EFFECT OF N-ACETYLCYSTEINE ON OXIDATIVE STRESS AND Bax AND Bcl2 EXPRESSION IN THE KIDNEY TISSUE OF RATS EXPOSED TO LEAD

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Received: 30 May 2020; Accepted: 17 December 2021

This study aimed to consider the lead-induced oxidative damage of the kidney of male rats and the role of antioxidant N-acetylcysteine (NAC) in preserving cells against Pb toxicity. Rats were randomly divided into five groups including G1 (control), G2 (single 70 mg/kg dose of Pb), G3 (continuous daily 2 mg/kg dosing of Pb for 4 weeks), G4 (single dose of Pb + 50 mg/kg NAC), and G5 (continuous daily dosing of Pb + 50 mg/kg NAC). The level of malonic dialdehyde (MDA) and total antioxidant capacity were measured spectrophotometrically. The level of Pb in serum and kidney tissue was measured by atomic absorption spectroscopy. Expression of Bax and Bcl2 genes was estimated using RT-PCR. It was shown that single and continuous exposure to Pb caused a considerable increase of Pb content in serum and kidney tissue of rats in G2 and G3 groups compared to other groups. NAC treatment significantly improved TAC values and decreased MDA values in the serum of rats exposed to Pb. Single and continuous Pb dosing caused a 3.9-fold increase in Bax expression and 1.5-fold and 2.1-fold decrease in Bcl2 expression in a kidney tissue respectively. The current study revealed that single and especially continuous Pb exposure was strongly associated with Pb accumulation, antioxidant depletion, oxidative stress and kidney cells apoptosis. NAC can help protect kidney tissue against Pb by elevating antioxidant capacity, mitigating oxidative stress and normalizing Bax and Bcl2 genes expression.

Keywords: Pb, kidney, N-acetyl cysteine, oxidative stress, Bax, Bcl2, apoptosis.

Lead (Pb) is a natural and potent environmental toxicant which is widespread in our environment from the air to soil and water [1, 2]. Lead can be associated with a wide spectrum of complications or diseases such as neurological dysfunctions, hemolytic anemia, frank anemia, bone injury, renal failure, cardiovascular disease, cancers and reproductive problems [2-6]; however, the exact mechanism in which Pb mediates these abnormalities is not well-understood. Recent studies have proposed that Pb may induce cell injuries or toxicities through multiple mechanisms. Oxidative stress (OS) induced by overproduction of reactive oxygen species (ROS) and decreased effective concentration of antioxidants are considered as the main mechanisms of the Pb toxicity which can be ultimately associated with tissue cells apoptosis and injury [7]. Apoptosis is a programmed cellular death which is regulated by specific biochemical and molecular factors and occurs under both normal physiological conditions and physiological abnormalities. This process is controlled and induced by several internal and ex-
ternal factors. The internal pathway includes genes that express proteins which are involved for the initiation (e.g. Bax) and inhibition of apoptosis (e.g. Bcl2) [8]. Bax and Bcl2 are well-known apoptotic proteins. While Bax is pro-apoptotic regulator and upon initiation of apoptotic signaling activates apoptosis, Bcl2 inhibits the release of cytochrome C from mitochondria and inhibits apoptosis. Higher Bcl2/Bax ratio is associated with increased survival rate of cells, whereas lower Bcl2/Bax ratio causes cells death [9]. Since oxidative stress is considered as one of the major factors for the induction of apoptosis, antioxidant supplementation may decrease apoptosis via the inhibition of Pb-induced oxidative stress. N-acetylcysteine (NAC) is a potential antioxidant that its anti-apoptotic properties have been recently studied by many researchers [10, 11]. Given the potential role of Pb in tissues injury, it seems that oxidative stress induction and alterations in expression of Bax and Bcl2 genes may be a main mechanism of Pb toxicity. Based on reports, Bax and Bcl2 genes have important roles in the regulation of apoptosis and it seems that impaired expression of these proteins is one of the main causes of the negative effect of Pb on different tissues. As previous studies confirmed the antioxidant and anti-inflammatory effects of NAC, administration of this compound appears to reduce oxidative stress and reduce the expression of apoptotic biomarkers in various organs.

