

Immunomodulatory Effect of Curcumin in the Upregulation of Inflammasome Pathway Genes Induced by Sulfur Mustard Analog: An In-vitro Study

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ABSTRACT

Sulfur Mustard (SM) induces cell injury via exerting oxidative stress, protease-anti protease imbalance, and inflammation. Inflammasome as one part of innate immunity has a critical role in the recognition of cell injuries and the initiation of the inflammation process by releasing IL-1 β . Hence, the present study investigated the effects of the sub-lethal doses of 2-chloroethyl ethyl sulfide (CEES) as SM analog on the gene expression level of inflammasome-related genes as well as the potential protective effects of curcumin on this process.

The effects of sub-lethal doses (500, 1000, and 2500 mM) of CEES on pulmonary epithelial cell line (A549) were determined at various time points (12, 24, and 48 h). Following the treatment of cells with CEES, the kinetic alterations of the expression levels of *nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)*, *NLR family pyrin domain containing 1 (NLRP1)*, *Caspase-1 (Casp1)*, and *Interleukin-1 β (IL-1 β)* genes were analyzed; using real-time PCR. In addition, the concurrent protective effects of different doses of curcumin (20, 40, 80, and 160 mM) on modulating the effects of CEES were studied.

Although it was found that the lowest sub-lethal dose of CEES (500 mM) was able to up-regulate the inflammasome-related genes, the maximum alterations occurred 48 h after the treatment with the higher sub-lethal dose (2500 mM) of CEES. The maximum alteration occurred in *Casp1* (38 fold), *IL-1 β* (19 fold), and *NLRP1* (~4 fold) genes ($p < 0.0001$). However, the *NF- κ B* gene expression level did not alter following CEES exposure. Even though low doses of curcumin (20, 40, and 80 mM) were able to down-regulate the studied genes, it was found that the treatment of cells with 160 mM of curcumin for 48 h was able to normalize the expression level of all genes.

The present study concludes that curcumin as an anti-inflammatory agent may have beneficial immunomodulatory effects following CEES exposure.

Keywords: Curcumin; Genes; Inflammasomes; Sulfur mustard; 2-chloroethyl ethyl sulfide

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INTRODUCTION

Sulfur Mustard [bis-(2-chloroethyl) sulfide] (SM) or blister gas is an oily liquid compound with a strong alkylating property and has been used as a chemical weapon for nearly 100 years and during World Wars I and II. Moreover, during the recent decades, it was applied against some nations such as Iran and Syria, too.¹⁻³

Cellular and molecular biology-based studies have indicated that SM can alkylate DNA and proteins, destroy enzymes, induce free radicals, and finally can cause necrosis and apoptosis in cells. In addition to death and acute complications, SM can cause long-term side effects on the lungs, skin, and eyes of its victims.^{4,5} Accumulated data shows that chronic complications of the mustard-gas induced lung disease occur through three major axes of oxidative stress, proteases-anti-protease imbalance, and inflammation.⁶ However, it seems that the immune cells were recruited into the injured tissue for tissue repair after mustard injury; however, their presence resulted in a vicious repair-destruction cycle. Recently, studies have indicated the presence of innate and adaptive immune cells such as neutrophils, Th17/Treg cells, and their cytokines in the airway of SM-exposed patients.⁷ Furthermore, some signal transduction pathways such as Smad (an acronym from the fusion of *Caenorhabditis elegans* sma genes and the *Drosophila* Mad, Mothers against decapentaplegic) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) are activated in the airway and skin of the mustard-exposed patients.⁸

Generally, the inflammasome signaling pathway is one of the first signal transduction pathways to be activated during cell injury. The inflammasome pathway includes a group of multiprotein cytosolic sensors that recognize a diverse set of inflammatory inducers, i.e. pathogen-associated molecular patterns and damage-associated molecular patterns (PAMPs and DAMPs, respectively). This set of sensors and intermediate proteins, i.e. Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain containing 1 (NLRP1), NLRP3, NLRP6, NLRP7, Nucleotide-binding oligomerization domain, Caspase Activation and Recruitment domain-containing protein 4 (NLRC4), Apoptosis-Speck-like protein containing a Caspase-recruitment domain (ASC), and Absent in melanoma 2 (AIM2) are responsible for activating a cysteine protease, called caspase-1, which catalyzes immature IL-1 β and IL-18

cytokines into their mature form. Finally, the inflammasome can activate a form of cell death called pyroptosis by secreting these pro-inflammatory cytokines.⁹

