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Original Article

Protective effect of N-Acetyl-L-Cysteine (NAC) on endosulfan-induced liver and kidney toxicity in rats

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Abstract: Endosulfan-induced systemic toxicity arises from oxidative stress and glutathione depletion. This study aims to investigate the possible protective effect of N-acetyl-L-cysteine (NAC) against endosulfan-induced toxicity in the liver and kidney tissue of rats. Wistar albino rats were separated into 4 groups and administered saline, NAC, endosulfan and endosulfan + NAC for 5 days. After euthanizing the animals, trunk blood was collected, followed by the removal of the kidney and liver for histological observation, the determination of glutathione, malondialdehyde levels, collagen content and myeloperoxidase activity. Lactate dehydrogenase (LDH) activity, blood urea nitrogen, creatinine, alanine aminotransferase and aspartate aminotransferase levels were measured in serum samples, while 8-OHdG, IL-1b, IL-6 and TNF-α were analyzed in plasma. Endosulfan provoked a significant decrease in tissue glutathione, along with a significant increase in malondialdehyde and collagen status, as well as myeloperoxidase activity. In addition, the pro-inflammatory mediators, LDH activity, and 8-OHdG, aspartate aminotransferase, creatinine, alanine aminotransferase and blood urea nitrogen levels were significantly raised in the endosulfan group. The endosulfan + NAC group showed significant decreases in MPO activity ($P < 0.001$ in both liver and kidney) and MDA levels ($P < 0.01$ in the liver, $P < 0.05$ in the kidney) compared with the endosulfan only group, revealing the protective effect of NAC. Furthermore, the results show that NAC treatment prevents all of the biochemical and histopathological alterations induced by endosulfan. This data indicates that NAC administration effectively prevents the deleterious effects of endosulfan toxicity and attenuates oxidative hepato-renal oxidative damage. This is possibly the result of its antioxidant effects.

Keywords: Endosulfan, N-Acetyl-L-Cysteine, lipid peroxidation, glutathione, myeloperoxidase

Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a, 6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide) is used as an organochlorine pesticide throughout the world [1], and belongs to the class II group of moderately hazardous pesticides [2]. Due to its lipophilic structure, exposure to endosulfan can cause bioaccumulation and biomagnification; these occur due to several different mechanisms. Endosulfan is an acutely toxic chemical. Many cases of acute poisoning and death resulting from high level ingestion or inhalation have been reported [3]. Studies have suggested that the adverse health effects of endosulfan exposure include endocrine disruption [4], hepatotoxicity, neurotoxicity, genotoxicity, infertility and spontaneous abortions [5]. Endosulfan may exert these effects through various mechanisms in several species. A possible mechanism of toxicity is the decrease in antioxidant defenses causing oxidative stress induced by the accumulation of reactive oxygen species (ROS). Overproduction of free radicals that interact with cell membrane proteins and fatty acids permanently impairs their function. On the other hand, cytokine activity is thought to be an important mechanism in endosulfan toxicity [6, 7]. Oxidative stress and inflammation have been shown to occur in the pathophysiology of endosulfan toxicity. Acute and chronic

toxicity studies of endosulfan in animals report that the primary systemic target organs are the liver, kidney and testes [8].

A natural sulfur-containing compound, N-Acetyl-L-cysteine (NAC) is generated in living organisms from the amino acid cysteine [9]. NAC affects the body's general antioxidant activities by increasing glutathione levels. This notable metabolic role has been previously stated [10]. *NAC* is highly efficient in preventing oxidative-damage, and is commonly used in experimental models as a positive control forantioxidant-defense [11].