Although previous studies considered oxidative stress and expression of apoptosis makers in different models (e.g. rats with non-alcoholic fatty liver disease or diabetes) and tissues (e.g. liver), in this research we have focused on kidney tissue and effect of NAC supplementation at a same on these parameters which are not reported previously. Therefore, this study aimed to consider the effect of Pb on biomarkers of oxidative stress (e.g. total antioxidant and malondialdehyde levels) and expression of Bax and Bcl2 in Pb-treated rats. The therapeutic effect of NAC on these parameters will also be considered.

**Materials and Methods**

**Animals.** Thirty male Wistar rats with 8-10 weeks of age and body weight of 150-200 g were bought from the laboratory animal research center at Pasteur Institute of Iran (Tehran). This research was approved by the animal care and use committee at the Tehran Medicine Sciences Islamic Azad University. All rats were adapted with lab environment for one week and then randomly divided into 5 groups, including control (G1), G2 (single dose of Pb), G3 (continuous daily dosing of Pb), G4 (single dose of NAC + Pb), and G5 (continuous daily dosing of Pb). Rats in each group were housed 3 per cage (30×15×15 cm) in a standard climate room (with temperature of 22 ± 2°C, humidity 50 ± 5%, and a 12:12 light/dark cycle) and had free access to food (10g/kg/day) and tap water.

**Treatments.** Rats in the control group were fed with normal pellet and water for 4 weeks. Rats in G1 group received a single gavage of Pb solution (70 mg/kg) on the first day of examination, while animals in G2 group received a continuous gavage of Pb solution (2 mg/kg) every other day for 4 weeks. Rats in G3 group received a combination of Pb (70 mg/kg) and NAC (50 mg/kg) solutions at same time on the first day of examination, and rats in G4 group gained a continuous administration of Pb (2 mg/kg) and NAC (50 mg/kg) solutions every other day for 4 weeks.

**Tissues and blood samples collection.** 48 hours after the final treatment, rats were anesthetized with xylazine (3-5 mg/kg) and ketamine (30-50 mg/kg) [12]. Blood samples were collected from the abdominal aorta for the assessment of serum Pb content. For histological study, kidney tissues were removed and fixed in 10% formalin for at least 48 hours. Fragments were dehydrated in graded series of ethanol, embedded in paraffin and sectioned using an automatic microtome at 4-5 mm thickness. The sectioned tissues were stained with haematoxylin-eosin (H&E) and evaluated for morphological and histological parameters by light microscope. A fragment of kidney tissue (~100 mg) was separated and homogenized in phosphate buffer (with pH 7.0) at 4°C with homogenizer (Hielscher, UP100H). The homogenized tissue was centrifuged at 12000 rpm/4°C for 15 min [13]. The supernatants were then collected and stored at -80°C for further analysis.

**Oxidative stress biomarkers.** Total antioxidant capacity (TAC) in supernatants was determined by ferric reducing of antioxidant power (FRAP), which discussed previously by Benize et al. [14]. Malondialdehyde (MDA) level was measured using the thiobarbituric acid (TBA) method [15].

**Measurement of Pb.** For the Pb analyses, kidney tissues (~100 mg) were dried overnight at 75°C and then digested in approximately 10× the dry tissue mass of nitric acid. The digested samples were diluted 5-fold by deionized water. For the analysis of Pb in serum, blood samples were centrifuged at
600 g for 10 min. After centrifugation, supernatants were diluted 5-fold by deionized water. Eventually, level of serum and kidney tissue Pb was measured by atomic absorption spectroscopy (AAS; Perkin Elmer model 2380). For Pb analysis, different concentrations of Pb (from 0.01–0.8 mg/l) and for tissue Pb analysis, concentrations of Pb (from 0.01–0.8 μg) were used to plot the standard curve.

