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EVALUATION OF CYTOTOXICITY OF GLUTATHIONE DERIVED β-LYASE METABOLITES OF SULFUR MUSTARD ON FOUR DIFFERENT TYPES OF IN VITRO CELL CULTURE MODEL

SÜLFÜR MUSTARDIN GLUTATYON BAĞIMLI β-LİYAZ METABOLİTLERİNİN DÖRT FARKLI TİP IN VITRO HÜCRE KÜLTÜR MODELİ ÜZERİNDEKİ SİTOTOKSİK ETKİLERİNİN DEĞERLENDİRİLMESİ

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Keywords: Sulfur mustard, cytotoxicity, β-lyase metabolites, SBMTE, MSMTESE, SBMSE

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ÖZ

Giriş: *Sülfür mustard (SM) alkilleyici özelliği çok güçlü bir yakıcı ajan ve aynı zamanda yasaklanmış bir kimyasal harp maddesidir. SBMTE, MSMTESE ve SBMSE; SM'ın glutatyon bağımlı β-liyaz metabolitleridir. Bu çalışmanın amacı; β-liyaz metabolitlerinin sitotoksik etkilerini dört farklı tip in vitro insan hücre hattı üzerinde değerlendirmektir.*

Gereç ve Yöntem: *İnsan retina pigment epitel hücre hattı, insan umbilikal ven endotel hücre hattı, insan oral keratinosit hücre hattı ve insan akciğer fibroblast hücre hattı beş farklı konsantrasyondaki (10, 50, 100, 500, and 1000 ng/mL) SBMTE, MSMTESE ve SBMSE'ye maruz bırakılmıştır. β-liyaz metabolitlerinin sitotoksik etkisi; maruziyetten 24, 48 ve 72 saat sonra yapılan MTT testlerinde yaşayan hücrelerin yüzdelerine göre tespit edilmiştir.*

Bulgular: *SBMTE, MSMTESE ve SBMSE'nin farklı konsantrasyonlarının dört farklı hücre kültür modelinde 24, 48 ve 72 saatteki sitotoksik etkileri değerlendirildiğinde, yaşayan hücrelerin yüzdeleri anlamında istatistiki olarak anlamlı bir fark bulunmamıştır (p> 0.05).*

Sonuç: *β-liyaz metabolitlerinin; SM'ın ana hedefi olan bu grup hücrelerde ortaya çıkan sitotoksik etkilerden doğrudan sorumlu olmadığı değerlendirilmektedir. Bulgularımız; SM maruziyeti sonrasında β-liyaz metabolitlerinin oksidan/antioksidan denge üzerindeki etkilerini anlamak için hayvan modellerinde kullanılabilir. Böylece SM kurbanlarının tedavisinde kullanılabilecek potansiyel antioksidan ajanların etkinlikleri değerlendirilebilir.*

SUMMARY

Introduction: *Sulfur mustard (SM) is an extremely alkylating vesicant agent and it is also a banned chemical warfare agent. SBMTE, MSMTESE, and SBMSE are glutathione derived β-lyase metabolites of SM. The aim of this study was to evaluate the cytotoxic effects of β-lyase metabolites on four different types of in vitro human cell lines.*

Material and Methods: *The human retinal pigmented epithelium cell line, human primary umbilical vein endothelial cell line, human oral keratinocytes cell line, and human lung fibroblast cell line were exposed to five*

different concentrations (10, 50, 100, 500, and 1000 ng/mL) of SBMTE, MSMTESE, and SBMSE. The cytotoxic effects of β-lyase metabolites were determined at 24, 48, and 72 hours (h) after the exposure by MTT assay as viability rate "%".

Results: *When the cytotoxic effects of different concentrations of SBMTE, MSMTESE, and SBMSE on four cell culture model at 24th, 48th, and 72th h were evaluated, there was no statistically significant difference found in mean of cell viability (p> 0.05).*

Conclusion: *It is concluded that β-lyase metabolites are not directly responsible for the toxicity occurring in these group of cells which are main targets of SM. Our findings could be used for understanding the effects of β-lyase metabolites on oxidant/antioxidant balance after SM exposure in animal models. By this way, effectiveness of potential antioxidant agents for the treatment of victims of SM exposure could be evaluated.*

