ORIGINAL RESEARCH

Protective effect of erdosteine against naphthalene-induced oxidative stress in rats

Özer Şehirli[1], Göksel Şener[1]

ABSTRACT: In this study the role of free radicals in naphthalene-induced toxicity and the protection by erdosteine are investigated. Female Sprague-Dawley rats were treated with a single oral dose of 1100 mg naphthalene/kg in corn oil. Erdosteine was given 50 mg/kg/day orally for 3 days before naphthalene treatment and rats were decapitated 24 hours after naphthalene administration. Liver and kidney tissue samples were taken for determination of malondialdehyde (MDA), glutathione (GSH), Na+, K+-ATPase and myeloperoxidase (MPO) activities. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine levels and lactate dehydrogenase (LDH) activity were measured in the serum samples, while TNF-α, IL-1β, IL-6, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and total antioxidant capacity (AOC) were assayed in plasma samples. Naphthalene administration caused a significant decrease in tissue GSH levels, Na+, K+-ATPase activity and plasma AOC levels, which was accompanied with significant increases in tissue MDA levels and MPO activity. Moreover the pro-inflammatory mediators (TNF-α, IL-β, IL-6), 8-OHdG, LDH activity, AST, ALT, creatinine and BUN levels were significantly increased in the naphthalene group. On the other hand erdosteine treatment prevented all these biochemical changes induced by naphthalene. In conclusion, it seems likely that erdostein protects tissues by inhibiting neutrophil infiltration, balancing the oxidant–antioxidant status and regulating the generation of inflammatory mediators.

KEY WORDS: Naphthalene; erdosteine; lipid peroxidation; glutathione; myeloperoxidase

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Received:
December 09, 2009

Accepted:
December 15, 2009

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are the main toxic and persistent compounds present in most crude oils. As a result of the release of petroleum oils to the sea PAHs are now ubiquitous contaminants of aquatic ecosystems (1). Some of these chemicals have been demonstrated to have mutagenic/carcinogenic (2, 3), genotoxic (4) and cytotoxic (5) properties (6). Naphthalene, a congeneric form of polycyclic aromatic hydrocarbons (PAHs), which is widely used commercially in moth repellents, lavatory scent disc and soil fumigants. It is also used in the manufacturing of naphthylamines, anthranilic and phthalic acids, and synthetic resins (7, 8).

The toxic manifestation induced by naphthalene appears to involve the conversion of naphthalene to naphthoquinone, as well as hydroxylated products including 1-naphthol, 2- naphthol and 1,2-dihydroxynaphthalene (9, 10) which cause oxidative damage. It has been demonstrated that naphthalene exposure resulted in elevated levels of serum and liver lipid peroxides (11), and decreased hepatic selenium dependent glutathione peroxidase activity (12). Naphthalene exposure is associated with the development of hemolytic anemia in humans and rats. Similarly administration of naphthalene (1100 mg/kg) to female Sprague-Dawley rats resulted in 2.5-fold increases in lipid peroxidation in liver and brain mitochondria 24 h after treatment indicated that the toxicity of naphthalene is at least in part related to free radicals and free radical-mediated oxidative stres (8).

Erdosteine [*N*-(carboxymethylthioacetyl)-homocysteine thiolactone] is a novel mucoactive agent that contains two blocked sulphydryl groups, one of which is present in an aliphatic side-chain and the other is enclosed in the heterocyclic ring (13). These chemically blocked sulfhydryl groups are liberated following hepatic metabolism and thereby the molecule subsequently exerts its free radical scavenging and antioxidant properties (14). Based

on its free radical scavenging activity, its protective effects against oxidant-induced tissue damage have been demonstrated in various inflammation models (15-18). Similarly we have also demonstrated the protective effect of erdosteine against colitis induced oxidative colonic tissue injury (19). On the basis of this background, using biochemical examination, we aimed to study the putative protective effects of erdosteine on the hepatic and renal tissues in the rats exposed to naphthelene.

MATERIALS AND METHODS

Animals

All experimental protocols were approved by the Marmara University School of Medicine Animal Care and Use Committee. Female Sprague-Dawlwey rats (200-250 g) were kept at a constant temperature (22 $\pm$ 1^o C) with 12 h light and dark cycles and fed a standard rat chow.