The major impacts of endosulfan toxicity have been reported as neurotoxicity, immunotoxicity, systemic organ deficiency and impairment of the antioxidant defense system [8]. Antioxidant supplementation such as vitamin C, E, or beta-carotene could help to prevent the occurrence of complications induced by the exacerbated oxidative stress that endosulfan can cause. Therefore the current study was designed to determine the possible preventative effect of NAC against endosulfan-based toxicity in the liver and kidney tissue of rats, using biochemical and histological parameters, and inflammatory responses. To accomplish this, we evaluated lactate dehydrogenase (LDH) activity, aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine levels in serum samples, 8-hydroxy-2'-deoxyguanosine (8-OHdG), the proinflammatory mediators TNF-α, IL-1β and IL-6 levels in plasma samples and malondialdehyde (MDA, a marker of oxidative lipid injury), glutathione (GSH, an antioxidant molecule) levels, collagen content, and myeloperoxidase (MPO) activity in the liver and kidney tissue of rats treated with endosulfan. Further, we focused on the protective effect of NAC on the biochemical parameters along with endosulfan induced histopathological variations.

Material and methods

Endosulfan

Endosulfan (technical grade, molecular weight: 407) is a compound consisting of α- and β-stereoisomers. The endosulfan used in this study was a generous gift from Hektaş, Istanbul, Turkey.

Animals

Ethical approval for the experimental protocols was obtained from the Marmara University School of Medicine Animal Care and Use Committee. Both genders of Wistar albino rats (250-300 g) were included in the study. The rats were housed at a temperature of 22 ± 2°C, in a 12 hour light-dark cycle, with free access to standard pellet food and water.

Experimental groups

Wistar albino rats (250-300 g) were randomly separated into four groups, each consisting of 8 animals (4 males and 4 females). The groups were administered the folowing different treatments for 5 days: Group C: endosulfan dissolved in corn oil, orally administrated at a dose of 2 ml/kg/day before intraperitoneal (i.p.) sterile 0.9% saline treatment in the control group; NAC Group: N-acetyl-L-cysteine at a dose of 150 mg/kg/day i.p.; Endo Group: endosulfan dissolved in corn oil, at a dose of 22 mg/kg/day orally (medium dose, 20% LD_{50}); Endo + NAC group: NAC (at a dose of 150 mg/kg/day i.p.) and endosulfan (at a dose of 22 mg/kg orally). The application dose of endosulfan was prepared following Siddiqui et al. [12] and NAC following Sehirli et al. [13].

After 5 days of treatment, the rats were euthanized, the kidney and liver tissue was removed gently, and the trunk blood was collected to obtain serum and plasma samples. All tissues, plasma and serum samples were stored at -80°C.

Meanwhile, tissue collagen content was determined to show oxidant-induced tissue fibrosis. Renal and liver tissue samples were fixed in 10% buffered p-formaldehyde to prepare for histological evaluations.

Biochemical analysis

To assess hepatic and renal functions, serum ALT, AST status, BUN, and creatinine concentrations, LDH activity was analyzed with an automated analyzer (Opera Technican Bayer Autoanalyzer, Germany).

Plasma levels of TNF-α, IL-1β and IL-6 were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits according to the

Table 1. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine and serum lactate dehydrogenase (LDH) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in the Control, Endo, saline-NAC and Endo + NAC groups (n=8 per group)

Groups	*Control*	*NAC*	*Endo*	*Endo + NAC*
AST (mg/dL)	220 ± 21	222 ± 22	326 ± 18**	221 ± 18^{++}
ALT (mg/dL)	73.5 ± 7.7	66.7 ± 6.8	108.7 ± 7.5**	74.8 ± 4.5^{++}
BUN (mg/dl)	22.5 ± 1.9	20.8 ± 2.3	39.7 ± 5.9*	23.2 ± 1.4^{+}
Creatinine (mg/dl)	0.51 ± 0.08	0.53 ± 0.07	0.89 ± 0.05**	0.59 ± 0.05^{+}
LDH (U/L)	1769 ± 152	1778 ± 140	4449 ± 196***	2613 ± 136$^{**,+++}$
8-OHdG (mg/dl)	0.64 ± 0.13	0.60 ± 0.15	2.78 ± 0.42***	1.08 ± 0.11^{+++}

$^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the control group. $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ compared to the untreated Endo group.

manufacturer's instructions and guidelines (Biosource International, Nivelles, Belgium).