Moreover, there is no effective antidote against SM poisoning and injury. However, a variety of antioxidant drugs such as N-acetylcysteine and anti-inflammatory drugs like corticosteroids have been used in this regard although these compounds have not been very effective.^{2,3,7} Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl) -1, 6-heptadiene-3, 5-dione] is a low-molecular-weight, a hydrophobic polyphenolic compound found in the underground stem of *Curcuma longa* that is generally called turmeric. Curcumin has many health benefits including antioxidant, antimicrobial, anti-inflammatory, anti-viral, and anti-cancer effects that have been approved by the FDA, FAO, and WHO.¹⁰ Based on the anti-inflammatory properties of curcumin and, this compound has been used in some inflammatory and autoimmune diseases.¹¹ In addition, several studies have indicated that curcumin exerts a significant protective effect against some herbicide, pesticide, and fungicide poisonings such as paraquat, malathion, and mancozeb in animal models.¹²⁻¹⁵

This study for the first time sought to investigate the effects of mustard gas analog 2-chloroethyl ethyl sulfide (CEES) on the inflammasome pathway. Then, the study examined the immunomodulatory role of curcumin in reducing the injuries caused by CEES that were followed by inhibiting the inflammasome pathway activation.

MATERIALS AND METHODS

Chemicals

CEES (Sigma, Schnelldorf, Germany, 242640) was prepared in 1 Methanol and then stored at -20°C. Due to the toxicity of CEES, all actions involving the mentioned dangerous substance were performed using both chemical and biological hoods along with protective clothing and mask with consideration of all safety precautions. All disposable items contaminated with CEES were removed with bleach before disposal. Curcumin (Merck, NJ, USA, 820354) was dissolved in corn oil.

Ethical Considerations

The project was performed considering ethical issues and obtaining a license from the local Ethics

Committee of the Baqiyatallah University of Medical Science, Tehran, Iran (IR.BMSU.REC.2999).

Pulmonary Epithelial Cell Culture

Pulmonary epithelial cells were obtained from the National Cell Bank of Iran (A549, Pasteur Institute, Tehran, Iran). These cells were cultured in T-25 flasks (Corning Inc., Corning, NY) containing RPMI-1640 medium with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (100 units/mL penicillin G and 100- μ g/mL streptomycin) at 37°C in a humidified atmosphere containing 5% CO₂ according to the supplier protocol.

Determination of Sub-lethal Doses of CEES at Different Time Points

Different concentrations of CEES (500, 1000, 2500, 3500, and 5000 mM as well as 0 mM as control) were added to the cell line at a different time (12-, 24-, and 48 h), and cell viability was measured using MTT assay. For this purpose, the cells were cultured in 96-wells plates and were allowed to fill at least 95% of the plate. Then, different concentrations of CEES, as well as the solvent (as the control), were added into different wells. After 12, 24, and 48 hours, the viability was measured using the MTT assay. Briefly, after exposure to CEES and media change, the cells were incubated at 37°C for 4 h with MTT solution (0.5 mg/mL final concentration). After four hours, the supernatants were removed, and MTT formazan was extracted by acidic isopropanol (0.04 N HCl). Measurement of the optical density was performed at 492 nm with the Bio-Rad microplate reader, and isopropanol was used as the blank reference.

Kinetics of any Changes in the Expression Levels of Inflammasome Genes after CEES Treatment

To determine when and which lowest dose of CEES can up-regulate the inflammasome genes, different sub-lethal doses of CEES (500, 1000, and 2500 mM) were added to the pulmonary epithelial cell line at a different time (12, 24, and 48 h). Cells were plated in a T-25 flask and grown approximately 95% before the treatment. Finally, the cells were removed and washed twice with RPMI (centrifuge, 8 min/1500 rpm, 4°C). Then, mRNA was extracted, and the gene expression level was evaluated by the method that will be described in the following section.

Evaluation of the Role of Different Concentrations of Curcumin in Inhibiting the Effects of CEES on the Expression of *NF- κ B*, *NLRP1*, *Casp1*, and *IL-1 β* Genes

Based on the results and with consideration of the maximum up-regulation of genes at 48 h, different concentrations of curcumin (20, 40, 80, 160, 320, and 400 mM) along with 2500 mM of CEES were added to the cell line for 48 h. Then, the cells were washed twice with RPMI (centrifuge, 8 min/1500 rpm, 4°C). Then, mRNA was extracted, and the gene expression level was evaluated; using the method that will be described in the following section.

