

# The Effect of Nitrogen Mustard on the Rat Brain and the Therapeutic Value of Proanthocyanidin

## Nitrogen Mustard'ın Rat Beyni Üzerine Etkisi ve Proanthocyanidin'in Tedavi Değeri

### 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 the electron microscope to investigate the effect of NM gas administered through the dermal and respiratory routes to rats on the brain cortex and also tried to show whether the antioxidant Proanthocyanidin (PC) could decrease this effect.

**MATERIAL and METHODS:** A total of 32 rats were randomized into four groups: Group I: Control group, Group II: PC group, Group III: NM group, Group IV: NM + PC group. The rats were sacrificed 3 days after NM gas exposure. A segment of the cortical tissue was prepared for electron microscopy.

**RESULTS:** We used the electron microscope for cellular analysis of NM on cortical neural cells. These investigations revealed degeneration of the cortical neural cell nuclei together with oedema and axonal degeneration in the subcortical neural tissue. The group receiving antioxidants was found to have less oedema and degeneration.

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

**KEYWORDS:** Nitrogen Mustard, Proanthocyanidin, Rat brain

### ÖZ

**AMAÇ:** Nitrogen Mustard (NM) dokuya penetre olduktan sonra hücresel nükleer DNA'ya hasar veren alkilleyici bir ajandır. Bu sitostatik, mutagenik ve sitotoksik etkilere neden olur. Cilt ve respiratuar yoldan uygulanan NM gazının beyin korteksine olan etkisini araştırmak ve aynı zamanda antioksidan Proanthocyanidin'in (PC) bu etkiyi azaltıp azaltamayabileceğini göstermek için elektron mikroskopu kullandık.

**YÖNTEM ve GEREÇ:** Toplam 32 rat dört gruba randomize edildi: Grup I: Kontrol grubu, Grup II: PC grubu, Grup III: NM grubu, Grup IV: NM+PC grubu. Ratlar NM gazı uygulamasından 3 gün sonra sakrifiye edildiler. Elektron mikroskop için kortikal bir doku segmenti hazırlandı.

**BULGULAR:** NM'in kortikal nöral hücreler üzerindeki hücresel etkisi için electron mikroskop kullandık. Bu incelemeler, ödemle birlikte kortikal nöral hücre nükleuslarının degenerasyonunu ve subkortikal nöral dokudaki aksonal degenerasyonu ortaya koydu. Antioksidan alan grupta daha az ödem ve degenerasyon gözlemlendi.

**SONUÇ:** Bu bulgular mustard gazının indüklediği yapısal değişikliklerin proanthocyanidin tedavisi ile önlenbildiği ve onarılabildiğini ima etmektedir.

**ANAHTAR SÖZCÜKLER:** Nitrogen Mustard, Proanthocyanidin, Rat beyini

Ayhan TEKİNER<sup>1</sup>

Orhan YUCEL<sup>2</sup>

Ayşe KOSE SARGIN<sup>3</sup>

Onur GENÇ<sup>4</sup>

Belgin CAN<sup>5</sup>

Turhan KARAYILANOĞLU<sup>6</sup>

Jale KARAKAYA<sup>7</sup>

Mehmet Akif BAYAR<sup>8</sup>

<sup>1,8</sup> Ministry of Health, Training and Research Hospital, Department of Neurosurgery, Ankara, Turkey

<sup>2,4</sup> Gülhane Military Medical Academy, Department of Thoracic Surgery, Ankara, Turkey

<sup>3,5</sup> Ankara University School of Medicine, Department of Histology and Embryology, Ankara, Turkey

<sup>6</sup> Gülhane Military Medical Academy, Department of Nuclear, Biologic and Chemical Warfare, Ankara, Turkey

<sup>7</sup> Hacettepe University, School of Medicine, Department of Biostatistics, Ankara, Turkey

Received : 08.05.2009

Accepted : 31.08.2009

Correspondence address:

Ayhan TEKİNER

E-mail: tekinerayhan@hotmail.com

## INTRODUCTION

Nitrogen Mustard (NM) is a structural analog of sulfur mustard, a potent warfare agent that affects the skin, eyes, lungs and the neuromuscular, hematological, gastrointestinal, endocrine and immune systems (3,5,7,10). It was first used as a chemical warfare agent during World War I and caused severe casualties. It was banned when the Geneva Convention signed in 1925 banned the use of all chemical gases in wars. It has not been used to a marked degree in World War II. Recent uses were during the Iran-Iraq war during 1982-1987 and against the civilian population in Halpece by the Iraqi government. 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 (9). 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 (11). This results in cytostatic, mutagenic and cytotoxic effects.

The interaction between NM and cellular structures is through ethylene imonium. 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 (3). 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 (3). The poly (ADP-ribose) polymerase (PADRPR) enzyme is activated on exposure to mustard gas, thus decreasing NAD<sup>+</sup> levels. This causes cell death. A normal NAD<sup>+</sup> concentration ensures continuity of the energy-providing system and prevents blister formation (3). 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<sup>+</sup> as a substrate. Mustard also inhibits glycolysis and stimulates the NADP<sup>+</sup>-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 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 subject via the transdermal and inhalation routes under conditions simulating the battlefield in this study. The combined effect of firearms and chemical weapons is usually made use of in current terrorist attacks. We investigated the harmful effect of mustard gas on neural tissue using an electron microscope in this study and also evaluated the efficacy of Proanthocyanidin (PC) as we thought it might be beneficial in subjects exposed to mustard gas.

## MATERIAL and METHODS

### I. Material:

#### a- Animals:

This experimental study took place at the Gulhane Military Medical Academy (GATA) Animal Experiments Laboratory. The Gulhane Military Medical Academy Animal Care and Usage Committee approved the experimental protocol. A total of 32 male *Rattus Norvegicus* weighing 140-160 g were used. These rats were randomized into four groups, each with eight rats.

#### b- Chemicals:

All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany) and all organic solvents from Merck KGaA (Darmstadt, Germany). All reagents were of analytical grade, were prepared fresh each day (except the phosphate buffer) and stored in a refrigerator at +4 C. The reagents were equilibrated at room temperature for 0.5 h before use when the analysis was initiated or reagent containers were refilled. Phosphate buffers were stable at +4 C for 1 month. 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. 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. A microscope (Zeiss OpMi 99) and microsurgical instruments were used during the surgical procedures and decapitation. The electron microscopy investigation was performed at the laboratory of the Ankara University School of Medicine, Department of Histology and Embryology.

## II. Methods:

### Experimental design:

#### A- Groups:

These rats were randomized into four groups, each with eight rats as follows: Group I: Control group (no trauma and no NM). Group II: (PC group) The subjects were fed a diet including PC (100 mg/kg/day). Group III: (NM group) A toxic dose of vaporized 8 mg NM dissolved in 5 ml of distilled water was used for 10 minutes ( $800 \text{ mg/m}^3/\text{min}$ ) on the NM group that was exposed to NM only. Group IV: (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 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 İlaç Sanayii, İstanbul with Parke-Davis license) - 35 mg/kg and Rompun (Xylazine 2% solution, Bayer, İstanbul) - 1.5 mg/kg. The rats in Group III and Group IV were administered 0.5 mg/kg NM gas. The subjects were placed in the chamber (Figure 1). The chamber was heated using a tungsten electric bulb (100/220V) with an average temperature of  $22^\circ\text{C} \pm 2^\circ\text{C}$ . The subjects were directly exposed to vaporized NM. Rats in the PC Group were fed with PC beginning 8 h before the NM gas application and continuing 72 h afterwards. We assessed the rectal temperature, the number of breathing in one minute and the number of heart beats in one minute of the subjects 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