The plasma 8-OHdG content was measured by the ELISA method (Highly Sensitive 8-OHdG ELISA kit, Japan Institute for the Control of Aging, Shizuoka, Japan).

Glutathione (GSH) and Malondialdehyde (MDA) analyses

The modified Ellman procedure was conducted to measure GSH levels [14]. Results are given as µmol/g tissue. The MDA levels were analyzed by monitoring thiobarbituric acid reactive substance formation as previously described [15]. Liver and kidney MDA levels are given as nmol/g tissue.

Measurement of myeloperoxidase (MPO) activity

Hepatic and renal MPO activities were determined as previously described by Hillegass et al. and are expressed in units per gram of tissue [16].

Tissue collagen measurement and histological analysis

Buffered formalin (10%) fixed tissue samples were embedded in paraffin and 15 µm sections were obtained. The collagen content was assessed based on the procedure published by Lopez De Leon and Rojkind [17]. 6 mm Paraffin sections were stained with Periodic acid-Schiff reaction (PAS) in the kidney tissues and Hematoxylin and eosin in the liver tissues, both of which were investigated with a light microscope (Olympus BX51). The severity of tissue damage was scored according to previously defined criteria [18].

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 4.0 (GraphPad Software, San Diego; CA; USA). All data is expressed as the means ± SEM. Comparison between Groups of data were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The level of significance was tested at $P < 0.05$.

Results

In the endosulfan treated group, serum ALT and AST levels were significantly higher ($P < 0.01$) than those of the Control Group, demonstrating the occurrence of hepatic damage. The Endo + NAC group had lower ALT and AST levels than the Endo Group group ($P < 0.01$, **Table 1**).

Creatinine and BUN levels, which are indicators of renal function, showed significant increases ($P < 0.05$ and $P < 0.01$, respectively) in the EndoGroup than in the Control Group. Creatinine and BUN levels in the Endo + NAC group were closer to the Control Group, whereas a significant difference was observed between the Endo and Endo + NAC groups ($P < 0.05$, **Table 1**).

Serum LDH activity assay was also performed in order to show generalized tissue damage. LDH activity was significantly higher in the Endo Group ($P < 0.001$) than in the control group; NAC treatment protected this effect significantly ($P < 0.001$ compared to the Endo group, **Table 1**).

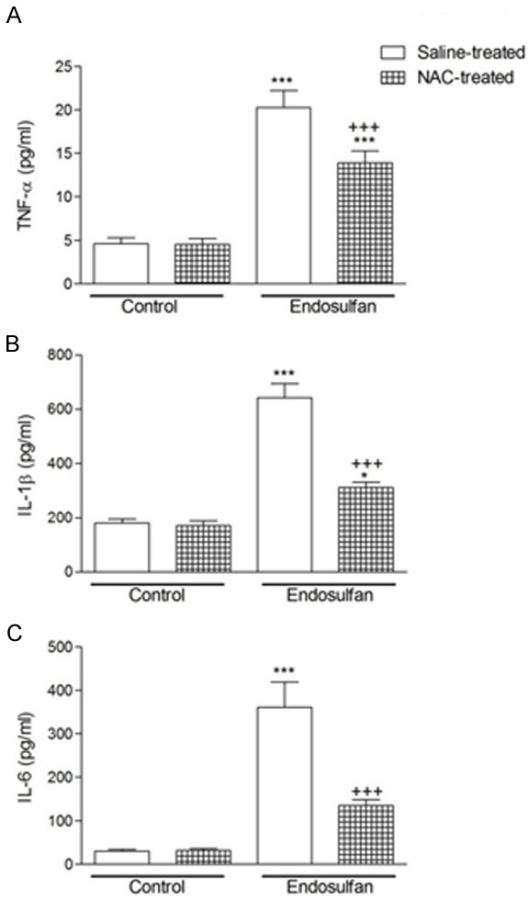

Figure 1. Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) in the saline- or NAC, treated Control and Endo groups. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to the saline-treat-Control group. $^{+++}P < 0.001$ compared to the saline-treated Endo Group. Each group consisted of 8 rats.

