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Influence of proanthocyanidin on blunt traumatized lungs after nitrogen mustard exposure (real terror attack simulation)

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SUMMARY

In our study, the deconstructive effect of nitrogen mustard on the lung exposed to blunt thorax trauma and the role of proanthocyanidine in preventing this scene were investigated. The rats were separated into four groups by the simple random sampling method and each group contained 15 rats. In all the groups cases were followed up for three days, and sacrificed after the follow up period. Samplings from the tissue were carried out for histopathological and biochemical parameter measurements [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and malondialdehyde (MDA)], and recorded. In the control group nothing was administered. In the second (proanthocyanidine) group proanthocyanidine was administered to the subjects during the follow up period. In the third group nitrogen mustard following blunt thorax trauma application before the 3-days follow up period was administered. In the fourth treatment group nitrogen mustard following blunt thorax trauma application before the 3-days follow up period was performed with proanthocyanidine administration 8 hours before the blunt thorax trauma and nitrogen mustard application. Proanthocyanidine treatment continued during the 3 days of follow up. Alveolar capillary damage, elevated leukocyte infiltration at alveolar area and fibrosis were found in the histologic investigation of the third group. Histological findings of the proanthocyanidine and treatment groups were similar to those of the control group ($p>0.05$). Exposure to nitrogen mustard gas caused an elevation in MDA levels and decrease in the GPx and SOD activities ($p<0.05$). MDA levels, GPx and SOD activities were the same in the lung tissues of the proanthocyanidine and control groups ($p>0.05$). In the treatment group, decrease in the MDA levels and increase in the CAT and GPx activities were observed ($p<0.05$). Nitrogen mustard administered with blunt thorax trauma caused oxidative stress and tissue damage, and proanthocyanidine improved the scene in this study.

Key words: *Blunt thorax trauma, chemical warfare agent, nitrogen mustard, proanthocyanidin, pulmonary contusion*

ÖZET

Proantosiyanidinin nitrojen mustarda maruz bırakılmış künt travma oluşturulmuş akciğerlerdeki etkisi (Gerçek terör saldırı simülasyonu)

Çalışmamızda nitrojen mustardın künt toraks travmasına maruz kalmış akciğerde yıkıcı etkisi ve proantosiyanidinin bu tabloyu önlemedeki rolü araştırıldı. Ratlar basit randomizasyon yöntemiyle her biri 15 rat içeren 4 gruba ayrıldı. Tüm gruplarda denekler üç gün takip edildi ve takip süresinden sonra sakrifiye edildi. Dokudan histopatolojik ve biyokimyasal parametre ölçümleri için [süperoksid dismutaz (SOD), glutatyon peroksidaz (GPx), katalaz (CAT), malondialdehid (MDA)] örnekleme yapıldı ve kaydedildi. Kontrol grubunda hiçbir tedavi uygulanmadı. İkinci (proantosiyanidin) grubunda deneklere üç günlük takip boyunca proantosiyanidin verildi. Üçüncü grupta denekler üç günlük takip süresinin öncesinde künt toraks travması ve ardından nitrojen mustarda maruz bırakıldı. Dördüncü tedavi grubunda deneklere künt toraks travması ve nitrojen mustard uygulanmadan 8 saat önce proantosiyanidin tedavisine başlandı. Üç günlük takip süresince proantosiyanidin tedavisine devam edildi. Üçüncü grubun histolojik incelemesinde alveolar kapiller hasar, alveolar alanda artmış lökosit infiltrasyonu ve fibrozis saptandı. Proantosiyanidin ve tedavi gruplarının histolojik bulguları kontrol grubuna benzerdi ($p>0.05$). Çalışmamızda nitrojen mustarda maruziyet akciğer dokusunda MDA düzeylerinde artmaya, GPx ve SOD aktivitelerinde azalmaya sebep oldu ($p<0.05$). MDA düzeyi, GPx ve SOD aktiviteleri proantosiyanidin akciğer dokusu ile kontrol grubunda aynıydı ($p>0.05$). Tedavi grubunda MDA düzeylerinde azalma, CAT ve GPx aktivitelerinde ise artma gözlendi ($p<0.05$). Çalışmamızda künt toraks travmasıyla birlikte akciğere uygulanan nitrojen mustard, oksidatif strese ve doku harabiyetine yol açmıştır ve proantosiyanidin tedavisi bu tabloyu azaltmada etkili olmuştur.