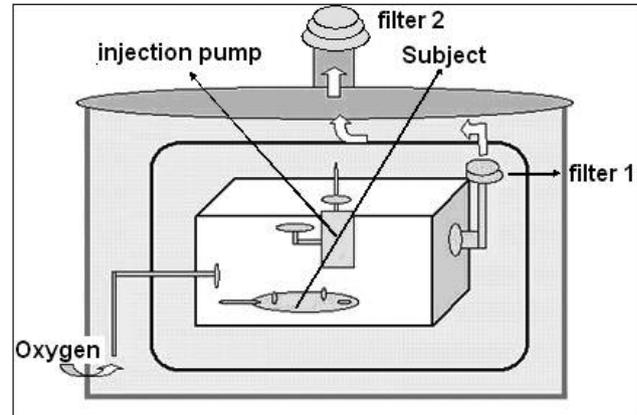


Figure 1: The chamber

the third day (with a lethal dose of Ketamin + Xylazine). Buterphenol (0.5 mg/kg sc) was administered to the subjects to provide analgesia during this process. The parietal brain cortex (4x4x4 mm.) of the sacrificed subjects was removed for histological studies (macroscopic and electron microscopic).

**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.

## III. Tissue preparation:

### Procedure for Histological Examination

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, sectioned into small pieces (4x4x4mm) and fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer, pH 7.2, at  $+4^\circ\text{C}$  for 2-4h and then post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7). The materials were later dehydrated in serially increasing concentrations of alcohol. The tissues

were then washed with propylene oxide and embedded in Araldite 6005 (Ciba-Geigy, Summit, N.J., USA). Semi-thin sections of 0.8 μm were cut with a glass knife on a Leica Ultracut R ultramicrotome (Leica, Solms, Germany), stained with toluidine blue azur II and then examined under a Zeiss Axioscope photomicroscope (Thornwood, N.Y., 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.

**IV. Statistical Analysis:**

The Fisher-Freeman-Halton exact test was used to compare categorical variables among groups. p values less than 0.05 was evaluated as statistically significant. Statistical analysis was performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL).

**RESULTS**

All slides obtained from all groups in this experimental study were studied with light and electron microscopy. These images obtaining from subjects of all groups were assessed for nucleus, mitochondria and perineural edema. The groups were compared in terms of pathological features. There were statistically significant differences between the groups (p<0.05). The comparison results are shown in (Table I). When NM group was compared with the control group and other groups according to nuclear degeneration, mitochondrial degeneration and edema, the NM group was found to different (p<0.05). Control, PC and NM+PC groups were found to be similar (p>0.05).

While the ratio of the nuclear and mitochondrial degenerations was 100 % in the NM group, the ratio fell to 12.5 % in the fourth group. While the ratio of edema was 87.5 % in the NM group, the ratio fell to 12.5 % in the fourth group. These results show the recovery.

In the control group (Group I), typical normal structural findings were seen in both light and electron microscopic observations. The neurons showed large euchromatic nuclei with prominent nucleoli and basophilic cytoplasm on photomicroscopy. Small blood vessels could be seen. The perikaryon of neurons was surrounded by a feltwork of axons and dendrites (Figure 2A). The neurons of the control group showed a fairly large nucleus with a distinct nucleolus on electron microscopy. Some dense chromatin margination could be seen in the nucleus. The cytoplasm around the nucleus contained parallel arrays of rough endoplasmic reticulum. Mitochondria were commonly observed. Many nerve fibers could be seen just outside the neuron body in the cross section. An electron micrograph of the cross section of brain cortex from the control group shows part of the cell body of a neuron including a portion of the nucleus (Figure 2B). Light and electron microscopic investigation showed that treatment of normal rats with PC (Group II) did not affect the structure of the brain significantly. There was no significant difference between the control and PC groups (Figures 2C,D). Photomicroscopy of the NM group (Group III) showed nerve cells with diverse shapes, many of which had a heterochromatic nucleus. Additionally; a perineuronal oedema was seen around the neurons (Figure 2E). Capillary dilatation and erythrocyte plugging in capillaries were observed. The feltwork of axons and dendrites surrounding the perikaryons was severely damaged (Figure 2E). The ultra-structural investigation of brain preparations correlated with the photomicroscopic findings (Figures 2F,G,H). The most striking change was severe disruption of the cytoplasmic matrix. Damage to cellular organelles and particularly severe damage to mitochondria were observed throughout the cortex. Mitochondria seemed to be variable in shape and size in the neurons of the NM group. Most of them were