Concurrently, the 8-OHdG levels, markers of oxidative DNA damage caused by ROS [19], were dramatically increased in the Endo Group ($P < 0.001$ versus the Control Group) whereas the 8-OHdG levels in the Endo + NAC Group remained at lower levels than those of the Endo Group, revealing the protective effect of NAC (**Table 1**).

IL-1β, TNF-α and IL-6 (pro-inflammatory cytokines) levels showed a significant increase in the Endo Group when compared with the Control Group ($P < 0.001$, **Figure 1**), and NAC administration prevented this increase.

In the Endo Group, the kidney and liver GSH levels were significantly lower than those of the Control Group ($P < 0.001$, **Figure 2**).

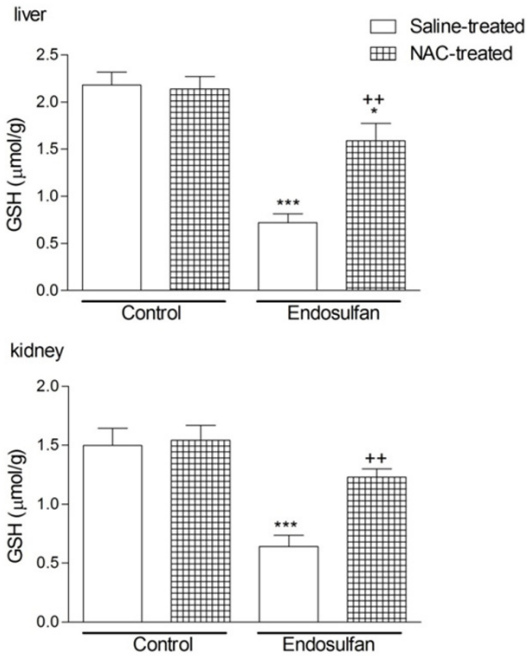

Figure 2. Glutathione (GSH) levels in the liver and kidney samples of the saline- or NAC-treated Control and Endo groups. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to the saline-treated Control Group. $^{++}P < 0.01$ compared to the saline-treated Endo Group. Each group consisted of 8 rats.

Nonetheless, the kidney GSH levels of the Control Group were similar to those of the Endo + NAC Group ($P > 0.05$), while the NAC and Control groups showed no significant differences ($P > 0.05$, **Figure 2**). The liver and kidney GSH levels of the Endo + NAC Group differed significantly from the Endo group ($P < 0.01$, **Figure 2**).

A good indicator of lipid peroxidation, MDA levels increased in all tissue samples in the Endo Group ($P < 0.001$, compared with the group (C), **Figure 3**). The Endo + NAC Group displayed a significant decrease in the MDA levels when compared to the EndoGroup ($P < 0.01$ in liver, $P < 0.05$ in kidney); the NAC and Control groups showed no effect.

When compared with the Control Groups, the Endo Group displayed increased MPO activity in all tissue specimens ($P < 0.001$) whereas this effect was prevented in the NAC Group ($P < 0.001$). The NAC and Control groups treatments showed no effect (**Figure 4**).