Experimental Design

Rats were given orogastrically either erdosteine (50mg/kg/ml n=16) or saline (n=16) for 3 consecutive days. On the fourth day, after an overnight fasting with free access to water, half of the saline or erdosteine-treated rats were given 1100mg/kg/ml of naphthalene in corn oil by gavage(naphtehelen groups), while the other half of the saline or erdosteine-treated group was given orogastrically corn oil (control groups). All rats were decapitated at 24 hour of naphthalene or corn oil administration. After decapitation of the animals, trunk blood was collected and liver and kidney were carefully dissected and stored at –70 C^o for the determination of tissue malondialdehyde (MDA) and glutathione (GSH) levels, Na^+-K^+ ATPase and myeloperoxidase (MPO) activities.

Biochemical analysis

Blood urea nitrogen (20) and serum AST, ALT (21) and creatinine (22) concentrations and LDH levels (23) were determined spectrophotometrically using an automated analyzer. Plasma levels of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 were quantified according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits specific for the previously mentioned rat cytokines (Biosource International, Nivelles, Belgium). The total antioxidant capacity in plasma were measured by using colorimetric test system (ImAnOx, cataloge no. KC5200, Immunodiagnostic AG, D-64625 Bensheim), according to the instructions provided by the manufacturer. The 8-OHdG content in the extracted DNA solution were determined by enzyme-linked immunosorbent assay (ELISA) method (Highly Sensitive 8-OHdG ELISA kit, Japan Institute for the Control of Aging, Shizuoka, Japan). These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intra-assay precision, and small amount of plasma sample required conducting the assay.

Malondialdehyde and glutathione assays

Tissue samples were homogenized with ice-cold 150 mM KCl for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (24). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} and results are expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure (25). Briefly, after centrifugation at 3000 rev./min for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/l $Na_2HPO_4.2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of 1.36 x 10^4 M^{-1} cm^{-1}. Results are expressed in μmol GSH/g tissue.

Myeloperoxidase activity

Myeloperoxidase (MPO) is an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear leukocytes (PMN). Tissue MPO activity is frequently utilized to estimate tissue PMN accumulation in inflamed tissues and correlates significantly with the number of PMN determined histochemically in tissues. MPO activity was measured in tissues in a procedure similar to that documented by Hillegass et al. (26). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41.400 g (10 min); pellets were suspended in 50 mM PB containing 0.5 % hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41.400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Measurement of Na^+,K^+-ATPase activity

Measurement of Na^+,K^+-ATPase activity is based on the measurement of inorganic phosphate that is formed from 3 mM disodium adenosine triphosphate added to the medium during the incubation period (27). The medium was incubated in a 37^o C water bath for 5 min with a mixture of 100 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 30 mM Tris HCl (pH 7.4). Following the preincubation period, Na_2ATP, at a final concentration of 3 mM was added to each tube and incubated at 37^o C for 30 min. After the incubation, the tubes were placed in an ice bath, and the reaction was stopped. Subsequently, the level of inorganic phosphate was determined in a spectrophotometer (Shimadzu, Japan) at excitation wavelength of 690 nm. The specific activity of the enzyme was expressed as μμol Pi mg^{-1} protein h^{-1}. The protein concentration of the supernatant was measured by the Lowry method (28).

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego; CA; USA). All data were expressed as means ± SEM. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p<0.05$ were regarded as significant.

RESULTS

As shown in the AST and ALT levels in the saline treated naphthalene group were found to be significantly higher that of saline treated control group ($p<0.001$), however treatment with erdosteine caused significant reduction in both AST and ALT levels ($p<0.01$-0.05). Similarly, BUN and creatinine levels, which significantly increased in the saline treated naphthalene group ($p<0.001$-0.01), were also reversed back to the control levels by erdosteine treatment ($p<0.01$-0.05). Serum lactate dehydroge-

nase activity, as a marker of generalized tissue damage, showed a significant increase in the saline treated naphthalene group ($p<0.001$), while erdosteine administration prevented this effect ($p<0.001$). In the saline-treated naphthalene group, proinflammatory cytokines, TNF-α, IL-1β and IL-6, were significantly increased ($p<0.001$) when compared to control group, while this naphthalene-induced rise in serum cytokine levels were abolished ($p<0.001$) with erdosteine treatment. Plasma 8-OHdG levels, as a marker of oxidative DNA damage, were significantly higher in the saline treated naphthalene group than in control groups ($p<0.001$) and erdosteine treatment reduced the elevated plasma 8-OHdG levels ($p<0.001$). The total plasma antioxidant capacity is decreased significantly due to naphthalene administration ($p<0.01$), and this decrease was prevented by erdosteine treatment ($p<0.05$, Table 1).