Gene expression analysis. RNX-Plus (SinaClon; RN7713C) Kit was used for total RNA extraction from homogenized kidney tissues. A Nanodrop ND-1000 spectrophotometer (Thermo Sci., Newington, NH) was applied to consider the quantity and quality of extracted RNAs. Electrophoresis on 1% agarose gel was also performed to determine the quality of extracted RNAs. Revert Aid Reverse Transcriptase (Thermo science, Germ any) and random hexamer primers (Thermo science, Germ any) were used for cDNA synthesis at 42°C for 1 h. A Rotor Gene 6000 (Corbett Research, Australia) thermocycler in 40 cycles was applied for amplifications. Each reaction included 5 μl master mix and 100 nM primers. Primer sequences are as follow: Bax, 5’-GAGGATATTGCTGATGGATA-3’ (forward), 5’-CAGTTGAAGTGGGCTCTG-3’ (reverse); Bcl2, 5’-GAGGCCGCAAGGAGATG-3’ (forward), 5’-ACAGCCAGGAATACTCAAACAGA-3’ (reverse); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5’-AAGTTCAACGGCAGAT-3’ (forward); 5’-CATACTGACCGCATCACC-3’ (reverse). The levels of mRNA were normalized relative to the amount of GAPDH mRNA. The relative expression of studied genes was calculated using 2^–ΔΔCt method.

Statistical analysis. All data are presented as means ±SD. One-Way ANOVA: Post Hoc-Tukey test was used to compare the mean of all data between groups. Data were analyzed using SPSS software (version 19). A P < 0.05 was considered as significant.

Results and Discussion

Histopathological examination of kidney tissue in each group revealed that there were no abnormalities in the control group (G1), while sections of kidney tissue from rats in G3 group showed increased elevated inflammation. Although the sections of kidney from rats treated with NAC (G4 and G5) showed abnormalities, these concrete disorders were lower compared to the rats in G2 and G3 groups. Combined therapy with NAC declined number of inflammatory cells along with inflammation in kidney of rats exposed to Pb (Fig. 1). Single and continuous treatments with Pb caused a significant increase of Bowman capsule and glomerulus area. The mean of Bowman capsule and glomerulus area in control group was 8257.03 ± 1548.50 μm² and 6882.43 ± 1398.21 μm², respectively. NAC supplementation significantly decreased Bowman capsule (from 11266.70 ± 377.17 μm² to 9771.50 ± 383.95 μm²) and glomerulus area (from 8609.60 ± 152.16 μm² to 7930.80 ± 251.65 μm²) of rats exposed to single dose of Pb. The mean concentration of Pb in the kidney and serum of rats treated with single dose of NAC + Pb was relatively similar to that in control.

Comparison of FRAP value between all groups can be seen in Fig. 2. Rats treated with continuous dose of Pb had significantly lower mean values of FRAP value (234.12 ± 35.82 μg/ml) compared to the other groups, while the mean level of FRAP in the kidney of rats in control group (543.05 ± 73.02 μg/ml) was significantly higher than that in other groups. NAC treatments significantly improved FRAP values in rats that exposed to single (from 335.43 ± 35.09 μg/ml to 473.72 ± 70.35 μg/ml; P = 0.002) and continuous dose (from 234.12 ± 35.82 μg/ml vs 291.75 ± 41.87 μg/ml; P = 0.043) of Pb.

Fig. 3 shows comparison of MDA mean levels between all groups. While rats in control group had significantly lower mean levels of MDA (17.26 ± 3.22 μg/ml) compared to the other groups, rats exposed to continuous dose of Pb had significantly higher MDA contents (50.51 ± 2.55 μg/ml) than other groups. NAC treatments significantly decreased MDA values in rats that exposed to single (from 31.55 ± 1.74 μg/ml to 23.81 ± 1.46 μg/ml; P < 0.001) and continuous dose (from 50.51 ± 2.55 μg/ml to 39.07 ± 1.79 μg/ml; P < 0.001) of Pb.

A significant difference was found in expression pattern of Bax and Bcl2 between groups (Fig. 4 and 5). Rats treated with continuous and single dose of Pb showed significantly downregulation of Bcl2 and overexpression of Bax compared to the other groups. However, NAC treatments significantly improved the expression Bcl2 and decreased the expression of Bax in rats exposed to single or continuous Pb.

Compared to control group, exposure to single and continuous dose of Pb caused a significant increase in Bax expression by 3.9-fold (P = 0.004)
Fig. 1. Sections of kidney tissue from different groups. The kidney of rats in control (G1) were normal in structure, while sections from rats in continuous group (G2 and G3) showed increased elevated inflammatory cells, enlargement of bowman capsule and glomerulus area. Combined therapy with NAC declined number of inflammatory cells along with bowman capsule and glomerulus area in Pb exposed groups (G4 and G5). X20 magnification.