**Table I:** Comparison of the groups in terms of nucleus, mitochondrial and edema

	Control (n=8)		PC (n=8)		NM (n=8)		NM+PC (n=8)		p value
	f	%	f	%	f	%	f	%	
Nucleus	0	0.0	0	0.0	8	100	1	12.5	<0.05
Mitochondrial	0	0.0	0	0.0	8	100	1	12.5	<0.05
Edema	0	0.0	1	12.5	7	87.5	1	12.5	<0.05

swollen. There were varying degrees of disorientation of cristae and vacuolization of mitochondrial matrix in these sections. Loss in matrix density was probably due to the loss of matrix substance. Intracellular vacuoles were present in the cytoplasm of the neurons (Figure 2G). Condensation and marginalization of many small heterochromatin aggregates were observed in the nucleus of most neurons (Figure 2G). Additionally severe perivascular oedema was seen around the blood vessels in this group (Figure 2H). Light micrographs of the NM-induced and PC-treated groups (Group IV) demonstrated that the mesh of neuronal and glial processes was normal in appearance, similar to the control group findings. The abnormal appearance of the cortex tissue in the NM group was mostly but not fully corrected by PC (Figure 1). The ultra-structural investigation of brain preparations from the treated group by electron microscopy provided results that were similar to the control group (Figure 2J).

**DISCUSSION**

Mustard gas functions as a highly cytotoxic blistrogen both for humans and animals (12). General local mordant effects of NM have been reported in many articles but there is only a limited number of studies reporting systematic effects. The autopsy of an Iranian legion exposed to a vesicant agent attack in Munich in 1985 revealed molecular ions belonging to mustard at a concentration, in mg/kg of wet weight of tissue, of 10.7 in the brain, 1.9 in the CSF, 2.4 in liver tissue, 5.6 in the kidney, 1.5 in the spleen, 0.8 in the lung, 3.9 in the muscle, 15.1 in lipid, 8.4 in skin tissue, 118 in subcutaneous lipid tissue+skin and under limit in excreta (6). In light of the literature, we created an experimental model to determine the effect of NM on brain tissue at the cellular level. The choice of subject is very important for an experimental study. Many experimental animals such as dogs, rats and pigs have been used in other articles. Some factors to take into consideration regarding the chosen subject are whether its anatomical and physiological characteristics are as close to man as possible, whether it is possible to obtain a sufficient number of subjects to produce meaningful statistical results, whether it is easy to care for and apply anesthesia to the subjects and whether ethic committee consent can be obtained. After analysing these factors and the subjects used for similar studies in the literature,