TABLE 1. Effects of erdosteine (50 mg/kg) treatment on some biochemical parameters in the serum of experimental groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

Parameters	Control Groups		Naphthalene Groups	
	Saline treated	Erdosteine treated	Saline treated	Erdosteine treated
AST (U/l)	246±10	250±12	368±18***	257±16++
ALT (U/l)	76.2±7.7	73.8±7.8	154±14.6***	110±6.3+
Creatinine (U/l)	0.7±0.1	0.6±0.1	1.8±0.2***	0.8±0.1+++
BUN (U/l)	25.7±2.1	23.5±2.1	40.2±2.5**	28.5±2.6++
LDH (U/l)	1799±135	1794±242	4759±243***	2416±101+++
TNF-α (pg/ml)	7.6±1.1	8.1±1.3	30.9±3.1***	13.6±2.1+++
IL-1β (pg/ml)	15.5±1.7	16.5±1.9	34.2±3.8***	18.8±2.2++
IL-6 (pg/ml)	4.9±0.9	6.5±1.2	16.1±1.5***	9.9±1.2+
8-OHdG (ng/ml)	0.8±0.1	0.9±0.1	6.6±0.9***	2.1±0.5+++
AOC (pg/ml)	447±49	424±53	187±21**	362±32+

Data are mean ± SEM. ** $p<0.01$, *** $p<0.001$ compared to saline treated control group. + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$compared compared to saline treated naphthalene group.

In accordance with these findings, the major cellular antioxidant GSH levels of liver and kidney samples in saline treated naphthalene group were significantly lower than those of the control groups ($p<0.001$). On the other hand, erdosteine treatment to naphthalene group restored the GSH levels in both tissues ($p<0.01$, Fig. 1).

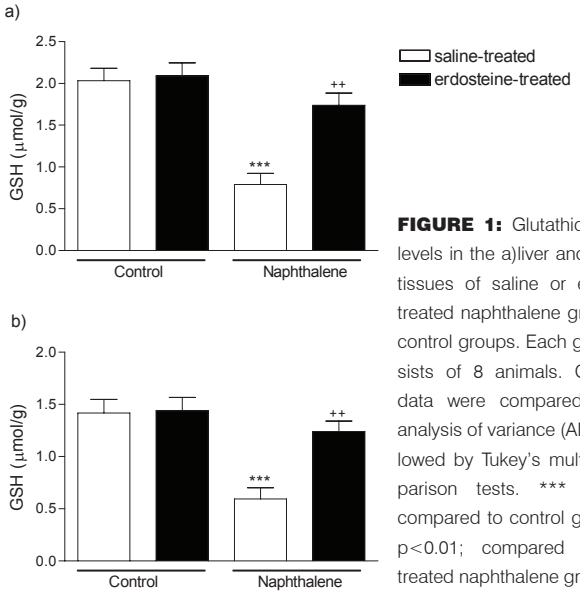

FIGURE 1: Glutathione (GSH) levels in the a)liver and b)kidney tissues of saline or erdosteine treated naphthalene groups and control groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *** $p<0.001$; compared to control group. ++ $p<0.01$; compared to saline treated naphthalene group.

The mean level of MDA, which is a major degradation product of lipid peroxidation, was increased in the liver and kidney tissues after naphthalene administration when compared with the control groups ($p<0.001$), while erdostein treatment to the naphthalene group caused a marked decrease in MDA levels ($p<0.01$, Fig. 2).

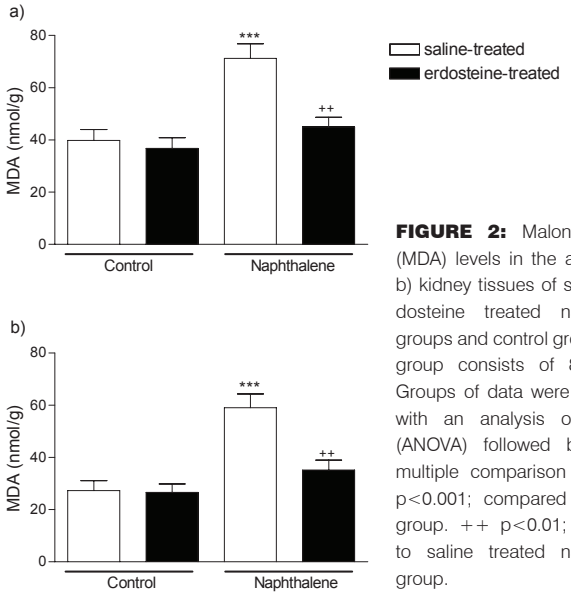

FIGURE 2: Malondialdehyde (MDA) levels in the a) liver and b) kidney tissues of saline or erdosteine treated naphthalene groups and control groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *** $p<0.001$; compared to control group. ++ $p<0.01$; compared to saline treated naphthalene group.

