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The Effect of Nitrogen Mustard on the Enzymatic Antioxidant Defense of Rat Brain Tissue and the Therapeutic Value of Proanthocyanidin

Nitrogen Mustard'ın Rat Beyin Dokusunda Enzimatik Antioksidan Savunma Üzerine Etkisi ve Proantosiyanidin'in Tedavi Değeri

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ABSTRACT

AIM: Nitrogen Mustard (NM) is an alkylating agent that damages cellular nuclear DNA after penetrating tissue. This results in cytostatic, mutagenic and cytotoxic effects. We used biochemical analyses to investigate the effect of NM gas administered through the dermal and respiratory routes, on the brain cortex of rats and also tried to show whether the antioxidant Proanthocyanidin (PC) could decrease this effect.

MATERIAL and METHODS: A total of 30 rats were randomized into three groups: Group I: Control group, Group II: NM group, and Group III: NM + PC group. The rats were sacrificed 3 days after NM gas exposure. A segment of the cortical tissue was prepared for biochemical analyses.

RESULTS: Biochemical analyses of cortical neural tissue regarding the Enzymatic Antioxidant Defense against NM were performed. The results of these analyses implied that PC was effective for healing of cortical neural tissue.

CONCLUSION: These findings imply that structural changes induced by mustard gas can be prevented and restored by proanthocyanidin treatment.

KEYWORDS: Nitrogen mustard, Antioxidants, Proanthocyanidin, Rat brain

ÖZ

AMAÇ: Nitrogen Mustard (NM) dokuya penetre olduktan sonra hücrede mutagenik ve sitotoksik etkilere neden olan alkilleyici bir ajandır. Cilt ve respiratuvar yoldan uygulanan NM gazının, rat beyin dokusunda enzimatik antioksidan savunma mekanizmaları üzerine etkisi ve proantosiyanidin'in (PC) tedavi değerinin araştırılması amaçlandı.

YÖNTEM ve GEREÇLER: Toplam 30 rat üç gruba randomize edildi: Grup I: Kontrol grubu, Grup II: NM grubu, Grup III: NM+PC grubu. Ratlar NM gazı uygulamasından 3 gün sonra sakrifiye edildiler. Biyokimyasal analizler için kortikal bir doku segmenti hazırlandı.

BULGULAR: NM için kortikal nöral dokunun Enzimatik Antioksidan Defans mekanizmaları ile ilgili biyokimyasal analizler uygulandı. Analiz sonuçları kortikal nöral dokunun iyileşmesi üzerine PC'nin etkili olduğunu ima etmektedir.

SONUÇ: Bu bulgular mustard gazının indüklediği yapısal değişikliklerin proantosiyanidin tedavisi ile önlenebildiği ve onarılabildiğini ima etmektedir.

ANAHTAR SÖZCÜKLER: Nitrogen mustard, Antioksidanlar, Proanthocyanidin, Rat beyni

INTRODUCTION

Nitrogen Mustard (NM) is a potent warfare agent that affects the skin, eyes, lungs and the neuromuscular, hematological, gastrointestinal, endocrine and immune systems (4,18). NM is absorbed via the skin or the anterior surface of the eyes, and by inhalation (2). Its most common effect on the eye is conjunctival irritation with lacrimation (17). Although these agents have been studied for years, the main events that initiate cell death and the cytotoxic mechanisms induced by NM have still not been fully elucidated. The mechanism of mustard injury is thought to be associated with target alkylation (19). This results in cytostatic, mutagenic and cytotoxic effects. The interaction between NM and cellular structures is through ethylene immonium. The molecule creates a ring structure and binds to macromolecules. This ring alkaline structure affects the nucleophilic parts of intracellular macromolecules. The major alkylating reaction in nucleic acids is through the 7th nitrogen of guanine. It thus

leads to the formation of cross-links between or within DNA helices. The 3rd nitrogen of adenine and the 6th oxygen atom of guanine are other regions where the alkylation reaction can be seen. It may also cause damage to RNA, proteins and the cell (4). These effects may lead to chromosomal aberrations in addition to inhibiting DNA, RNA and protein synthesis with the cells pausing at the G2-M phase of the cycle. The result is severe damage, especially in cells with high mitotic activity (4). The poly (ADP-ribose) polymerase (PADRPR) enzyme is activated on exposure to mustard gas, thus decreasing NAD+ levels. This causes cell death. A normal NAD+ concentration ensures continuity of the energy-providing system and prevents blister formation (4). Nicotinamide may be used as a reversible PADRPR inhibitor and this agent has been reported to decrease cytotoxicity when used within 24 hours of exposure to NM. DNA alkylation by mustard causes breaks in the DNA chain, stimulating the DNA repair mechanisms and the activation of the PADRPR enzyme, which uses NAD+ as a substrate. Mustard also inhibits glycolysis and stimulates the NADP+-dependent hexose monophosphate shunt. These changes in cellular metabolism are reported to cause death especially in basal epidermal cells.