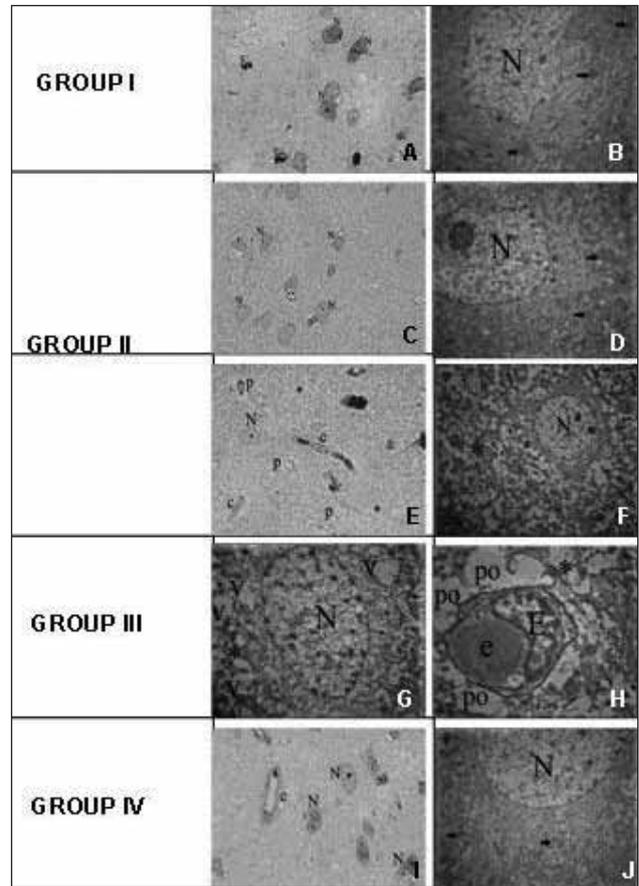


Figure 2: A,B: Group I; Figure C,D: Group II; Figure E,F,G,H: Group III; Figure I,J: Group IV  
 N: nucleus, c: capillary, \*: mesh of neuronal and glial processes, arrow: mitochondrion, P: perineuronal oedema, V: intracellular vacuole, po: perivascular oedema  
 Figure A, C, E, I, Toluidine blue-Azur II x250  
 Figure B, D, F, G, H, J, Uranyl Acetate-Lead citrate  
 Figure B, D, G, J x2784;  
 Figure F x1293; Figure H x3597

the rats of Sprague Dawley species of Rattus Norvegicus were selected for our study as they can be obtained and cared for easily, they breed quickly, a sufficient number of subjects can be used for statistical studies and they are commonly preferred for such experimental studies (7). The next step of our study was applying mustard gas, which showed some important differences when compared to the literature. In this experimental study, considering the recovery time and in the light of a literature about this subject (14) the subjects were sacrificed 72 h later. A literature search revealed mostly experimental study models where mustard gas had

been applied via the intratracheal route. Yaren et al administered the liquid form of mustard gas to the subjects through the intratracheal route in their 2006 study (14). Shandon et al also administered mustard gas through the intratracheal route (8). The subjects were directly exposed to vaporized NM during our experimental study simulating a real battlefield. In 1947, Kinsey and Grant determined that mustard creates a conjugate with glutathione while Davison et al in 1961 reported that mustard creates a conjugate with glutathione and is detoxified in the mercapturic acid pathway to be excreted as a glutathione metabolite. The biochemical mechanism behind this destructive effect has not been well understood. It has been suggested that the activation of proteolytic enzymes contributes to this process. We added the anti-oxidant PC in this study. The anti-oxidant proanthocyanidin can be extracted from grape pip extract, huckleberry, cola hazelnut and some other vegetables and fruits. PC especially protects the cell membrane from damage due to free radicals (4). The aim was to investigate the effect of antioxidants in treatment by adding PC to the diet of the subjects. In the experimental study by Yaren et al, the effects of NM administered to lung tissue only were investigated histopathologically (14). We investigated the histopathological effects of NM on neural tissue with the light microscope and electron microscope. When the results were assessed, it is obvious that NM causes degeneration in the neural nuclei and mitochondria and perineural edema and PC can be prevent this harmful effect.

Photomicroscopy of the NM group (Group III) showed nerve cells with diverse shapes, many of which had a large nucleus. Capillary dilatation and erythrocyte plugging in capillaries were observed. The feltwork of axons and dendrites surrounding the perikaryons was severely damaged (Figure 2E). The ultra-structural investigation of brain preparations correlated with the photomicroscopic findings (Figures 2F,G,H). The most striking change was severe disruption of the cytoplasmic matrix. Damage to cellular organelles and particularly severe damage to mitochondria were observed throughout the cortex. Mitochondria seemed to be variable in shape and size in the neurons of the NM group. Most of them were swollen. There were varying degrees of disorientation of cristae and vacuolization of mitochondrial matrix in these sections. Loss in matrix density was probably due to