INTRODUCTION

Sulfur mustard (SM) is an extremely alkylating vesicant agent (1, 2). It is also a banned chemical warfare agent that was first used during the First World War in Ypres/Belgium in 1917 by the German's army against allied troops (3). SM was used several times against civilians in Syria between 2013 and 2019 by non-state actors (4-6). The pathological effects of SM exposure on various organ systems have reported in many studies (7). SM could affect almost all cell constituents, though the eyes, the skin, and the pulmonary system are the primary targets (8, 9). Major clinical symptoms of human SM exposure are presented after a symptom-free latency period up to 24 hours (h) as bilateral conjunctivitis associated with blepharospasm, corneal erosion, temporary blindness, nasopharyngeal irritation, sore throat, coughing, pulmonary edema, erythema, and blisters. Moreover, hematological, immunological, and neuropsychiatric disorders are other main pathological findings of SM exposure (10-12).

Four main metabolic pathways have been determined in rats following SM exposure. One of them is producing of glutathione (GSH) derived β-lyase metabolites including 1,1-sulfonylbis[2-(methylthio) ethane] (SBMTE), 1-methyl-sulfinyl-2-[2-(methylthio) ethylsulfonyl] ethane (MSMTESE), and 1,1-sulfonylbis [2-(methylsulfinyl) ethane] (SBMSE) as shown in Figure 1 (13). Even though the shortage of an entire understanding of SM metabolism, the detection of β-lyase metabolites of SM in urine indicates some similarities between the metabolism of SM in humans and rats. β-lyase metabolites are known as the main unique urinary indicator of SM exposure in humans as they have not been detected in non-exposed individuals. After SM exposure, detectable amounts of β-lyase metabolites are excreted, especially early after in the first two weeks (14). They are analyzed quantitatively by using advanced gas chromatographic methods after performing some specific pretreatment procedures like reducing SBMSE and MSMTESE to SBMTE with titanium trichloride ($TiCl_3$) (15). In our recent study, we developed a rapid and cost-effective gas chromatography-tandem mass spectrometry (GC-MS/MS) method for the quantification of β-lyase metabolites as SBMTE in human urine by using $TiCl_3$ and urinary SBMTE was detected at a concentration of 1 ng/mL (5, 13).

There are some clues that SM oxidation products may exhibit vital toxicity (16). Besides, it is stated that the depletion of GSH, which is an essential intracellular antioxidant could increase oxidative stress (17). However, there is no detailed study in the literature with regard to the toxicity of β-lyase metabolites. In our study, we aimed to evaluate the cytotoxic effects of SBMTE, MSMTESE, and SBMSE at five different concentrations (10, 50, 100, 500, and 1000 ng/mL) on four different types of in vitro human cell lines which are the main targets of SM exposure including the eyes, the oropharynx, and the skin.

MATERIALS AND METHODS

SBMTE, MSMTESE, and SBMSE at a purity of ≥98%, were purchased from Spiez Laboratory/Switzerland. Initially, stock solutions (1 mg/mL) prepared by dissolving neat SBMTE, MSMTESE and SBMSE in methanol (MeOH). Then, dilution solutions for each metabolite were prepared from these stock solutions at a concentration of 10, 50, 100, 500, and 1000

ng/ml in 1% MeOH. The cell culture medium used for achieving 1% MeOH solution.

The human retinal pigmented epithelium cell line (Arpe-19) and human primary umbilical vein endothelial cell line (HUVEC) were obtained from ATCC, USA. Human oral keratinocytes cell line was obtained from ScienCell Research Laboratories, USA. The human lung fibroblast cell line (MRC-5) was obtained from ATCC, USA. Arpe-19, Keratinocyte, and MRC-5 cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Biological Industries, Israel). HUVEC cell line cultivated in Roswell Park Memorial Institute Medium (RPMI 1640) (Corning, Manassas). All cell lines were supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel), 1% L- Glutamine (Biological Industries, Israel), and 1% Pen-Strep Solution (Penicillin 10.000 units/ml, Streptomycin 10 mg/ml) (Biological Industries, Israel).