The aim of this experimental study was to show the effects of mustard gas at the intracellular level and determine the effect of strong antioxidants such as Proanthocyanidin (PC) in order to elucidate the diagnostic and therapeutic process to contribute to other studies aiming to decrease morbidity and mortality (1,13). The warfare agent mustard gas was administered to the subjects via the transdermal and inhalation routes under conditions simulating the battlefield in this study. We investigated the harmful effect of mustard gas on neural tissue in this study and also evaluated the efficacy of PC as we thought it might be beneficial in subjects exposed to mustard gas.

MATERIAL and METHODS

The study was performed at the Gülhane Military Medical Academy Animal Research Laboratory, and it was approved by this Academy's Ethics Committee.

I. Material:

a- Animals: A total of 30 male Rattus norvegicus weighing 140-160 g were used. These rats were randomized into three groups, each with ten rats. The animals were taken care of according to the relevant articles of the Helsinki Declaration and the guidelines of the U.S. National Institutes of Health.

b- Chemicals: NM and the chemicals required for the oxidative stress analysis were obtained from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany) and the organic solvents were bought from Merck KGaA (Darmstadt, Germany). A commercially available grape seed PC extract was purchased from GNC Bakara A.Ş. (Proantosiyanidin: GN 6018, 100 mg, 90 capsules, Istanbul, TR). The mustard gas was administered to the subjects at the Nuclear Biologic and Chemical (NBC) laboratory and the sacrifice was performed at the Experimental Animals Breeding and Research Laboratory.

II. Methods:

Experimental design:

A- Groups: These rats were randomized into three groups, each with ten rats as follows: Group I: Control group (CG, no trauma and no NM). Group II: (NM group) A toxic dose of vaporized 8 mg NM dissolved in 5 ml of distilled water was used for 10 minutes (800 mg/m3/min) on the NM group that was exposed to NM only. Group III: (Treatment Group, TMG, NM + PC group). These subjects were exposed to the same dose of NM and were fed a diet containing 100 mg/kg/day PC, administered orally via an orogastric injection. All exposures were carried out in a chamber 100 L in volume equipped with chemical, biological and radiological (CBR) filters. All rats were sacrificed with a lethal dose of xylazine and ketamine 72 hours later.

B- Trauma induction by Mustard Gas administration and Perfusion-Decapitation-Obtaining tissue samples.

1. Anesthesia: Anesthesia was provided before the surgical procedure to the subjects in each group with an intramuscular injection of Ketamine hydrochloride(Ketalar 5% solution, Eczacıbaşı İlac Sanayii, İstanbul with Parke-Davis license)–35 mg/kg and Rompun (Xylazine 2% solution, Bayer, Istanbul)-1.5 mg/kg. The rats in Group III were administered 0.5 mg/kg NM gas. The subjects were placed in the chamber. The chamber was heated using a tungsten electric bulb (100/220V) with an average temperature of 22ºC +/- 2ºC. The subjects were directly exposed to vaporized NM. Rats in the PC Group were fed PC beginning 8 h before the NM gas application and continuing 72 h afterwards. We assessed subjects' rectal temperature, and the number of breaths and heart beats per minute twice a day for 72 hours. The activities and the neurological deficits of the subjects were also evaluated. The subjects were allowed to survive for an additional 72 h. All subjects were sacrificed on the third day with a lethal dose of Ketamine + Xylazine. Buterphenol (0.5 mg/kg sc) was administered to the subjects to provide analgesia during this process. The parietal brain cortex (4x4x4mm) of the sacrificed subjects was removed for biochemical studies.