Fig. 2. Comparison of the mean of FRAP value in serum of rats in different groups. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of TAC between all groups. *P < 0.001; **P < 0.01; ***P < 0.05 compared to control group.

Fig. 3. Comparison of the mean of MDA value in serum of rats in different groups. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of MDA between all groups. *P < 0.001; **P < 0.01; ***P < 0.05 compared to control group.
and 13.14-fold ($P < 0.001$), respectively. In contrast, rats that treated with a combination of NAC and Pb showed a mild decrease in Bax expression rather than to those treated with single or continuous dose Pb. Bax expression in NAC + continuous Pb was significantly decreased by 1.98-fold compared to the continuous Pb group ($P < 0.0001$; Table 1).

Single and continuous exposures to Pb significantly decreased the expression of Bcl2 compared to control by 1.49-fold ($P = 0.042$) and 2.08-fold ($P < 0.001$), respectively (Table 2). However, NAC treatments significantly improved Bcl2 expression in the continuous group by 1.41-fold ($P = 0.042$).

The mean of Pb concentrations in the blood and kidney tissue of rats exposed to single dose of Pb were $1.12 \pm 0.15$ mg/l and $0.021 \pm 0.003$ μg/g tissue, respectively, while the mean of Pb contents in the blood and kidney tissue of rats exposed to continuous Pb were $2.30 \pm 0.3$ mg/l and $0.047 \pm 0.006$ μg/g tissue, respectively (Fig. 6 and Fig. 7). NAC supplementation significantly decreased Pb concentrations in the both serum (from $1.12 \pm 0.15$ mg/l to $0.65 \pm 0.11$ mg/l) and tissue samples (from $0.021 \pm 0.003$ μg/g tissue to $0.018 \pm 0.001$ μg/g tissue) of rats exposed to single dose of Pb. The mean concentration of Pb in the kidney and serum of rats treated with NAC + single Pb dose was relatively similar to that in control. NAC supplementation significantly decreased Pb concentrations in the both serum (from $2.30 \pm 0.3$ mg/l to $1.10 \pm 0.14$ mg/l) and tissue samples (from $0.047 \pm 0.006$ μg/g tissue to $0.032 \pm 0.003$ μg/g tissue) of rats exposed to continuous dose of Pb.

In this study, we evaluated the effect of NAC supplementation on histological changes, oxidative stress biomarkers as well as expression patterns of Bax and Bcl2 genes in the kidney tissue of rats exposed to single or continuous dose treatment of Pb. Our findings have revealed that Pb exposure, especially continuous exposure, is associated with a significant depletion of total antioxidants and increased levels of MDA in the kidney tissue of study rats. Cd exposure, especially continuous exposure, was significantly associated with accumulation of kidney and blood Pb in exposed animals. We also found that Pb administration, especially at continuous dosing, significantly caused an overexpression of Bax and down-regulation of Bcl2 genes in the kidney tissue of exposed rats. Our findings support the idea that toxicological effect of Pb on kidney tissue is mediated through the induction of oxidative stress and renal cells apoptosis. Several lines of studies have demonstrated that Pb exposure has the both genotoxicity and cytotoxicity effects and induces oxidative stress and inflammation in different tissues [16]. For example, Kumar, et al., [17] demonstrated that Pb caused a significant increase in oxidative stress biomarkers in the liver of chicken. Pb exposure significantly increased the level of TBAR, and activity of glutathione peroxidase (GPX), GSH reductase, and catalase (CAT), but decreased the mean levels of glutathione (GSH) in the liver of exposed poutries [17]. Vitamin E + Selenium supplementation

Fig. 4. Comparison of the mean mRNA levels of Bax. Gene expression was detected by Real-Time PCR. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of Bax expression pattern between all groups. *$P < 0.001$; **$P < 0.01$; ***$P < 0.05$ compared to control group