Evaluation of the Expression Levels of Inflammasome Genes; Using Real-time PCR

After treatment of the cells with CEES or CEES concurrent curcumin, the expression level of *NF- κ B*, *NLRP1*, *Casp1*, and *IL-1 β* genes was evaluated using real-time RT-PCR. First, the total RNA of the cells was extracted, and cDNA was synthesized using the Superscript III reverse transcriptase (GeneAll, Korea). qRT-PCR was carried out; using SYBR green master mix reagents (GeneAll, Korea) in Mini-8 Real-time PCR system (Coyote Biotech-Korea) with specific primers (Table 1).⁹

A total reaction volume of 25 μ L was obtained by mixing 2 μ L of cDNA template that corresponded to 50 ng of the total RNA, 12.5 μ L of SYBR Green PCR Master Mix (1.5 mmol/MgCl₂), 1 μ L forward primer (10 pmol/L), 1 μ L reverse primer (10 pmol/L), and 8.5 μ L ddH₂O. PCR condition was initially denaturated at 95°C for 5 minutes and was followed by 37 amplification cycles, which consisted of denaturation at 94°C for 30 seconds, annealing at a suitable temperature for 30 seconds, and extension at 72°C for 30 seconds. Threshold cycle values were normalized by GAPDH expression. After calculating the CT of samples, the changes in the gene expression were calculated; using the relative method, the formula $\Delta\Delta CT$, and relative quantification (RQ)=2^{- $\Delta\Delta CT$} .

Statistical Analysis

All tests were repeated three times, and the statistical analysis was performed by a paired-samples *t*-test analysis; using the GraphPad Prism 8.0 software (San Diego, CA). *p*<0.05 was considered to be statistically significant.

Table 1. Sequences of PCR primers used for assaying gene expression level of desired cytokines and genes

Designation		Sequence	Ann. Temp (°C)	Band length (bp)
<i>IL-1B</i>	Forward	5'- TCCGACCACCACTACAGCAA-3'	57	102
	Reverse	5'- ATCTTTCAACACGCAGGACA-3'		
<i>NF-κB1</i>	Forward	5'-TGCAGCAGACCAAGGAGATG-3'	60	140
	Reverse	5'-TGCATTGGGGGCTTACTGT-3'		
<i>NLRP1</i>	Forward	5'- CAGGCAGCACAGATCAACAT-3'	57.5	104
	Reverse	5'- GTGACCTTGAGGACGGAGAA-3'		
<i>Casp1</i>	Forward	5'- GCTTTCTGCTCTTCCACACC-3'	61	160
	Reverse	5'- GATCTGGCTGCTCAAATGAA-3'		
<i>GAPDH</i>	Forward	5'- TCGACAGTCAGCCGCATCTTCTTT-3'	62	98
	Reverse	5'- ACCAAATCCGTTGACTCCGACCTT-3'		

RESULTS

Determination of Sub-lethal Doses of CEES at Different Time Points

The MTT assay results showed that the high concentrations of the CEES (3500 and 5000 mM) were lethal for the pulmonary epithelial cell line at 48 h after the treatment. The CEES sub-lethal dose for this cell line was determined to be 2500 mM during 48 h.

Kinetics of the Effect of CEES on the Expression Levels of the Inflammasome Genes

For determining when and which lowest dose of CEES was capable of up-regulating the inflammasome genes, three different sub-lethal doses of CEES (500, 1000, and 2500 mM) were treated in the pulmonary epithelial cell during 12, 24, and 48h. The obtained results revealed that the sub-lethal doses of CEES (500, 1000, and 2500 mM) had no significant effect ($p < 0.05$) on *NF-κB* gene expression level 12, 24, and 48h after the treatment. However, 500 and 1000 mM of CEES could significantly up-regulate the *NLRP1* gene (1.5- and 3-fold ($p = 0.0003$ and 0.0009)) 48h after the treatment. Furthermore, 2500 mM of CEES could highly up-regulate *NLRP1* gene up to 3.6-fold at 48h after the treatment ($p = 0.0027$) (Figure 1A). Moreover, results showed that even though the lowest dose of CEES (500 mM) was able to up-regulate the *IL-1β* gene up to 4-fold and 11-fold at 12 and 24 h after the treatment, respectively ($p = 0.0003$ and 0.0001), the

2500 mM of CEES could highly increase the gene expression level (19-fold change, $p = 0.0001$) in the *IL-1β* gene at 48 h. *Caspase-1* gene was overexpressed by CEES poison, and the higher dose (2500 mM) at 48 h after the treatment induced a vigorous up-regulation to ~38-fold change ($p = 0.0001$) (Figure 1B).