2. Perfusion-Decapitation-Obtaining Tissue Samples: Subjects in all groups were put to sleep again with the same method following the specified duration (72 hours). A thoracotomy was performed and 1000 ml of 0.9% NaCl was administered at 100mmHg pressure through a catheter delivered from the left ventricle to the aorta. The right atrium was opened and the infused saline withdrawn. Perfusion was continued until the fluid was clear. The animals were then sacrificed by decapitation. The scalp was opened, craniotomy performed and the calvarium exposed in the middle. The brain and brainstem were removed intact. Tissue samples 4x4x4 mm in size were obtained from the hemisphere. A microscope and microsurgical instruments were used during these procedures. Amicroscope (Zeiss OpMi 99) and microsurgical instruments were used during the surgical procedures and decapitation.

III. Tissue preparation: Following a period of 72 hours, the animals were anesthetized with ketamine (85 mg/kg) and their craniums were opened by craniotomy. The brains were removed immediately and sectioned into small pieces (4x4x4mm). Tissues were cleaned with cold saline, wrapped in aluminum foil and immediately immersed in liquid nitrogen. The tissue samples were stored at -80°C until analyses. The samples were thawed, weighed and homogenized in phosphate-buffered saline, pH 7.4.

Oxidative stress status related parameter analysis

Tissue preparation for oxidative stress status: Tissue samples were homogenized in 1.5% KCl solution on ice using a homogenizer. Then homogenized samples were centrifuged at 5000 xg and 4°C for 10 min. The supernatant was used for the analyses.

Thiobarbituric Acid Reactive Substances (TBARS) level measurement: Malondialdehyde (MDA) levels in tissue homogenate samples were determined in accordance with the method described in our previous studies (24). MDA levels were expressed as TBARS. Following the reaction of thiobarbituric acid with MDA, the reaction product was measured spectrophotometrically. Tetramethoxy propane solution was used as the standard.

Superoxide Dismutase (SOD) activity measurement: CuZn-SOD activity in tissue homogenate was measured by the method described in our previous studies (24). Briefly, each homogenate was diluted 1:400 with 10 mM phosphate buffer, pH 7.00. Twenty-five µL of diluted hemolysate was mixed with 850 µL of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride(INT) in a buffer solution containing 50 mmol/L CAPS and 0.94 mmol/L EDTA pH 10.2. Then, 125 µL of xanthine oxidase (80 U/L) was added to the mixture and absorbance increase was followed at 505 nm for 3 min against air. Twenty-five µL of phosphate buffer or 25 µL of various standard concentrations in place of the samples were used as blank or standard determinations. CuZn-SOD activity was expressed in U/g tissue.

Glutathione Peroxidase (GPO) activity measurement: GPO activities in tissue homogenates were measured by the method described in our previous studies (24). The reaction mixture was 50 mmol/L tris buffer, pH 7.6 containing 1 mmol/L of Na_2EDTA, 2 mmol/L of reduced glutathione (GSH), 0.2 mmol/L of NADPH, 4 mmol/L of sodium azide and 1000 U of glutathione reductase (GR). Fifty µL of tissue homogenate and 950 µL of reaction mixture were mixed and incubated at 37°C for 5 min. The reaction was then initiated with 10 µL of t-butyl hydroperoxide (8 mmol/L) and the decrease in NADPH absorbance was followed at 340 nm for 3 min. Enzyme activities were reported as U/g in tissue.

Catalase (CAT) activity measurement: CAT activity in tissue homogenate was measured by the method of Aebi (24). The reaction mixture was 50 mM phosphate buffer pH 7.0, 10 mM H_2O_2 and homogenate. The reduction rate of H_2O_2 was followed at 240 nm at room temperature for 30 seconds. Catalase activity was expressed in U/g tissue.

IV. Statistical Analysis:

Descriptive statistics are expressed as mean, median and min-max. Differences among the groups were analyzed using the Kruskal-Wallis test followed by the multiple comparison procedure of Conover. Statistical analyses were performed via SPSS 15.0 statistical software. P values less than 0.05 were evaluated as statistically significant.

RESULTS

The biochemical variables (TBARS, SOD, GPO, CAT) in the three groups were examined and compared using the Kruskal-Wallis test. Oxidative stress status analysis included the TBARS level, and SOD, CAT and GPO activities. The TBARS levels, CAT and GPO activities in PC were different from control group. NM direct exposure caused significantly increased TBARS levels, and increased GPO and SOD activity in brain tissue. PC treatment decreased TBARS levels, but CAT and GPO activities were similar to those of the treatment group. The levels of biochemical parameters according to the study groups are presented in Table I.