Fig. 5. Comparison of the mean mRNA levels of Bcl2. Gene expression was detected by Real-Time PCR. One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of Bax expression pattern between all groups. *$P < 0.001$; **$P < 0.01$ compared to control group
caused a significant improvement in these parameters. More recently, Shraideh et al., [18] considered the relationship between occupational lead exposure and plasma levels of oxidative stress biomarkers. They found that Pb levels in the serum of these workers were significantly higher compared to control group (~4–5 times). While there was a significant decrease in the level of plasma GSH (16–25%) and TAC value (21–33%), the mean content of MDA (120–333%) was significantly increased in the case of workers compared to the control group. Dribben et al., [19] showed that Pb exposure triggers neuronal apoptosis in the developing mouse brain. Xu et al., [20] revealed that Pb exposure increases the level of histone acetylation and induces apoptosis in vascular and cardiac tissues. These data indicate that oxidative stress and overexpression of apoptotic mediators, which have been found in our study, seem to be a possible mechanism of Pb toxicity on kidney tissue; however, further studies are need to confirm this results.

According to these findings and the concepts of Pb pathogenesis on the kidney tissue, these might make a wise basis for the use of antioxidants that could protect renal cells from oxidative stress and apoptosis. Here, we considered the effect of NAC treatment to mitigate oxidative stress and renal cells

Table 1. Comparison of the fold change ratio of the Bax expression

| Experimental groups          | Fold-change ratio | Up-/down-regulation | P-value |
|------------------------------|-------------------|----------------------|---------|
| Single vs control            | 3.90              | Up-regulated         | 0.004   |
| Continuous vs control        | 13.14             | Up-regulated         | < 0.001 |
| NAC + single vs control      | 2.24              | Up-regulated         | 0.18    |
| NAC + continuous vs control  | 6.62              | Up-regulated         | < 0.001 |
| Continuous vs Single         | 3.37              | Up-regulated         | < 0.001 |
| Single vs NAC + continuous   | 5.87              | Up-regulated         | 0.006   |
| Single + NAC vs single       | 1.74              | Down-regulated       | 0.081   |
| Single + NAC vs continuous   | 5.87              | Down-regulated       | < 0.001 |
| Single + NAC vs NAC + continuous | 2.96          | Down-regulated       | < 0.001 |
| Continuous + NAC vs continuous| 1.98             | Down-regulated       | 0.038   |

*P < 0.05 is considered as significant; One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of Bax expression pattern between all groups

Table 2. Comparison of the fold change ratio of the Bcl2 expression

| Experimental groups          | Fold-change ratio | Up-/down-regulation | P-value |
|------------------------------|-------------------|----------------------|---------|
| Single vs control            | 1.49              | Down-regulated       | 0.042   |
| Continuous vs control        | 2.08              | Down-regulated       | < 0.001 |
| NAC + single vs control      | 1.01              | Down-regulated       | 0.38    |
| NAC + continuous vs control  | 1.47              | Down-regulated       | 0.028   |
| Continuous vs Single         | 1.40              | Down-regulated       | 0.09    |
| Single vs NAC + continuous   | 2.05              | Up-regulated         | 0.006   |
| Single + NAC vs single       | 1.47              | Up-regulated         | 0.081   |
| Single + NAC vs continuous   | 2.05              | Up-regulated         | < 0.001 |
| Single + NAC vs NAC + continuous | 1.46            | Up-regulated         | 0.033   |
| Continuous + NAC vs continuous| 1.41             | Up-regulated         | 0.042   |