Anahtar kelimeler: *Künt toraks travması, kimyasal savaş ajanı, nitrojen mustard, proantosiyanidin, pulmoner kontüzyon*

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Introduction

One of the most important chemical warfare agents is nitrogen mustard (NM) (1,2). It is also known as NM, and it is the most widely used agent in chemical weapons (3,4). Sulfur mustard (SM) has posed a military threat and this is still considered as a major threat agent for mankind (5,6). NM is a structural analogue of SM (1). Many agreements were spurred after the Second World War for the prohibition of the manufacture and use of NM. These are the 1993 Chemical Weapons Convention (CWC) and chemical warfare agent (CWA) destruction programs (7). Currently, instead of studying CWA many investigators have preferred dealing with their analogs due to the following reasons: 1- the toxicity of some analogs has been recognized to be similar or even greater than that of the parent CWA, and 2- the increased concern that CWA could be used by terrorists against civilians (8,9). In addition, chemical agents are used to amplify the injurious power of the destroying weapons. As chemical weapon-mediated terrorist attacks are common nowadays, experimental studies should be carried out for CWA-s.

The most destructive effects of mustards occur on the respiratory system, eyes and skin (5,7,10). Because of their significant effects on respiratory system, mustards are extremely relevant to trauma-dealing medical staff too. NM exposure and blunt thorax trauma (BTT) also cause inflammatory lung diseases, including acute respiratory distress syndrome (7,10). Excess production of free radicals, nitric oxide and superoxide is closely related to cell and tissue pathology caused by mustard (3). There is neither an effective treatment to toxic effects of mustards, nor a therapeutic antidote to them (5,6).

Proanthocyanidine (PC) is a free radical scavenger, and at the same time it has anti-inflammatory effects (11,12). The objective of the present study was to investigate the role of oxidative stress status in BTT

coexisting with mustard toxicity, and to determine the protective effect of PC. Up to date there are several studies related to NM (1,3), but this is the first study that reveals a developed war model in which both trauma and chemical weapons are applied at the same time, as in the case of real terror attacks.

Material and Methods

The study was performed in Gülhane Military Medical Academy Animal Research Laboratory, and it was approved by the Ethics Committee of Gülhane Military Medical Academy.

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).

Animals: Sixty adult Ratus Norvecus rats, weighing 160±10 grams were used. The rats were separated into four groups by the simple random sampling method and each group contained fifteen rats.

Experimental design: The first group was the control group (CG): Vaporized 5 ml distilled water was applied for 10 minutes and the rats were not exposed to trauma and NM. Subjects were sacrificed by anesthetic with lethal dose after a 3-days follow up period. Two samples were taken from the lungs, one of which was fixed in 2.5% buffered glutaraldehyde for histopathological examination and the other was kept in liquid nitrogen for biochemical analysis. MDA level, activities of superoxide dismutase (SOD), glutathion peroxidase (GPx) and catalase (CAT) were measured with biochemical analyses in the lung tissues of sacrificed subjects. With the method we mentioned briefly in the following part of the paper, histopathological and biochemical investigation were performed at the tissue samples, and results have been recorded.

Histological examination: The lung tissue was removed, sectioned into small pieces and fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.2 at + 4 °C for 2-4 h and post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7). Later the materials were dehydrated in serially increasing concentrations of alcohol. The tissues were then washed with propylene oxide and embedded in Araldite 6005 (Ciba-Geigy, Summit, NJ, USA). Semi-thin sections of 0.8 µm were cut with a glass knife on an ultramicrotome Leica Ultracut R (Leica, Solms, Germany) stained with toluidin blue azur II and then examined under a Zeiss Axioscope photomicroscope (Thornwood, NY, USA). Ultrathin sections of 60 nm were cut with a glass knife on a Leica Ultracut R ultramicrotome, stained with uranyl acetate and lead citrate and examined on a LEO 906 E (LEO Elektronenmikroskopie, Oberkochen, Germany) transmission electron microscope.