As seen in Table I, differences among the three groups were found to be statistically significant for all biochemical variables ($p<0.05$). TBARS, SOD and GPO values were highest in the NM group and lowest in the control group. The difference between each group was statistically significant for TBARS, SOD and GPO ($p<0.05$). CAT levels were similar in the control and treatment groups ($p>0.05$) but the NM groups had higher CAT levels than the other groups ($p <0.05$). The distributions of TBARS, SOD, GPO, CAT values are presented graphically in Figures 1-4.

Table I: The Comparison of the Biochemical Parameters for the Three Groups

	Control (n=10)		Nitrogen mustard (n=10)		Treatment (n=10)		p value
Variables	Mean± SD	Median (Min-Max)	Mean± SD	Median (Min-Max)	Mean± SD	Median (Min-Max)	
TBARS (nmol/g)	8.44±1.45	8.21 (6.40-11.29)	121.59±21.30	115.35 (101-167)	73.75±12.40	72.5 (51.02-93.74)	<0.001
SOD (U/g)	154±52.32	134 (114-262)	432.9±43.16	437 (382-500)	207.6±77.60	191 (131-401)	<0.001
GOP (U/g)	1.63±0.37	1.52 (1.13-2.39)	3.71±0.71	3.65 (3.11-5.53)	2.13±0.55	2.17 (1.34-3.11)	<0.001
CAT (U/g)	3.51±0.60	3.6 (2.20-4.20)	4.28±0.44	4.33 (3.59-5.21)	3.79±0.66	3.77 (2.92-4.91)	0.011

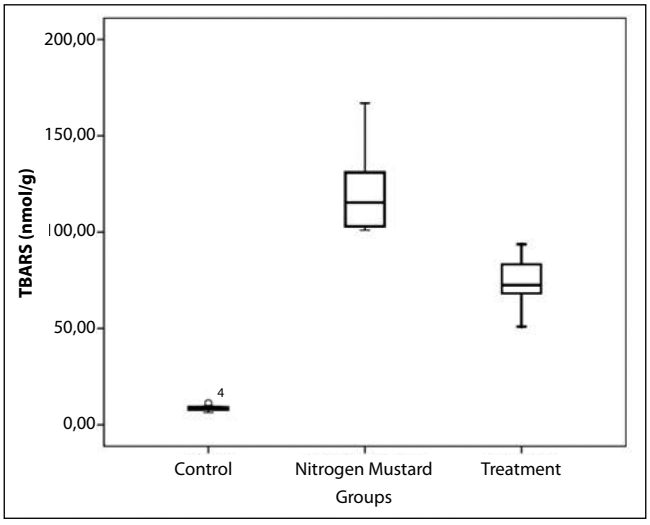

Figure 1: The distribution of TBARS values for the three groups.

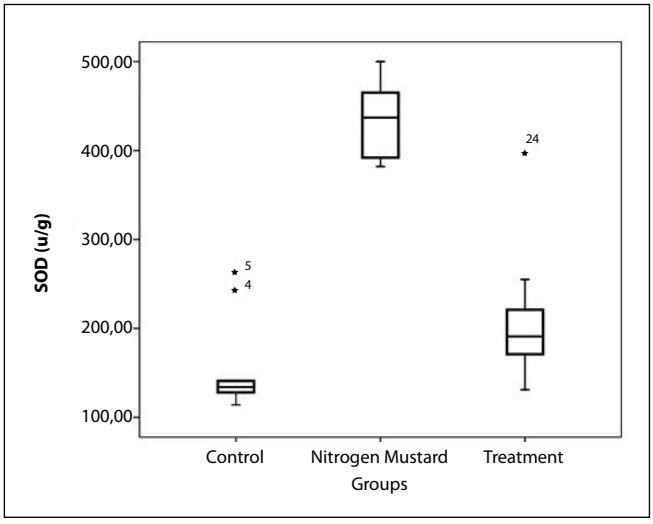

Figure 2: The distribution of SOD values for the three groups.