After the administration of endosulfan, the collagen content, an indicator of the level of fibrot-

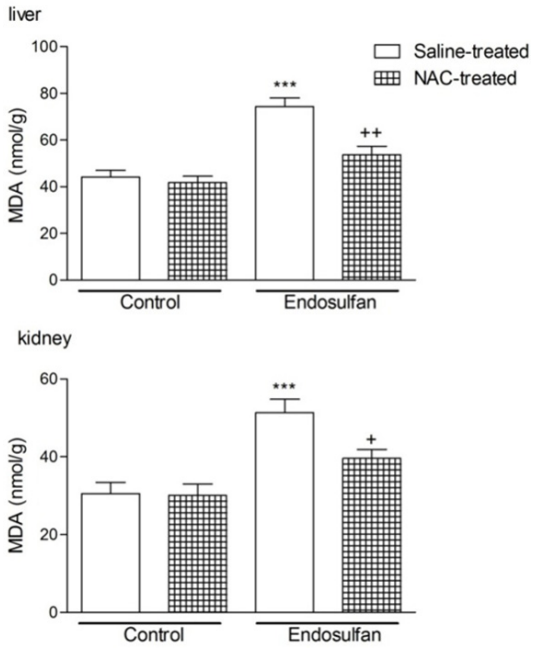

Figure 3. Malondialdehyde (MDA) levels in the liver and kidney samples of the saline- or NAC-treated Control and Endo groups. ***P < 0.001 compared to the saline-treated Control Group. +P < 0.05, ++P < 0.01 compared to the Endo Group. Each group consisted of 8 rats.

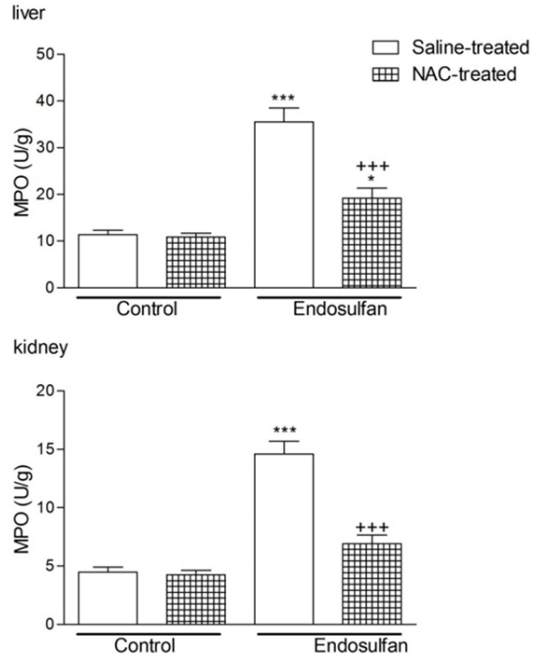

Figure 4. Myeloperoxidase (MPO) activity in the liver and kidney samples of the saline- or NAC-treated Control and Endo groups. *P < 0.05, ***P < 0.001 compared to the saline-treated Control Group. +++P < 0.001 compared to the saline-treated Edo Group. Each group consisted of 8 rats.

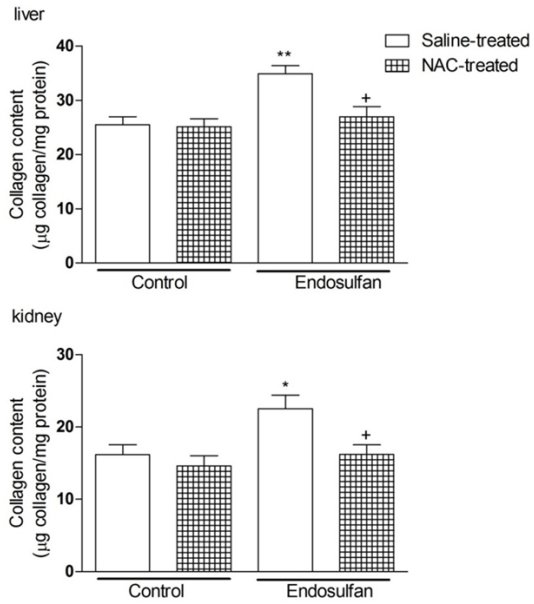

Figure 5. Collagen content in the liver and kidney samples of the saline- or NAC-treated Control and Endo groups. *P < 0.05, **P < 0.01, compared to the saline-treated Control Group. +P < 0.05, compared to the saline-treated Endo Group. Each group consisted of 8 rats.

ic activity, showed marked increases in liver and kidney tissues when compared to the control group (P < 0.01 and P < 0.05 respectively, **Figure 5**). NAC treatment of the Endo Group reversed this effect (P < 0.05); whereas the NAC and Control groups did not display a change the collagen content of these tissues.