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 glass homogenizer. Then homogenized samples were centrifuged for 10 min at 5000xg and 4°C. Supernatant was used for the analysis.

GPx activity measurement: GPx activities in tissue homogenates were measured by the method described in our previous studies (13,14). 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 for 5 min at 37 °C. Then the reaction was 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.

MDA level measurement: MDA levels in tissue homogenate samples were determined in accordance with the method described in our previous studies (13,14). MDA levels were expressed as TBARS. After the reaction of thiobarbituric acid with MDA, the reaction product was measured spectrophotometrically. Tetramethoxy propane solution was used as standard.

SOD activity measurement: CuZn-SOD activity in tissue homogenate was measured by the method described in our previous studies (13,14). 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 sample were used as blank or standard determinations. CuZn-SOD activity was expressed in U/g tissue.

CAT activity measurement: CAT activity in tissue homogenate was measured by the method of Aebi (13,14). 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 for 30 seconds at room temperature. Catalase activity was expressed in U/g tissue.

The second group was the PC group (PCG): The same protocol (three days follow up, vaporized distilled water application, sacrification, histopathological and biochemical sampling, histopathological and biochemical evaluation and recording) was applied with the control group. Differently from the CG, PC administration (100 mg/kg body weight/oral/via Gavage) has been started 8 hours before the three days of follow up period onset. PC treatment continued until the subjects were sacrificed.

The third group was the traumatized NM group (TMG): The same protocol (three days follow up, vaporized distilled water application, sacrification, histopathological and biochemical sampling, histopathological and biochemical evaluation and recording) was applied with the control group. Differently from the CG, 1- BTT was applied before the three days of follow up period, 2- Traumatized subjects were exposed to NM via vaporized distilled water. BTT and NM were administrated in the ways we described in our previous studies (13,15).

Blunt thorax trauma application: The BTT administration model that we developed before (GATA trauma model) was administered to the subjects in order to form a BTT. The BTT forming model is briefly is that: Rats were anesthetized with intraperitoneal Ketamine hydrocloride 90 mg/kg and Xylazine. After the anesthesia, rats were placed in right lateral decubitis position over the GATA trauma model's support part (Figure 1). Forty grams metal weight (0.004 joule) was used to form trauma on the subjects. Rats were placed in lateral decubitis position over the trauma model's support part. Metal weights were dropped from 1 m height onto rats through the plastic pipe for gaining BTT. BTT was applied at the right lateral axial axe, on the fourth intercostal space.

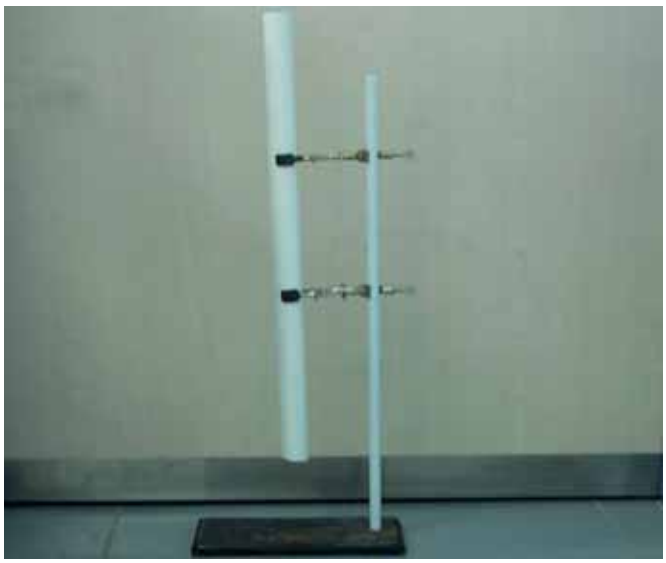

Figure 1. Blunt thorax trauma model (GATA trauma model). Metal weights (50 g) were dropped from 1 m height onto rats through the plastic pipe for gaining blunt thorax trauma

NM exposure: The subjects were exposed to NM via the method we described in our previous studies. In brief: after BTT application, the rats were placed in the chamber. Rats were directly exposed to toxic dose of vaporized 8 mg NM dissolved in 5 ml distilled water for 10 minutes, 800 mg/m^3/min. All exposures were performed in a 100 L volume chamber equipped with chemical, biological, radiological and nuclear filters.