the loss of matrix substance. Condensation and marginalization of many small heterochromatin aggregates and formation of apoptotic bodies were observed in the nucleus of most neurons (Figure 2G). Light micrographs of the NM and PC brain group (Group IV) demonstrated that the mesh of neuronal and glial processes were normal in appearance, similar to the control group findings. The abnormal appearance of the cortex tissue in the NM group was mostly but not fully corrected by PC (Figure 1). The ultrastructural investigation of brain preparations from the treated group by electron microscopy provided results that were similar to the control group (Figure 2J). These findings imply that the structural changes induced by NM can be prevented and restored to normal by PC treatment. On the other hand, electron microscopy showed that treatment of normal rats with PC did not affect the structure of the brain significantly.

## CONCLUSION

These findings may imply that structural changes induced by mustard gas can be prevented and restored by proanthocyanidin treatment although proanthocyanidin does not affect brain structure significantly in normal rats.

## 6. Acknowledgments:

The authors thank Yucel Kanpolat and Mustafa Sargon for their assistance in preparation of this manuscript, and the staff of the Gulhane Military Medical Academy Animal Experiments Laboratory for assistance in the irradiation of rats.

## REFERENCES

1. Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG: Free radicals and grape seed PC extract: importance in human health and disease prevention. *Toxicology* 148:187-197, 2000
2. Balali-Mood M, Hefazi M: Comparison of early and late toxic effects of sulfur mustard in Iranian veterans. *Basic Clin Pharmacol Toxicol* 99(4):273-282, 2006
3. Dacre JC, Goldman M: Toxicology and pharmacology of the chemical warfare agent sulfur mustard. *Pharmacological Reviews*: 289-326, 1996
4. Debasis B, Manashi B, Sidney JS, Dipak KD, Sidhartha DR, Charles AK, Shantaram SJ, Harry G: Free radicals and grape seed roanthocyanidin extract: Importance in human health and disease prevention, *Pruesse Toxicology* 148:187-197, 2000
5. De Keyser H, Geerts ML, Colardyn F, De Bersaques J: Skin damage caused by the effect of NM gas. *Hautarzt* 37(8):467-471, 1986
6. Drasch G, Kretschmer E, Küuert G, von Mayer, L: Concentrations of Mustard Gas (Bis (2-Chloroethyl) Sulfide) in the Tissues of a Victim of a Vesicant Exposure. *J Forens Sci* 32(6):1788-1793, 1987

7. Marrs TC, Maynard RL, Sidell FR: Chemical warfare agents: toxicology and treatment. Chichester: John Wiley and Sons, 1996
8. McClintock SD, Hoesel LM, Das SK, Till GO, Neff T, Kunkel RG, Smith MG, Ward PA: Attenuation of half sulfur mustard gas-induced acute lung injury in rats. *J Appl Toxicol* 26(2):126-131, 2006
9. Safarinejad MR, Moosavi SA, Montazeri B: Ocular injuries caused by mustard gas: Diagnosis, treatment, and medical defense. *Military Medicine* 166(1):67-70, 2001
10. Saladi RN, Smith E, Persaud AN: Mustard: A potential agent of chemical warfare and terrorism. *Clin Exp Dermatol* 31(1):1-5, 2006
11. Sawyer TW, Lundy PM, Weiss MT: Protective effect of an inhibitor of nitric oxide synthase on sulphur mustard toxicity in vitro. *Toxicology and Applied Pharmacology* 141: 138-144, 1996
12. Papirmeister B, Gross CL, Meier HL, Petralli JP, Johnson JB: *Fundam J: Appl Toxicol* 5:134, 1985
13. Pataki T, Bak I, Kovacks P, et al: Grape seed PCs improved cardiac recovery during reperfusion after ischemia in isolated rat hearts. *Am J Clin Nutr* 75:894-899, 2002
14. Yaren H, Mollaoglu H, Kurt B, Korkmaz A, Oter S, Topal T, Karayilanoglu T: Lung toxicity of NM may be mediated by nitric oxide and peroxy nitrite in rats. *Res Vet Sci* 83(1):116-122, 2007