Light microscopy revealed interstitial edema, inflammatory cell infiltration, and degenerated proximal and distal tubules along with disrupted glomeruli. The PAS reaction seemed to be elevated in tubular and glomerular basal membranes. Due to degenerated microvilli, the brush borders of proximal tubules demonstrated a decreased PAS reaction. Tissue sections from the Endo + NAC group presented minimal kidney parenchymal damage, resulting in good tissue integrity (**Figure 6**).

The result of the liver histopathological examination showed that the endosulfan induced hepatic vasocongestion, leading to mild liver damage. However, significant histopathological alteration was observed neither in the liver tissue of the Endo Group nor in the Endo + NAC Group (**Figure 7**).

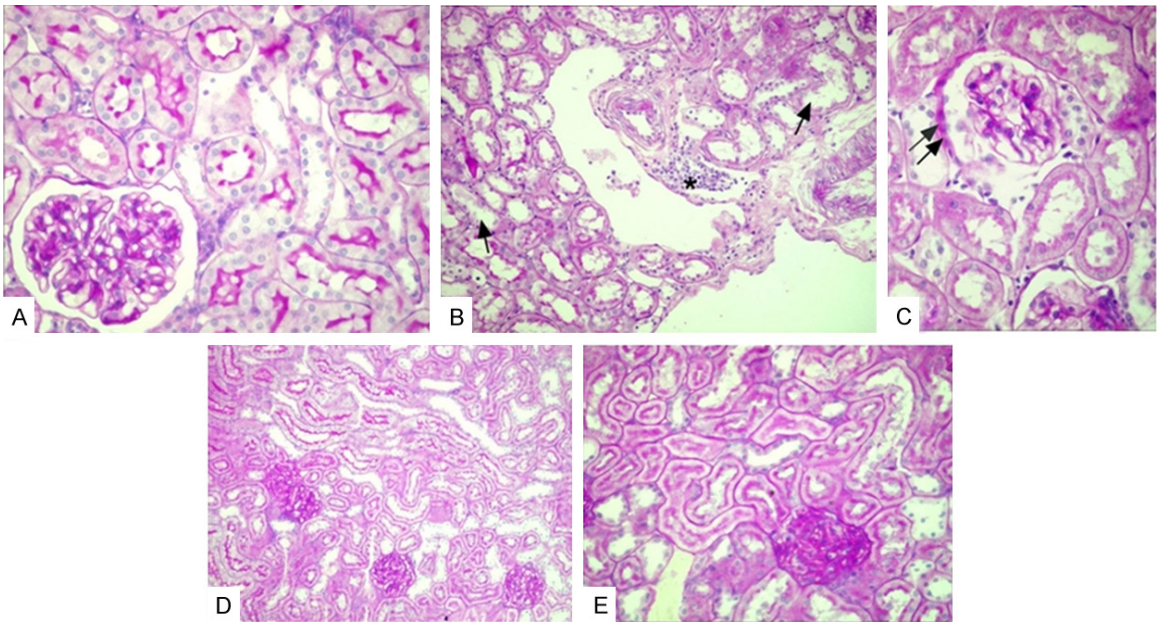

Figure 6. A: Control Group: normal kidney morphology. B, C: Endo group: extreme degenerations of kidney tubules (◊), inflammatory cell infiltration (*), disrupted parietal layer of glomerulus (double arrow) and prominent thickness in the glomerular and tubular basal membranes. D, E: Endo + NAC Group: the micrograph depicts a nearly regular kidney parenchymal histology. PAS reaction. (B, D ×200), (A, C, E ×400).