Fourth (treatment) group (TG): The same protocol (three days follow up, BTT, NM exposure, sacrification, histopathological and biochemical sampling, histopathological and biochemical evaluation and recording) was applied with the TMG. Differently from the TMG, PC administration (100 mg/kg body weight/oral/via gavage) has been started 8 hours before BTT administration and NM exposure. PC treatment continued until the subjects were sacrificed.

Statistical analysis: Statistical analysis was performed by using Kruskal-Wallis and Bonferroni-corrected Mann-Whitney U tests. All the results were assessed as the mean with min and max and $p < 0.05$ was accepted as statistically significant.

Results

Results of histological examination: In CG, typical normal structural findings were seen in both light and electron microscopic observations. The alveolar wall consisted of surface epithelium, supporting tissue and blood vessels. Most of the alveolar surface area was covered by large squamous type I pneumocytes which was seen to have densely stained nuclei in histological section. Type II pneumocytes had large round nuclei with a prominent nucleolus and vacuolated cytoplasm. It was seen that capillary blood vessels had formed an extensive plexus around each alveolus. Alveolar macrophages could be found on the surface of alveolar lining cells as well as in the supporting tissue of the alveolar septa. Light micrographs and electronmicrographs of TMG capillary dilatation and eythrocyte plugging in capillaries were observed. The most striking change to be noticed was numerous numbers of alveolar macrophages. Light micrographs of the PCG were normal in appearance, similar to the CG findings. Furhermore, light micrographs of the TG were normal in appearance, similar to the CG findings too. The abnormal appearance of the lung tissue in NM were mostly but not completely corrected by PC. These findings can im-

ply that the structural changes induced by NM could be partially prevented and restored by PC treatment. Representative histopathological pictures of the study groups are demonstrated in Figures 2 and 3.

MDA levels, and GPX, SOD and CAT activities in tissue: Oxidative stress status analysis included MDA level, and SOD, CAT and GPx activities. MDA levels, CAT and GPx activities in PCG were similar to CG. NM direct exposure caused increased MDA levels, and decreased GPx and SOD activity significantly in lung tissue. PC treatment decreased MDA levels, but CAT and GPx activities were similar to those of TMG group. The levels of histochemical parameters according to the study groups are presented in Table 1.

Discussion

NM is the most widely used chemical agent in war and terror attacks (1,16). It affects many organs such as respiratory tract, eyes, skin, gastrointestinal and central nervous systems (1,3). Because of their significant effects on respiratory system, mustards are extremely relevant to trauma-dealing medical staff too. In addition, mustards are a mutagenic, carcinogenic and cytotoxic agent (17,18). It has been shown that mustard toxicity comes from oxidative as well as nitrosative stress leading to lipid, protein and DNA damage in the target cell (1). Yaren et al. have reported that peroxynitrite may be responsible, at least in part for NM-induced lung toxicity, and peroxynitrite scavengers may be useful in order to prevent mustard toxicity (1). Excess production of free radicals, nitric oxide and superoxide is closely related to cell and tissue pathology of mustard (3). NM exposure or BTT also causes inflammatory lung diseases, including acute respiratory distress syndrome (7,10).

Pulmonary contusion, parenchymal lung injury, hemothorax and pneumothorax are the most common pulmonary injuries after BTT (19). Good surgical