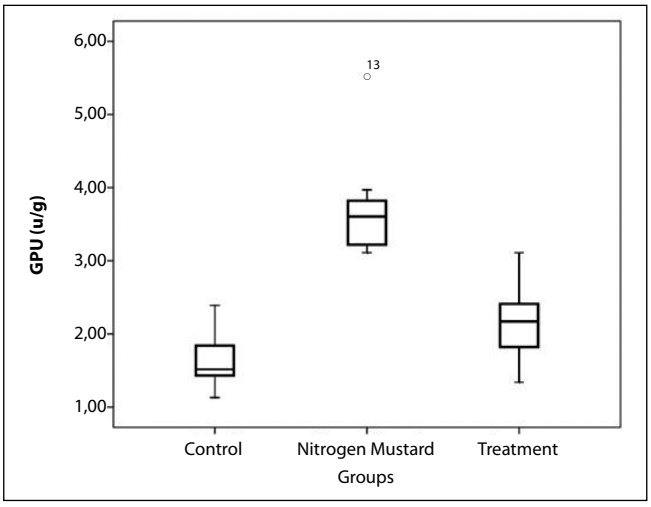

Figure 3: The distribution of GPO values for the three groups.

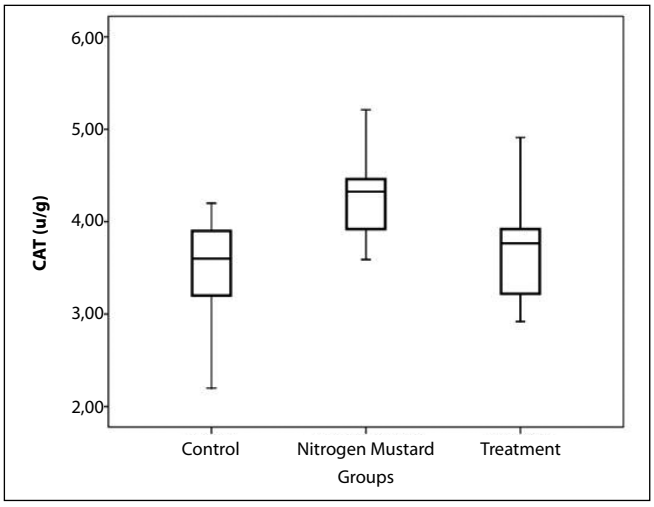

Figure 4: The distribution of CAT values for the three groups.

DISCUSSION

Nitrogen mustard (NM) is a chemical warfare agent that targets the eyes, skin, and lung. However, because of its hydrophobic characteristics, NM easily penetrates and accumulates in the lipid component of the exposed organism. Despite many years of research into NM, the toxicity induced by this agent is still not fully understood besides the alkylation of DNA. It was previously claimed by some studies (5,7,14) that the oxidative stress was likely involved in the pathogenesis of NM toxicity. Polyunsaturated fatty acids (PUFA) are very susceptible to oxidative stress and this process results in lipid peroxidation. The substantial PUFA content of the brain and its high oxygen consumption support the possible role of oxidative stress and lipid peroxidation in NM toxicity (12). In view of these considerations, we postulated that NM can affect brain tissue and studied the enzymatic antioxidant defense parameters such as SOD, GR, GPO, and CAT and the MDA, the end-product of lipid peroxidation. Additionally, we determined the antioxidant role of PC as a free radical scavenger in NM toxicity.

MDA, or more exactly thiobarbituric acid–reactive substances (TBARS), is the end-product of the lipid peroxidation of fatty acids containing two or more double bonds. Generally, TBARS measurement is a good index of lipid peroxidation. Sharma et al (20) have shown that liver glutathione, an important antioxidant, is depleted and liver MDA increased when NM is administered to animals. A previous study showed that intracellular peroxide levels were increased in human bronchial epithelial cells due to NM treatment (15). We also found that brain TBARS levels were increased in NM-administered rats. More importantly, PC treatment significantly decreased TBARS levels in NM-administered rats. These results indicate that lipid peroxidation is increased due to NM effects and free radical scavenger PC attenuates lipid peroxidation in the rat brain. As a result, lipid peroxidation in brain tissue appears to be a side effect of NM toxicity.