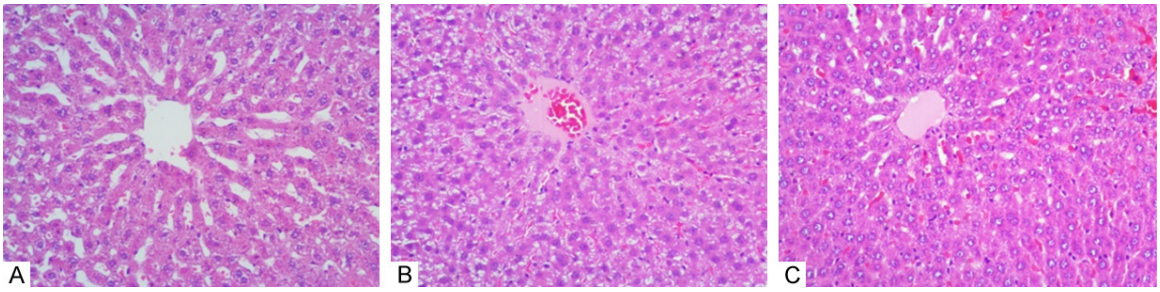

Figure 7. A: Control Group: normal liver morphology. B: Endo Group: vasocongestion in the liver. C: Endo + NAC Group: vasocongestion in the liver. Hematoxylin-Eosin. ×100.

Discussion

Our results indicate that oral administration of endosulfan increased MDA levels, MPO activity and collagen content, while reducing the GSH levels of kidney and liver specimens, resulting in lipid peroxidation and oxidative stress. NAC supplementation markedly reduced MPO activity and partially improved the collagen content induced by endosulfan. Degenerated microvillar structures and cytoplasmic damage at both proximal and distal tubular epithelial cells in the kidney tissues were coincident with increased levels of MDA and MPO activity. We assumed that N-acetylcysteine prevents the altered oxidative stress parameters related to endosulfan exposure by inducing antioxidant mechanisms.

Endosulfan has also been previously studied. In one study, endosulfan was administered torats at different doses and decreased GSH levels were observed in liver and kidney tissues [12]. In another study focusing on rat liver tissue, it was demonstrated that endosulfan causes an increase in thiobarbituric acid reactive substances, indicating lipid peroxidation in liver tissue [20]. In addition to these results, we focused on alterations in biochemical parameters, the measurement of tissue collagen and histological analysis, and we also considered the possible protective effect of NAC.

As averification, the study carried out by Ahmed et al. observed the how the treatment of cells with endosulfan caused a decrease in intracellular GSH levels, thus revealing a correlation between oxidative stress and the level of apoptosis of peripheral blood mononuclear cells. *In vitro* NAC co-treatment reduced GSH depletion and apoptosis [21]. The histopathological results of our study concluded by inhibiting the depletion of GSH in the endo + NAC treated group, which resulted in prominent cellular protection of kidney tubular epithelial cells.

Enzymatic and nonenzymatic antioxidants, e.g. reduced glutathione (GSH) and NAC, either repairs the oxidative damage or directly scavenges oxygen radicals, such as hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) and counteracts the damage caused by the intracellular ROS [22]. *N*-acetylcysteine, as a glutathione precursor (GSH), is an antioxidant that has a protective effect against oxidative stress, and prevents lipid peroxide production by scavenging free radicals in biological membranes [23].

It was well documented that 8-OHdG is an important biomarker for the evaluation of oxidative stress and the assessment of cancer risk caused by exposure to carcinogenic substances [24, 25]. The possible protective effect of NAC treatment was observed with the results of 8-OHdG levels, which were correlated with altered oxidative stress parameters. Similar findings were reported by Ahmed et al. [26].