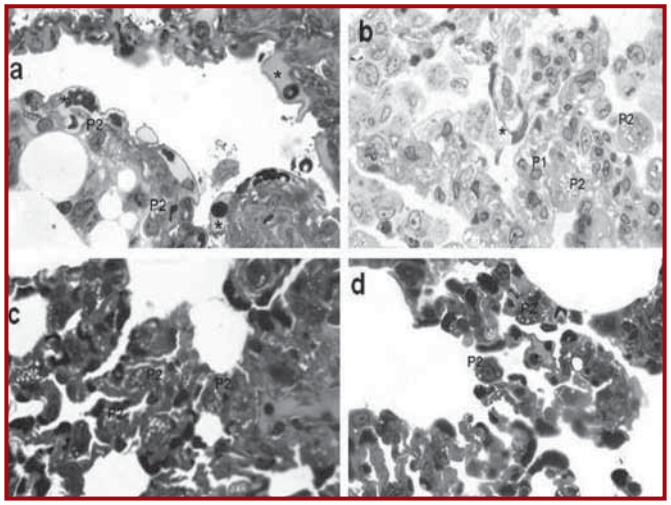

Figure 2. a. Control group: Toluidin blue-Azur IIx250, b. Proanthocyanidin group: Toluidin blue-Azur IIx250, c. Traumatized mustard group: Toluidin blue-Azur IIx250, d. Treatment group: Toluidin blue-Azur IIx250. *: capillary lumen, P1: type 1 pneumocyte, P2: type 2 pneumocyte, arrowheads: alveolar macrophages

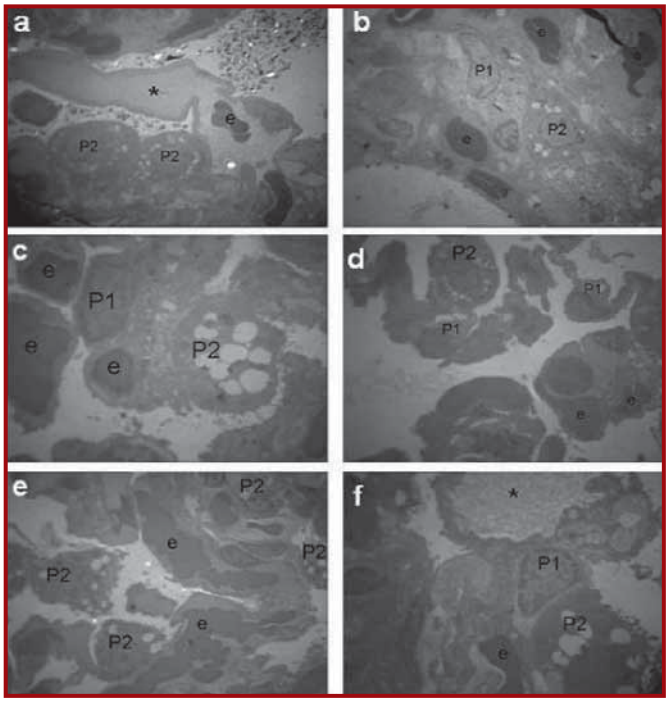

Figure 3. a. Control group x1000, b. Proanthocyanidin group x1000, c. Traumatized mustard group: x2156, d. Treatment group: Gx1000, e. Treatment group: x1000, f. Treatment group: x2156. For electron microscopic examination all of the figures are stained with uranil acetat-lead citrat. *: capillary lumen, P1: type 1 pneumocyte, P2: type 2 pneumocyte, arrowheads: alveolar macrophages, e: erythrocyte

Table I. Oxidative stress related parameters of the lung tissue after blunt thoracic trauma and mustard exposure and proanthocyanidin treatment in rats

Groups	(n)	Malondialdehyde (nmol/g)*	Glutathione peroxidase (U/g)*	Catalase (U/g)*	Superoxide dismutase (U/g)*
Control group	15	6.87 (6.71/8.01)	45.07 (43.58/48.19)	3.16 (3.02/3.24)	240.20 (224.28/250.89)
Proanthocyanidin group	15	6.77 (6.69/6.82)	43.17 (42.95/44.92)	3.36 (3.28/3.45)	247.24 (235.81/258.14)
Nitrogen mustard group	15	9.37 (9.24/9.48)#	28.85 (27.01/29.98) #	3.26 (3.16/3.75)	92.03 (85.02/94.44)#
Treatment group	15	7.07 (7.01/7.22)Φ	28.79 (26.51/30.14)	3.18 (3.01/3.22)	113.88(107.08/125.88)

*: Values are given as mean (min/max)
#: p<0.001, Nitrogen mustard group compared with control group
Φ: p<0.05, Treatment group compared with nitrogen mustard group

outcomes are reported about management of these pulmonary injuries, and in general, vascular repair, pneumoraphy and chest tube thoracostomy are sufficient treating procedures for them (19,20).