The experiment was performed in the laminar airflow cabinet (Bilser, Biosafety Cabinet, Ankara, Turkey) after the UV lamp operated for 10 minutes for the sterilization. Cells were suspended in a liquid nitrogen tank were dissolved in a 37º C water bath and mixed with Phosphate-Buffered Saline (PBS) (Biological Industries, Israel) after transferring to conical tubes. Cells were remaining in the pellet after centrifugation planted in flasks. The flasks were removed from the oven providing 37^0 C and 5% CO_2 medium. When the cells were seen to cover the surface of the culture vessels by 80%, the passaging was started. After the subsequent procedures, 20 µl of the cell suspension diluted with culture medium was taken and placed in the Thoma slide and the cells were counted. In order to evaluate the cytotoxic effects of SBMTE, MSMTESE, and SBMSE at 24, 48, and 72 hours (h) with MTT assay, 100 µl of cell solution was cultivated with 96 well-plates where there were 20000 cells in each well. The plates were left in the incubator for 24 h incubation. After the incubation, 100 µl of SBMTE, MSMTESE, and SBMSE in 1% MeOH methanol was added to each well with six repetitions at 10, 50, 100, 500, and 1000 ng/mL doses. Then, the plates were removed to the incubator for MTT assay. In our study, MTT assay which was based on metabolic activity measurement, was used in order to evaluate the cell proliferation, viability, and cytotoxicity. At the end of the procedure, the plates were scanned in an ELISA plate reader at 570 nm wavelength.

Statistical analysis was performed in GraphPad Prism Version 5.04 for Windows (GraphPad Software, San Diego, California, USA) by using one-way ANOVA, followed by Tukey's Multiple Comparison test. The level of significance was set at $p<0.05$.

RESULTS

After 10, 50, 100, 500, and 1000 ng/mL SBMTE, MSMTESE, and SBMSE in 1% MeOH were added to ARPE-19, HUVEC, Human Oral Keratinocyte, and MRC-5 cells, Average ± Standard Deviation values were determined according to the optical densities which were obtained with MTT assay. The cell culture medium was used as the control group. The cell viability of the control group was accepted as 100% and the viability rates of the applied metabolites on the cells following the optical density measurement were calculated as "%" by the formula below;

Viability Rate (%) = (Test well absorbance / Control well absorbance) x100

When the cytotoxic effects of different concentrations of SBMTE, MSMTESE, and SBMSE at 24 h was evaluated, there was no statistically significant difference found ($p> 0.05$) (Figure 2). When the cytotoxic effects of different concentrations of SBMTE, MSMTESE, and SBMSE at 48 h was evaluated, there was no statistically significant difference found ($p> 0.05$) (Figure 3). When the cytotoxic effects of different concentrations of SBMTE, MSMTESE, and SBMSE at 72 h was evaluated, there was no statistically significant difference found ($p> 0.05$) (Figure 4).

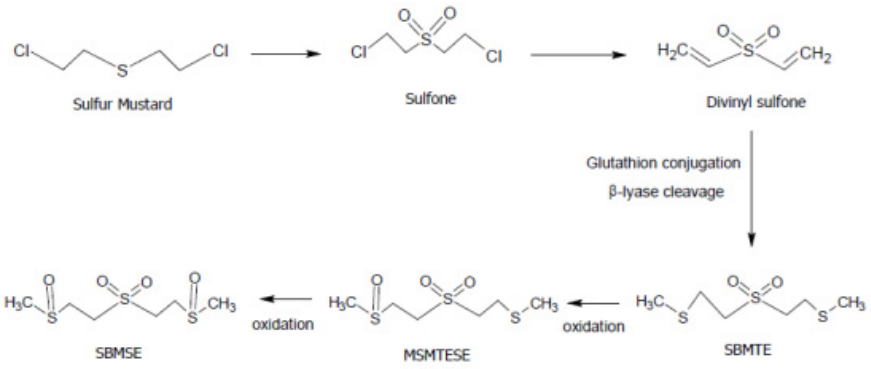

Figure 1. Major metabolic pathways of β-lyase metabolites.

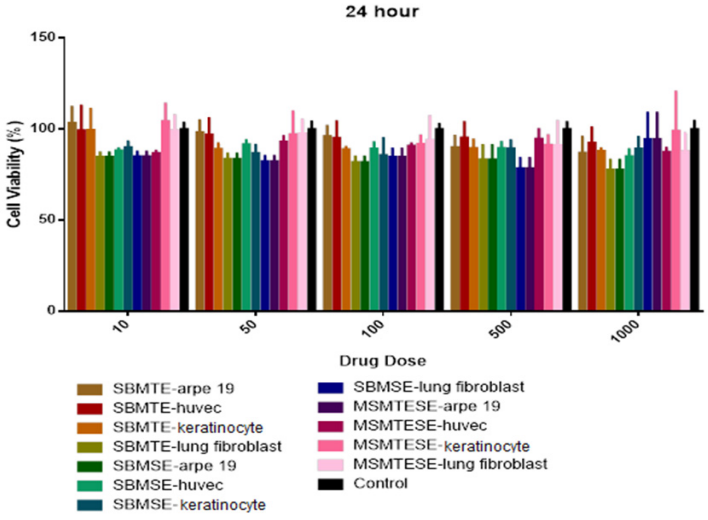

Figure 2. The cell viability (%) of ARPE-19, HUVEC, Human Oral Keratinocyte and MRC-5 cell lines at 24. h. after adding SBMTE, SBMSE, and MSMTESE at various concentrations (10, 50, 100, 500, and 1000 ng/mL).