Myeloperoxidase activity, as an indicator of neutrophil infiltration, was significantly higher in the liver and kidney tissues of the saline treated naphthalen group when compared to control groups ($p<0.001$). On the other hand, erdosteine treatment in naphthalene group significantly decreased the MPO levels both in liver and kidney ($p<0.001$-0.01, Fig. 3).

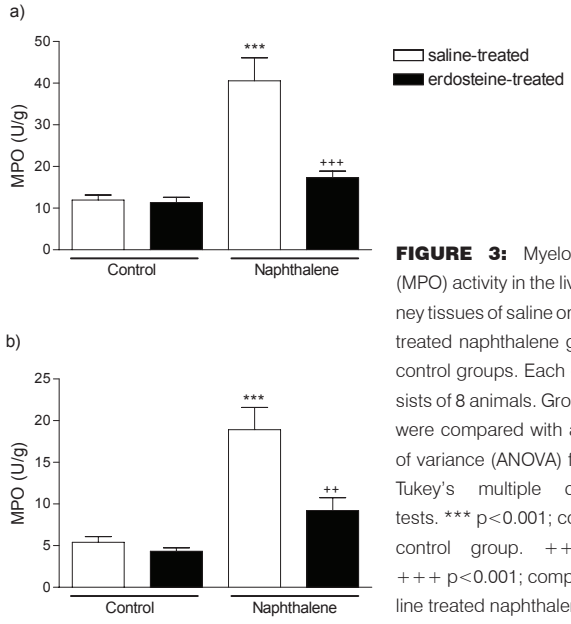

FIGURE 3: Myeloperoxidase (MPO) activity in the liver and kidney tissues of saline or erdosteine treated naphthalene groups and control groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *** $p<0.001$; compared to control group. ++ $p<0.01$, +++ $p<0.001$; compared to saline treated naphthalene group.

The activity of Na^+-K^+-ATPase was shown to be significantly decreased in the liver and kidney tissue of saline treated naphthalen group compared with control group; however, erdosteine

treatment in naphthalene group significantly increased all tissues Na^+-K^+-ATPase activity ($p<0.001$-0.01, Fig. 4).

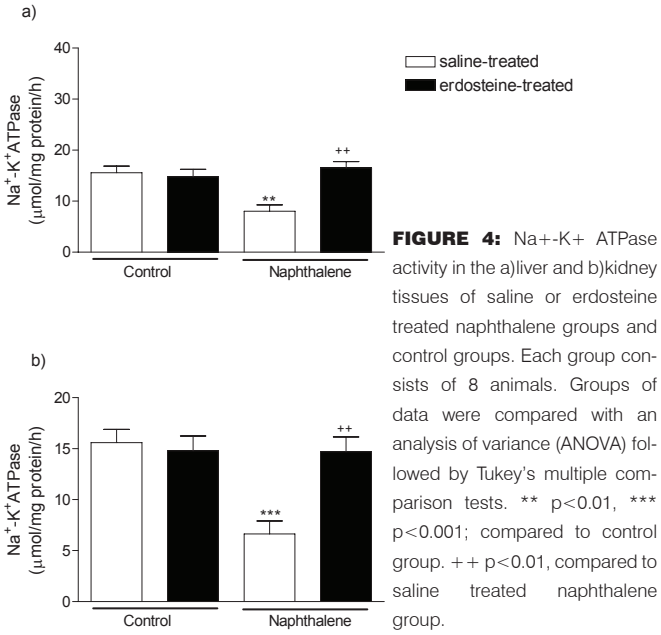

FIGURE 4: Na+-K+ ATPase activity in the a)liver and b)kidney tissues of saline or erdosteine treated naphthalene groups and control groups. Each group consists of 8 animals. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. ** $p<0.01$, *** $p<0.001$; compared to control group. ++ $p<0.01$, compared to saline treated naphthalene group.