*P < 0.05 is considered as significant; One-Way ANOVA: Post Hoc-Tukey test was applied to compare mean value of Bcl2 expression pattern between all groups
apoptosis caused by Pb effects. In our study, NAC treatment significantly decreased kidney cells injuries caused by Pb. This effect was associated with a significant increase in total antioxidant capacity and a significant decrease in MDA contents in the kidney tissue. Interestingly, we found that NAC treatment not only improves the total antioxidants capacity, but also it attenuates oxidative stress, expression of Bax and increases anti-apoptotic Bcl2 in the kidney tissue of Pb-treated rats. Although the level of oxidative stress biomarkers and expression of apoptotic factors in the kidney of rats exposed to continuous dose of Pb + NAC were somewhat high, NAC improved these abnormalities in this group compared to rats that only treated with continuous dose of Pb. These data indicate that NAC can be helpful in mitigating oxidative stress and renal cells apoptosis in subjects who chronically expose to Pb. To support these findings, many studies revealed that NAC attenuates inflammation and oxidative stress by declining ROS production and apoptosis, as well as down-regulation of inflammatory cytokines and increasing of anti-inflammatory mediators and antioxidants. A recent study has demonstrated that NAC treatment no only decreases Zearalenone (ZEN)-induced oxidative stress biomarkers and expression of Bax, Caspase 3 and Caspase 9, but also it improves the activity of GPX and glutathione reductase (GR) in vitro [21]. Yedjou et al., [22] reported that NAC supplementation protects hepatic cells against Pb-induced cellular injury, genotoxicity and oxidative stress. Shieh et al., [23] have revealed that NAC treatment reversed malathion-induced oxidative stress responses, and prevented malathion-evoked apoptosis by regulating apoptotic protein expressions (Bax, Bcl2, Caspases-3, and -9) in normal human astrocytes. Al-Nahdi et al., [24] revealed that NAC not only decreases streptozotocin-induced oxidative stress, but also inhibits DNA damage and expression of apoptotic proteins in pancreatic β-cells. In another study, Chen et al., [25] evaluated the effect of NAC treatment (150 mg/kg) against cadmium-induced neuronal apoptosis and oxidative stress in mice model. They found that continuous exposure to Cd (10-50 mg/l) is significantly associated with overproduction of ROS, and brain damage or neuronal cell apoptosis. NAC treatment significantly prevented Cd-induced ROS production and attenuated Cd-induced brain damage or neuronal cell death by increasing the activities of Cu/Zn-superoxide dismutase, catalase and glutathione peroxidase, as well as the level of glutathione in the brain. Our findings were in agreement with these results. According to previous accomplished data and our findings, oxidative stress and apoptosis serve as common mediator of Pb cytotoxicity on kidney tissue. On the other hand, NAC supplementation declines the toxicity effects of Pb exposure.

**Conclusion.** In conclusion, the findings of the current study revealed that Pb exposure, especially continuous exposure to Pb, is strongly associated with accumulation of Pb, oxidative stress, antioxidant depletion, and kidney cells apoptosis. NAC can help protect kidney tissue against Pb by elevating antioxidants capacity, mitigating oxidative stress, as well as down-regulating of apoptotic factors.
Відомості про конфлікт інтересів.
Автори завершують прочитання інтереси в зв'язку з формою конфлікту інтересів, доступною на сайті http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf и заявляють, що вони не мають конфліктів інтересів.

Знавство. Ця робота була підтримана AYA University of Medical Sciences, Tehran, Iran. Ми також хотіли б подякувати Mr. Asghar Beigi Harchegani за допомогу в завершенні цього проекту у рамках обмеженої термінової рамки.

Фінансування. Ця робота не була фінансово підтримана жодною організацією і не отримала жодного гранту.

**ВПЛИВ **
**N-АЦЕТИЛЦИСТЕЇНУ НА ОКСИДАТИВНИЙ СТРЕС ТА ЕКСПРЕСІЮ Bax I Bcl2 В ТКАНІ НІРКИ ЩУРІВ ЗА ДІЇ СВІНЕЦЯ**

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У роботі досліджували спричинене свинцем (Pb) окисне пошкодження нирок щурів та роль антиоксиданту N-ацетилцистеїну (NAC) у запобіганні токсичній дії Pb на клітини нирки. Щурів випадковим чином розділили на п'ять груп: G1 (контроль), G2 (разова доза Pb + 50 мг/кг NAC), G3 (щоденне введення Pb + 50 мг/кг NAC) та G4 (щоденне введення 2 мг/кг Pb протягом 4 тижнів), G5 (щоденне введення Pb + 50 мг/кг NAC). Рівень Pb у сироватці та тканині нирок вимірювали методом атомноабсорбційної спектроскопії. Рівень також наведено результати показують, що одноразова і більшою мірою тривала дія Pb пов'язана з накопиченням Pb, винищенням антиоксидантів, окисним стресом та апоптозом клітин нирок. Застосування NAC може допомогти захистити ниркову тканину від дії Pb, підвищуючи його антиоксидантну здатність, зменшуючи оксидативний стрес та нормалізуючи експресію генів Bax та Bcl2.

Ключові слова: свинець, нирки, N-ацетилцистеїн, оксидативний стрес, Bax, Bcl2, апоптоз.

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