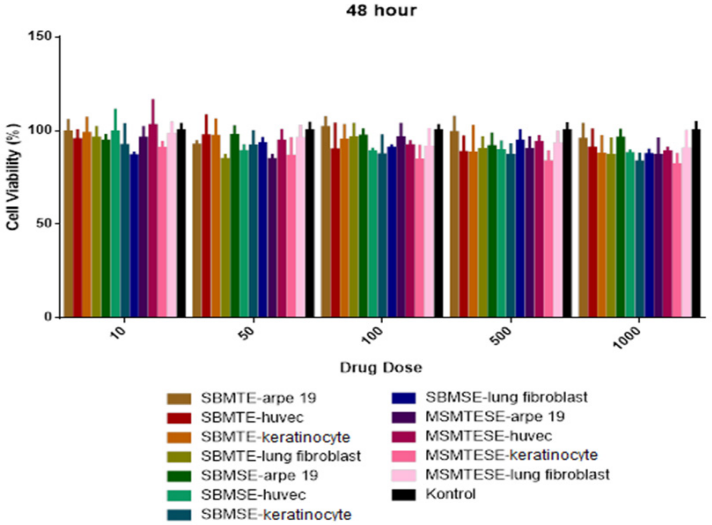

Figure 3. The cell viability (%) of ARPE-19, HUVEC, Human Oral Keratinocyte and MRC-5 cell lines at 48. h. after adding SBMTE, SBMSE, and MSMTESE at various concentrations (10, 50, 100, 500, and 1000 ng/mL).

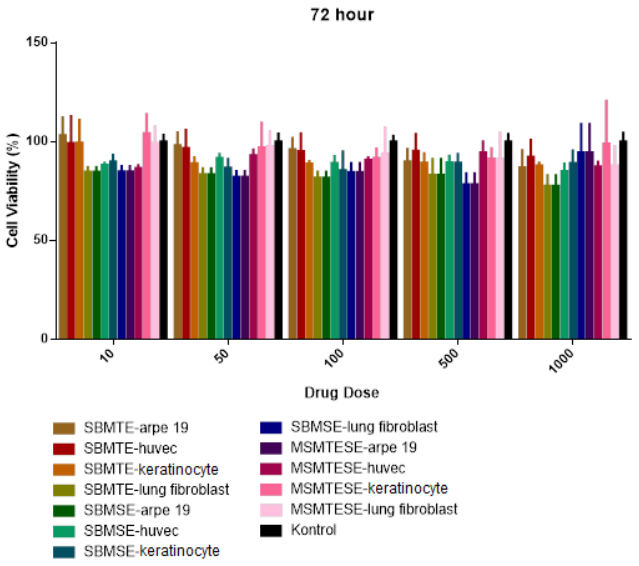

Figure 4. The cell viability (%) of ARPE-19, HUVEC, Human Oral Keratinocyte and MRC-5 cell lines at 72. h. after adding SBMTE, SBMSE, and MSMTESE at various concentrations (10, 50, 100, 500, and 1000 ng/mL).

DISCUSSION

SM which targets the eyes, the respiratory system and the skin causes oxidative stress and increased DNA alkylation. The result is tissue and organ damage which is triggered by the cell death due to the inflammation. In toxicokinetic studies, it was observed that the distribution of SM after intravenous and intraperitoneal exposure in animals was not similar. In this context, it was evaluated that the exposure route was an essential toxicokinetic factor for SM poisoning (8). SM tends to form a hyperactive compound in the intramolecular cycle. It is known that SM interacts with almost all of the cells and tissues after absorption and exerts its toxic effect through GSH depletion and DNA alkylation. Even low-dose SM exposure kills cells that divide especially during the late G1 or S phases, possibly by forming cross-links in the DNA helix. At high doses, SM causes sudden cell death that is expressed as acute damage to the cornea, mucous membranes, and the skin through different mechanisms. The first of these mechanisms is nicotinamide adenine dinucleotide (NAD) depression. As a result of the formation of DNA chain breaks, the poly (ADP-ribose) polymerase (PARP) enzyme activates and PARP quickly consumes cellular NAD pools (2). After all, low intracellular ATP levels cause acute cell death. The second mechanism is the inactivation of sulfhydryl containing proteins and peptides such as GSH. This inactivation leads to redox formation lipid peroxidation, membrane damage, calcium imbalance in the cell which is finalized as cell death (18). After the adsorption, a part of SM is metabolized to SBMTE, MSMTESE, and SBMSE via GSH and β-lyase enzymes. This transformation thought to have been carried out to reduce the genotoxic damage of SM. However, the cytotoxic effects of β-lyase metabolites production is still unknown.