DISCUSSION

The current data clearly demonstrates that treatment with erdosteine due to its free radical scavenging properties significantly inhibited napthelene-induced lipid peroxidation and neutrophil infiltration in the hepatic and renal tissues, while the depleted antioxidant GSH level and inhibited Na^+-K^+-ATPase activity were restored to control levels. In addition, elevated serum levels of LDH, AST, ALT, BUN creatinine and 8-OHdG, as well as reduced AOC demonstrate the severity of oxidative stress. The results also demonstrate that erdosteine treatment, as observed by the reversal of alterations in all the measured parameters in tissues, alleviated the naphtelene-induced plasma markers of oxidative stress and improved renal and hepatic functions.

Naphthalene exposure is associated with several toxic manifestations in humans and laboratory animals, with the lens of the eye and the lungs being the most sensitive (29). Most human toxicities involve low dose, chronic exposure to naphthalene (30). In mice, 200 mg/kg intraperitoneal naphthalene administration caused pulmonary damage (30). Koch et al. (31) reported the development of cataract following exposure to naphthalene. In tests with Chinese hamster ovary cells, naphthalene induced sister chromatid exchanges with and without exogenous metabolic activation. Naphthalene caused an increase in incidence and severity of olfactory epithelial metaplasia of respiratory epithelium in the nose and chronic inflammation in the lungs (32). Previously, studying the toxic effects of naphthalene in mice, we have demonstrated that when administered chronically, naphthalene caused multiorgan damage by increasing lipid peroxidation of the tissues. Furthermore when aqueous garlic extract was administered concomitantly with naphthalene, this damage was prevented which suggest that naphthalene caused oxidative injury and antioxidative agents could be beneficial against naphthalene toxicity (33).

Naphthalene undergoes extensive microsomal metabolism. The first step in the naphthalene metabolism is oxidative in nature and catalyzed by cytochrome P-450 oxygenases in the microsome that produces an electrophilic arene epoxide intermediate, 1,2-naphthalene oxide (34). The epoxides can spontaneously rearrange to form naphthols (predominantly 1-naphthol) which underwent further metabolism to naphthaquinons. Naphthaquinons are directly toxic to monunuclear leukocytes and depleted glutathione to 1% of the control levels (35). As the glutathione is a key antioxidant, depletion of glutathione may cause oxidant damage. Furthermore it has been demonstrated that cultured macrophage J774A.1 cells with naphthalene resulted in a concentration-dependent increase in the production of superoxide anion and hydroxyl radical production (36). Thus depletion of glutathione and tissue damage may occur not only as the result of metabolism of naphthalene to reactive intermediates, but also to the formation of reactive oxygen radicals (ROS). Similarly, in our study naphthalene administration significantly reduced the total antioxidant capacity of plasma with a concomitant decrease in tissue antioxidant, glutathione levels. Furthermore erdosteine treatment replenishes both of the plasma and the tissue antioxidant status and protected against this toxicity. The roles of oxidative stress and reactive oxygen species (ROS) and the critical role of glutathione in preventing naphthalene toxicity are well documented (37-40). Pretreatment of animals with the antioxidants caffeic acid and vitamin E, as well as the glutathione precursor N-acetylcysteine and the free radical spin trapping agent alpha phenyl-N-t-butylnitrone, also effectively decreased naphthalene-induced cataracts (29, 41).

The protective effects of erdosteine on inflammation and congestion due to hypoxic exposure indicate anti-inflammatory effects of erdosteine by scavenging inflammatory cells-derived ROS, because it has been demonstrated that erdosteine and its active metabolites significantly inhibit phorbol 12-myristate 13-acetate-induced luminol dependent chemiluminescence (42). In addition, erdosteine is effective in protecting against other inflammatory-associated lung diseases, like pulmonary fibrosis in rats (43-45). Experimentally studies examined the role of erdosteine in the protection of kidney after renal ischemia/reperfusion (I/R) in rats (46, 47). Pretreatment with erdosteine at doses of 10 mg kg^{-1} day^{-1} attenuated the increase of the level of MDA and oxidant enzymes activities (XO and myeloperoxidase) of kidney compared to I/R group (46). Moreover, oral administration of erdosteine (50 mg kg^{-1} day^{-1}) started 2 days before cisplatin intraperitoneal injection prevented the hepatic and kidney oxidative injury in rats (48). Concomitant treatment with erdosteine increased CAT, GSH-Px and SOD activities in liver and kidney tissues, compared with cisplatin group (49).

As a free radical generating system, lipid peroxidation has been suggested to be closely related to oxidant-induced tissue damage, and MDA is a good indicator of the degree of lipid peroxidation (8, 50, 51). In this study, we observed that naphthalene administration resulted in a significant increase in MDA levels in all the tissues (liver and kidney) compared with control animals. On the other hand erdosteine prevented MDA increases through its antioxidant effects.