The Inhibitory Effect of Different Concentrations of Curcumin on the Up-regulation of *NLRP3* and *IL-1β* Genes induced by CEES

The results showed that the low dose of curcumin (20 and 40 mM) could down-regulate the *IL-1β* gene expression up to 53% and 66%, respectively when simultaneously treated with CEES. Likewise, the *IL-1β* gene expression level indicated a reduction of 90% at 80 mM concentration of curcumin. Finally, 160 mM of curcumin was capable of normalizing *IL-1β* gene expression level. The low doses of curcumin (20 and 40 mM) could only reduce the *NLRP1* gene expression up to 20% and 45%, respectively. Furthermore, the *NLRP1* gene expression showed a reduction of 60% at 80 mM concentration of curcumin. Finally, 160 mM of curcumin could mitigate the *NLRP1* gene expression to a normal level. This situation is true for the *caspase* gene. Although 20, 40, and 80 mM of curcumin down-regulated this gene, 160 mM of curcumin was able to normalize the *caspase* gene expression. Finally, higher concentrations of curcumin (320 and 400 mM) indicated a toxic effect and caused pulmonary epithelial cell death (Figure 2A and B).

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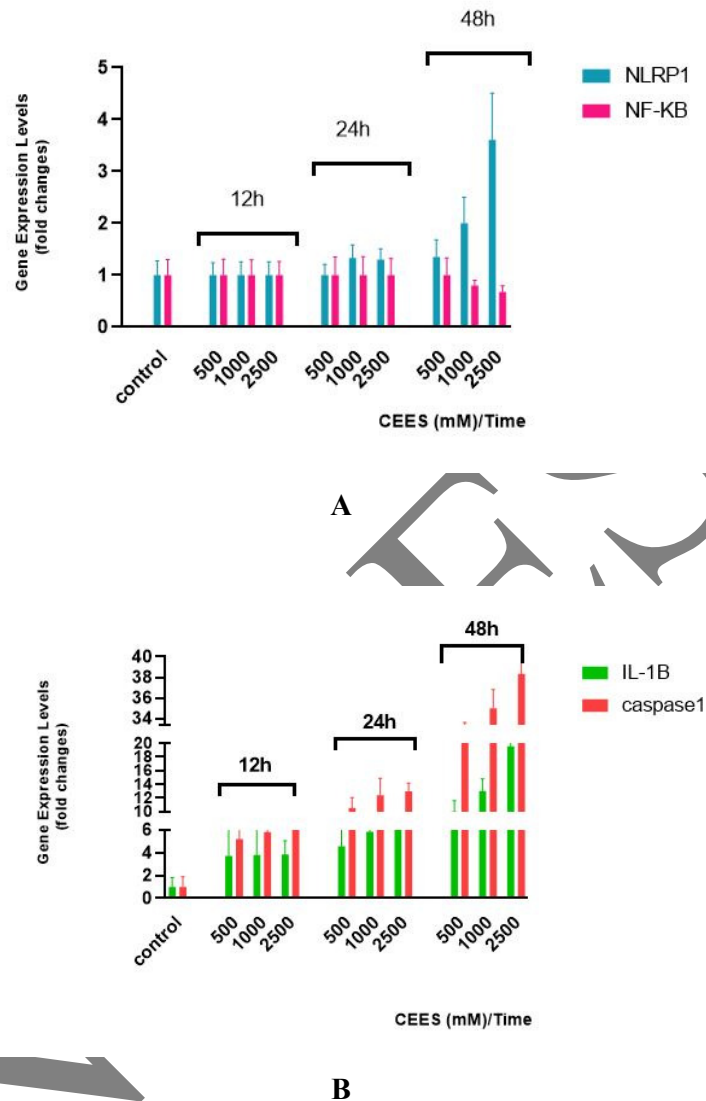


Figure 1. Alterations in inflammasome genes expression levels at various times (12-, 24- and 48 h) induced by three different sub-lethal doses of chloroethyl ethyl sulfide (CEES) (500, 1000, and 2500 mM). (A) The *Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain containing (NLRP1)* expression level was significantly increased at 24 h post-exposure to CEES and reaches the maximum level (~ 4-fold increase) ($p=0.0027$) at 48 h compared to the controls. The nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-κB*) gene expression was not altered upon CEES exposure. (B) *Interleukin-1β (IL-1β)* and *Caspase-1 (Casp1)* were influenced by CEES and began a trend toward the increased level at 12h that continued out to 48h (19- and 38-fold increase) ($p=0.0001$) compared to the controls. Data expressed as mean (SD, $n=3$).