β-lyase metabolites, especially urinary SBMTE is an unequivocal diagnostic biomarker for SM exposure (19). While β-lyase metabolites are detected at the highest level on the 3rd day after SM exposure, their urinary concentration shows a sharp decline in the time-concentration profile starting from the 4th day due to GSH depletion. Therefore, the relationship between GSH-dependent β-lyase metabolites urinary over time depends on individual metabolic rate and GSH amount (20). Within this information; by applying β-lyase metabolites to HUVEC, Arpe-19, oral mucosal keratinocytes, and MRC-5 cell lines in vitro, we aimed to monitor the cytotoxic effects on these four different cell lines at the 24., 48., and 72. h. Since the highest urinary SBMTE level that was detected in urine samples of SM victims is 797 ng/mL, we determined the maximum concentration in dilution solutions as 1000 ng/mL

which was above the maximum level of urinary SBMTE in victims of SM exposure (5).

There was no statistically significant difference found when cytotoxic effects were evaluated with MTT assay at the end of 24, 48, and 72 h following the application of various concentrations of SBMTE, MSMTESE, and SBMSE up to 1000 ng/ml on four different cell lines ($p > 0.05$). It is concluded that β-lyase metabolites are not directly responsible for the toxicity occurring in these group of cells which are main targets of SM as a result of the absence of a statistically significant increase in mean of cytotoxicity despite increasing metabolite application. Our findings support that excessive GSH depletion during the formation of β-lyase metabolites increases the free oxygen radicals which induces oxidative damage and cytotoxic effects.

Disruption of oxidative stress balance could be seen in the cell due to the decrease in cell viability after SM exposure. SM disrupts the oxidative stress balance and this finding has been reported in numerous cell culture studies (21). However, in vivo studies have not been able to distinguish to what extent β-lyase metabolites cause oxidative damage at the cell level. In this study, only the effects of β -lyase metabolites in isolated in vitro cell culture models were examined and this is the limitation of the current study.

Although Web of Sciences, Pubmed, Google Scholar, and Scopus were investigated, there was no cell culture study found about the effects of urinary β-lyase metabolites on the oxidant/ antioxidant balance after SM exposure so our aim for the next step is to use the results of this in vitro cell culture study for the evaluation of cytotoxic effects of β-lyase metabolites in animal models.

CONCLUSION

A group of products that are emerged after SM exposure form β -lyase metabolites including SBMTE, MSMTESE and SBMSE. It is concluded that β-lyase metabolites do not have a direct effect on cytotoxic mechanisms due to increased oxidative stress which expresses itself as the typical clinical picture of SM exposure. Our findings could be used for understanding the effects of β-lyase metabolites on oxidant/ antioxidant balance after SM exposure in animal models. Therefore, a more detailed investigation of the effect of β-lyase metabolites on the oxidative stress due to GSH depletion may help in determining the effectiveness of the possible antioxidant agents for the treatment of victims of SM exposure.