In a recent study of rats, endosulfan exposure caused a significant biochemical imbalance in blood parameters, due to a decrease in antioxidant levels, e.g. superoxide dismutase (SOD) and glutathione S-transferase, while glutathione peroxidase, catalase and lipid peroxidation were significantly increased in a dose dependent manner in the heart and liver [27]. Our results are also in agreement with other recently published studies [7, 28].

Various studies demonstrate that NAC exhibits anti-inflammatory properties. NAC modulates the inflammatory markers induced by lipopolysaccharides in animal models [10]. In our study, the increase in the level of pro- inflammatory cytokines in the Endo + NAC Group was inhibited by NAC, something which does not happen in the Endo group.

In the present study, endosulfan induced renal damage, including tubuler degeneration and dysfunction, were achieved by reducing lipid peroxidation. Moreover, NAC treatment was effective in protecting the kidneys against endosulfan-induced degeneration. Similarly, Batca Kayhan et al. investigated the damage in rat kidney tissue by endosulfan administration. Histological techniques allowed them to observe distinct structural changes such as tubular dilation, hydropic degeneration in tubular epithelium, and hemorrhage in the cortical and medullaof the kidney [29]. Karaoz et al. also observed that endosulfan caused perivascular and peritubular mononuclear cell infiltrations, glomerular and tubular degenerations as the main microscopical outcomes in the kidneys of rats [30]. In a combined chronic toxicity/carcinogenicity study, adverse effects of endosulfan were noted. These included an increased number of enlarged kidneys in females as well as an increased incidence of aneurysms in blood vessels and progressive glomerulo-nephrosis in males [31].

The BUN, creatinine, ALT and AST levels were significantly higher in the Endo Group than in the Control Group, and our results suggest that the administration of NAC was sufficient to reduce the extent of hepatic and renal damage caused by endosulfan. Based on the histopathological examination of the renal specimens in these groups, renal tissue damage in the Endo Group was observed, and this damage was reversed in the Endo + NAC Group. Prominent cellular damage at both proximal and distal tubular epithelial cells in the NAC Group was thought to have caused the disrupted levels of BUN and creatinine. On the contrary, significant histopathological alteration was observed in the liver tissue of neither the Endo Group nor the Endo + NAC Group.

Xenobiotics and intermediates may disturb the redox balance and provoke excessive production of ROS in hepatocytes, which oxidizes lipids, proteins, DNA, and other macromolecules, disrupts cell processes, and injures hepatocytes [32, 33]. ROS production might be enhanced by covalent binding or oxidative effects on electron transport. Therefore, ROS-mediated oxidative stress may play a pivotal role in hepatotoxicity [34]. Recent studies high-

light the importance of oxidative stress and the role of ROS in toxicity diseases [6, 31, 35, 36].

Although the use of endosulfan is banned in many countries, studies show that endosulfan is still in use [37]. Therefore, endosulfan is a threat to the environment, wildlife, and human health. NAC may be used to prevent oxidative damage by minimizing the harmful effects of endosulfan. Understanding the mechanism of the exposure-antioxidant effect relationships is necessary.

This study had several limitations that deserve comment. The dose dependent effect of endosulfan could be analyzed by assaying different doses, and the measurement of antioxidant enzymes (such as superoxide dismutase and catalase) would be more useful for showing the preventative effect of NAC. By the same token, the urine could be analyzed to determine if there was any decrease in the presence of toxic products after NAC treatment, in order to support our results.

In conclusion, the results of this study demonstrate that in endosulfan toxicity, NAC balances the oxidant-antioxidant status and protects tissue, regulates the generation of inflammatory mediators, and inhibits neutrophil infiltration. The tissue protection effect of NAC was clearly evident in histopathological sections of the NAC treated group which displayed a prominent decrease in kidney tissue damage.

Disclosure of conflict of interest

None.

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