Reactive oxygen species (ROS) are constantly formed in the animal body and removed by an antioxidant defense system. In healthy animals, the generation of ROS appears to be approximately in balance with antioxidant defenses. An imbalance between ROS and antioxidant defenses in favor of the ROS has been described as oxidative stress. A substantial part of intracellular antioxidant defense is based on the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. These enzymes eliminate toxic reduction intermediates of oxygen inside the cell, prevent radical formation and so restrict oxygen toxicity. Therefore, the protection of cells from damage by ROS may be evaluated on the basis of tissue activities of these antioxidant enzymes. SOD, one of the most prominent intracellular antioxidant enzymes, is present in all aerobic cells and has an antitoxic effect against superoxide anion. It removes superoxide anion catalytically by promoting the dismutation of superoxide to hydrogen peroxide and molecular oxygen. Hydrogen peroxide, a reaction product of the superoxide dismutation reaction, inactivates SOD, and in the presence of hydrogen peroxide SOD acts as a prooxidant. Hydrogen peroxide should therefore be scavenged by other antioxidant mechanisms. On the other hand, oxidative stress induces a protective compensatory increase in the synthesis of antioxidants (3) and tissues may respond to oxidant stress with an enzyme induction. We found a significantly increase in SOD activities against NM administration in this study. We believe this increase results from an enzyme induction response to oxidative stress due to NM administration. The decreased SOD activity after PC treatment confirms this postulate. Hydrogen peroxide is also a reactive oxygen species and can be scavenged by different antioxidant mechanisms. The most important enzymatic scavenging mechanisms are based on catalase and glutathione peroxidase activities. Catalase removes hydrogen peroxide when in high concentrations while GPO removes hydrogen peroxide when present in low concentrations (6). The activities of both enzymes were increased in NM-administered rats and we think this increase was the result of enzyme induction. Although catalase induction was weaker than those of other antioxidant enzymes, it was statistically significant and this could result in increased oxidation stress. PC treatment also decreased catalase activity and this decrease was also statistically significant. In contrast to our findings, Ucar et al (11) recently reported depressed GPO activity after NM administration in the lung tissue. There is no study reporting oxidative effects of NM in brain tissue in the literature. We believe our results show that brain tissue has different antioxidant potential from lung.

Glutathione reductase (GR) is another cellular antioxidant enzyme and cooperates with GPO. GPO can remove hydrogen peroxide and organic hydroperoxides by conversion of reduced glutathione (GSH) to the oxidized form (GSSG) and GR substitutes the GSSG for the reaction of GPO. GR levels were also increased after NM administration. The GR increase completes the antioxidant efficiency of the GPO increase, and this may be a result of the induction of antioxidant enzymes. Another consequence of oxidative stress may be a change in nitric oxide (NO) metabolism. Elevated levels of reactive oxygen metabolites, especially superoxide anion, deplete bioavailable NO and exacerbate local oxidative stress by reacting with NO to form peroxynitrite (8,10,23). Peroxynitrite, a reactive nitrogen metabolite, can readily cross cell membranes, so traveling significant distances and damaging tissues. The increased TBARS levels in our study show increased oxidative/nitrosative stress due to NM administration. The NM damage in the brain may therefore arise partly in relation to peroxynitrite. Antioxidant enzyme expression can be induced by antioxidant response element activation under oxidative stress conditions (3). The antioxidant response element is a regulatory enhancer found in the 5′-flanking region of many detoxification enzymes. SOD and enzymes that play a role in glutathione metabolism are among these detoxification enzymes (9,22). The upregulation of antioxidant response element–dependent target genes is known to have neuroprotective effect. However, the mechanism of activation is currently unknown (3). We believe that antioxidant enzymes in the brain could be upregulated for antioxidant defense against oxidative stres under oxidative stress conditions caused by NM.

One limitations of the study is that we aimed to investigate the potential the enzymatic antioxidant defense system against oxidative stress in the brain caused by nitrogen mustard. However, investigating thiol groups and GSH and GSSG levels could be valuable particularly for NM. GSH and GSSG are substrates of GPO and GR, respectively, and it has been reported that GSH is depleted by NM (16,21).

In conclusion NM administration increases oxidative stress and lipid peroxidation in the rat brain. Significantly increased TBARS levels reveal an accelerated lipid peroxidation process. The increase of antioxidant enzyme activities after NM administration shows the high antioxidant potential of brain tissue probably based on induction of enzyme activities by antioxidant response elements. PC administration can alleviate oxidative stress and the lipid peroxidation process in animals exposed to NM. Effects of PC may ensure a sustained outcome in limiting oxidative stres on lipid peroxidation. Therefore PC can be used in clinical setting in the future.

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