth performance of healthy animals, with protective effects observed at the tissue level against PNP-induced liver injury, but without significant effects at both the intrahepatic oxidative level and the gene level, which has the potential to be related to the dose of Arg, and possibly not affect inflammation initiation by reducing oxidation levels.

Funding

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IRB approval

The experimental protocols were approved by Animal Care and Use Committee of Nanjing Agricultural University.

Ethical statement

The experimental protocols were approved following the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

Statement of conflict of interest

The authors have declared no conflict to interest.

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