In contrast, pulmonary contusion is a challenge for a thoracic surgeon and intensive care specialist. Pulmonary contusion represents a spectrum of lung injury characterized by the development of parenchymal infiltrates and various degrees of respiratory dysfunction. There is a spectrum of injury severity, ranging from localized consolidation with little clinical impact, to acute lung injury and ARDS (20-22). Currently, it is believed that pulmonary contusion is the most common potentially life-threatening pulmonary injury (20,22).

As we demonstrated, NM and BTT have individually important hazardous effects on lungs. It is easy to predict that patients with blunt traumatized lungs after NM exposure will have increased mortality and morbidity rates. There are numerous experimental studies about BTT and NM (1,3,23-26). However, to our knowledge there is no experimental study concerning BTT and chemical weapons effects together. In this study we simulated the scenario of a terror attack to patients with blunt traumatized lungs after chemical gas exposure.

There is still no beneficial treatment or therapeutic antidote available to toxic effects of mustards (5,27). In addition, pulmonary contusion treatment is generally supportive (20). There are ongoing researches into such agents that may have antioxidant properties (28,29). It is essential that further thorough investigation is done in this area.

It has been revealed that PC is a free radical scavenger and it has also anti-thrombotic and anti-inflammatory effects (11,12,30,31). It has been shown that, in addition to its antioxidative property, it enhances low-level production of intracellular NO in primary rat astroglial cultures. Moreover, PC pretreatment protects the microglial GSH pool during high output NO production and results in an elevation of the H_2O_2 tolerance in astroglial cells (17). It has been stated that IH636 GSPE provides superior antioxidant efficacy as compared to Vitamins C, E and β-carotene (13). It is clear that novel antioxidants have sufficient effects against free radicals and cardiovascular disease to provide organism. (13,31). In particular, novel antioxidants can neutralize harmful free radicals and their damaging effects on tissue and organs as well as increasing the body's antioxidant status (13). PC is a combination of biologically active polyphenolic flavonoids including oligomeric PCs. Their biological, pharmacological, therapeutic, and chemoprotective properties against oxidative stress and oxygen free radicals have already been demonstrated (13,31). PC is a potent bioavailable free radical scavenger that provides significant protection over multiple target organs against structurally diverse drug and chemically induced toxic manifestations in rats (13). The mechanistic pathways performed by PC to provide cardioprotectin includes: (a) potent hydroxyl and other free radical scavenging abilities, (b) anti-apoptotic, anti-necrotic and anti-endonucleolytic potentials, (c) modulatory effect on apoptotic regulatory bcl-XL, p53 and c-myc genes, (d) cytochrome P450 2E1 inhibitory activity, (e) inhibitory effects on adhesion molecules, (f) modulatory effects on proapoptotic and cardioregulatory genes c-JUN, JNK-1, and CD36 (13,30,32,33).

The histological examination and oxidative stress status related parameters of our study showed similar findings in PCG and CG. These findings support that PC has no unpredicted side effects. Furthermore, due to its obvious antioxidant effect, PC can be an efficient protector against blunt traumatized lungs after chemical gas exposure. PC decreased MDA levels when compared to non-PC given group. However GPX and CAT activities were not significantly different between PCG and TMG groups. In addition, increased CAT activities were reported in PCG compared with TMG group ($p<0.05$). This demonstrated that free radicals were scavenged by PC but SOD, CAT and GPx activities were still lower than CG. It can be concluded that free radicals were not removed completely.

In this study light micrographs and electronmicrograps of TMG capillary dilatation and eythrocyte plugging in capillaries were observed. The most striking change was numerous numbers of alveolar macrophages. PC treatment decreased histopathological changes in TG. Light micrographs of the TG were normal in appearance, similar to the CG findings. The abnormal appearance of the lung tissue in TMG were not completely but mostly corrected by PC. It was shown that free oxygen radial damage has an important role in mustard toxicity.

These findings can imply that the structural changes induced by blunt traumatized lungs after NM exposure can be partially prevented and restored by PC treatment. PC is a strong free oxygen radial scavenger and because of this property it can be an option for decreasing toxic patients' mortalities and morbidities. Further clinic and experimental studies are needed to prove PC and other antioxidant agent's probable protective roles against mustard toxicity.

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