REFERENCES

1. Lin Y, Dong Y, Chen J, Li CZ, Nie ZY, Guo L, et al. Gas chromatographic-tandem mass spectrometric analysis of beta-lyase metabolites of sulfur mustard adducts with glutathione in urine and its use in a rabbit cutaneous exposure model. J Chromatogr B 945 (2014): 233-239.
2. Ruszkiewicz JA, Burkle A, Mangerich A. NAD(+) in sulfur mustard toxicity. Toxicol Lett 324 (2020): 95-103.
3. Mangerich A, Esser C. Chemical warfare in the First World War: reflections 100 years later. Arch Toxicol 2014; 88(11): 1909-11.
4. Kilic E, Ortatatli M, Sezigen S, Eyison RK, Kenar L. Acute intensive care unit management of mustard gas victims: the Turkish experience. Cutan Ocul Toxicol 2018; 37(4): 332-7.
5. Sezigen S, Eyison RK, Kilic E, Kenar L. Evidence of sulfur mustard exposure in victims of chemical terrorism by detection of urinary beta-lyase metabolites. Clin Toxicol (Phila) 2020; 58(1): 36-44.
6. Sezigen S, Ivelik K, Ortatatli M, Almacioglu M, Demirkasimoglu M, Eyison RK, et al. Victims of chemical terrorism, a family of four who were exposed to sulfur mustard. Toxicol Lett 303 (2019): 9-15.
7. Hassan ZM, Ebtekar M, Ghanei M, Taghikhani M, Noori Daloii MR, Ghazanfari T. Immunobiological consequences of sulfur mustard contamination. Iran J Allergy Asthma Immunol 2006; 5(3): 101-8.
8. Balali M. Clinical and laboratory findings in Iranian fighters with chemical gas poisoning. Arch Belg 1984; supp: 254-9.
9. Khateri S, Ghanei M, Keshavarz S, Soroush M, Haines D. Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustard agent. J Occup Environ Med. 2003;45(11):1136-43.

10. Rowell M, Kehe K, Balszuweit F, Thiermann H. The chronic effects of sulfur mustard exposure. Toxicology. 2009;263(1):9-11.

11. Ghabili K, Agutter PS, Ghanei M, Ansarin K, Shoja MM. Mustard gas toxicity: the acute and chronic pathological effects. J Appl Toxicol 2010; 30(7): 627-43.

12. Beigi Harchegani A, Mirnam Niha M, Sohrabiyan M, Ghatrehsamani M, Tahmasbpour E, Shahriary A. Cellular and molecular mechanisms of sulfur mustard toxicity on spermatozoa and male fertility. Toxicol Res (Camb) 2018; 7(6):1029-35.

13. Eyison RK, Sezigen S, Ortatatli M, Kenar L. Optimized Gas Chromatography-Tandem Mass Spectrometry for 1,1'-sulfonylbis[2-(methylthio) ethane] Quantification in Human Urine. J Chromatogr Sci 2019; 57(5): 397-402.

14. Black RM, Hambrook JL, Howells DJ, Read RW. Biological fate of sulfur mustard, 1,1'-thiobis(2-chloroethane). Urinary excretion profiles of hydrolysis products and beta-lyase metabolites of sulfur mustard after cutaneous application in rats. J Anal Toxicol 1992;16(2):79-84.

15. Young CL, Ash D, Driskell WJ, Boyer AE, Martinez RA, Silks LA, et al. A rapid, sensitive method for the quantitation of specific metabolites of sulfur mustard in human urine using isotope-dilution gas chromatography-tandem mass spectrometry. J Anal Toxicol 2004; 28(5): 339-45.

16. Anslow WP, Jr., Karnofsky DA, et al. The intravenous, subcutaneous and cutaneous toxicity of bis (beta-chloroethyl) sulfide (mustard gas) and of various derivatives. J Pharmacol Exp Ther 1948; 93(1):1-9.

17. Paromov V, Qui M, Yang H, Smith M, Stone WL. The influence of N-acetyl-L-cysteine on oxidative stress and nitric oxide synthesis in stimulated macrophages treated with a mustard gas analogue. BMC Cell Biol 2008; 9(1): 33.

18. Ghabili K, Agutter PS, Ghanei M, Ansarin K, Panahi Y, Shoja MM. Sulfur mustard toxicity: History, chemistry, pharmacokinetics, and pharmacodynamics. Crit Rev Toxicol 2011;41(5): 384-403.

19. Sezigen S, Eyison RK, Kenar L. Bioanalytical verification of sulfur mustard exposure in a Syrian family. Gulhane Medical Journal 2019; 61(2);64-8.

20. Xu H, Nie Z, Zhang Y, Li C, Yue L, Yang W et al. Four sulfur mustard exposure cases: Overall analysis of four types of biomarkers in clinical samples provides positive implication for early diagnosis and treatment monitoring. Toxicol Rep 2014;1:533-43.

21. Menacher G, Steinritz D, Schmidt A, Popp T, Worek F, Gudermann T et al. Effects of anti-inflammatory compounds on sulfur mustard injured cells: Recommendations and caveats suggested by in vitro cell culture models. Toxicol Lett 2018; 293 :91-7.

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