This increase in lipid peroxidation may partly be due to the free radicals generated by neutrophils. Because; besides their direct damaging effects on tissues, free radicals seem to trigger the ac-

cumulation of leukocytes in the tissue involved, and thus cause tissue injury also indirectly through activated neutrophils. Activated neutrophils are known to induce tissue injury through the production and release of reactive oxygen metabolites and cytotoxic proteins (e.g. proteases, myeloperoxidase, lactoferrin) into the extracellular fluid. When neutrophils are stimulated by various stimulants, MPO, as well as other tissue-damaging substances are released from the cells. Thus, it is an index of neutrophil infiltration. Since the neutrophil infiltration is an important event for the acute inflammation, increase in MPO activity due to naphthalene may cause inflammation and damage in the organs. On the other hand, proinflammatory cytokines TNF-alpha, IL-1β and IL-6 were found to be significantly increased which also verifies that naphthalene toxicity is closely related with inflammatory mechanisms and oxidative damage. Since erdosteine treatment significantly decreased these cytokines and prevented the infiltration of neutrophil into the damaged tissue, our results suggest that the protective effects of the erdosteine is mediated in part by blocking plasma cytokines and tissue neutrophil infiltration.

The effect of erdosteine has been also tested during testicular torsion and detorsion in rats (52). Erdosteine was applied 50 mg kg^{-1} day^{-1} for 2 days before rats were subject to left unilateral testicular torsion/detorsion. Administration of erdosteine reduced the histological damages associated with testicular torsion and detorsion by preventing the accumulation of free oxygen radicals (49).

In conclusion, the present study clearly demonstrates that oxidative metabolism of the naphthalene is one of the principal mechanism leading to multiple organ damage and the protective effects of erdosteine can be attributed, at least in part, to its ability to inhibit neutrophil infiltration, to balance oxidant-antioxidant status, and to regulate the generation of inflammatory mediators, suggesting a future role in the treatment of organ failures due to drug or chemical toxicities.

Sıçanlarda naftalen ile oluşturulan oksidatif hasara karşı erdostein'in koruyucu etkileri

ÖZET: Bu çalışmada naftalen aracılı toksisitede serbest radikallerin rolü ve erdosteinin koruyucu etkisinin incelenmesi amaçlanmıştır. Dişi Sprague-Dawley sıçanlara mısır yağ içerisinde hazırlanan 1100 mg/kg naftalen tek doz oral olarak uygulandı. Naftalen uygulamasından once 3 gün boyunca 50 mg/kg dozunda oral olarak erdostein verildi ve sıçanlar naftalen uygulamasından 24 saat sonra dekapite edildi. Karaciğer ve böbrek dokularında malondialdehit (MDA), glutatyon (GSH), düzeyleri Na+, K+-ATPaz ve myeloperoksidaz (MPO) aktiviteleri incelendi. Serum örneklerinde aspartat aminotransferaz (AST), alanin aminotransferaz (ALT), kan üre azotu (BUN), kreatinin düzeyleri ve laktat dehidrogenaz (LDH) aktivitesi ölçüldü. Plazma örneklerinde TNF-α, IL-1β, IL-6, 8-hidroksi-2'-deoksiguanozin (8-OHdG) ve total antioksidan kapasitesi (AOC) değerlendirildi. Naftalen uygulaması doku GSH düzeylerinde, Na+, K+-ATPaz aktivitesinde ve plazma AOC düzeylerinde anlamlı olarak azalmaya, doku MDA düzeylerinde ve MPO aktivitesinde anlamlı olarak artışa neden oldu. Ayrıca pro-inflamatuvar mediyatörler (TNF-α, IL-β, IL-6) 8-OHdG, LDH aktivitesi, AST, ALT, kreatinin ve BUN düzeyleri naftalen grubunda anlamlı olarak yükseldi. Buna karşılık erdostein uygulaması naftalenin neden olduğu tüm biyokimyasal değişiklikleri anlamlı olarak önledi. Sonuç olarak erdosteinin nötrofil infiltrasyonunu inhibe ederek, oksidan-antioksidan dengeyi sağlayarak ve inflamatuvar mediyatörlerin salıverilmesini düzenleyerek doku koruyucu etki gösterdiği düşünülmektedir.

ANAHTAR KELİMELER: Naftalen; erdostein; lipit peroksidasyon; glutatyon; myeloperoksidaz

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