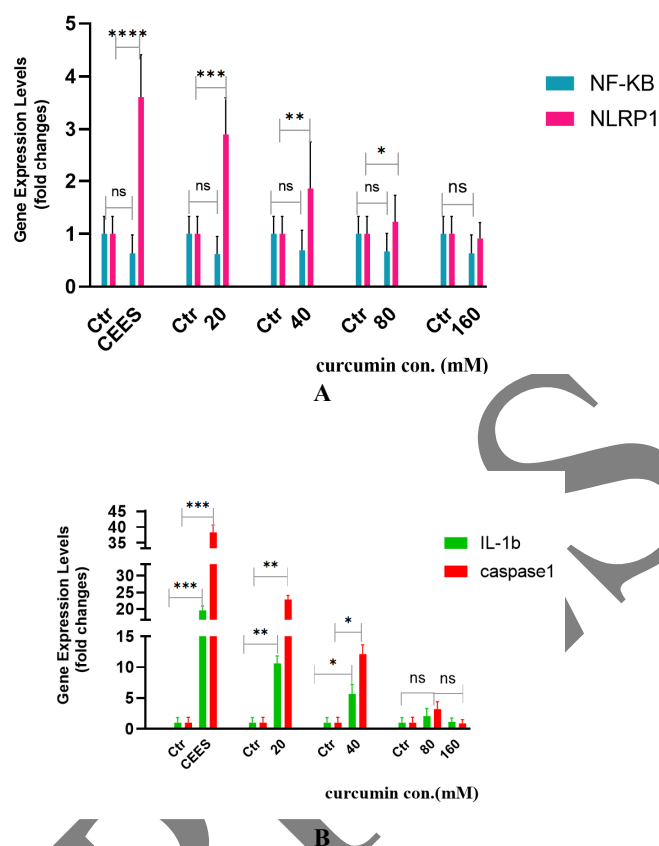


Figure 2. Modulatory effect of curcumin on any alterations in inflammasome genes expression levels induced by chloroethyl ethyl sulfide (CEES). Curcumin has an immunomodulatory effect when concurrently treated with CEES. 20, 40, and 80 mM of curcumin is capable of down-regulate the expression of the level of the studied gene, and 160 mM of curcumin normalizes the Nucleotide-binding oligomerization domain, Leucine-rich Repeat and Pyrin domain containing (*NLRP1*), Interleukine-1 β (*IL-1 β*), and caspase- 1 (*Casp1*) (gene expression level. Also, curcumin was able to reduce nuclear factor kappa-light-chain-enhancer of activated B cells (*NF- κ B*) gene expression level. Data expressed as mean (SD, n=3). * p <0.05, ** p <0.01, * p <0.001. ns: No significant.**

DISCUSSION

SM causes injuries to cells, especially lung, skin, and eye cells. SM as an alkylating agent can cause DNA breakage, protein degradation, cell membrane damage, and finally necrosis and apoptosis in the exposed cells. The oxidative stress, protease/anti-protease imbalance, and inflammation are three major pathophysiological mechanisms of SM.¹ In this study, for the first time, the researchers aimed at revealing whether mustard gas can activate the inflammatory pathway via NLRP1 sensor and whether curcumin can

modulate cell injury induced by mustard gas through this pathway.

First, it must be determined when and which concentration of CEES at various times (kinetic study) can activate the inflammasome pathway. CEES, a less toxic analog of SM used in this study has been applied in many studies to understand the molecular mechanisms of SM.¹⁶ SM can form carbonium and sulfonium ions and interact with adenine and guanine bases. This genotoxicity effect could activate the intracellular repair systems such as Poly (ADP-ribose) polymerase (PARP) that causes a rapid depletion of

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NAD⁺/ATP and lead to cell death.^{4,17} The present *in-vitro* experiment specified 500, 1000, and 2500 mM of CEES as the sub-lethal doses. In addition, higher doses showed necrosis and apoptosis in the pulmonary epithelial cell line. Moreover, the minimum time for the induction of the inflammasome gene expression was 12 h after the exposure.

In this study, all doses of CEES 12 h after exposure had no significant effect on *NLRP1* gene expression level; however, the up-regulation of this gene started 24h following the treatment with two mid and high doses of CEES (1000 and 2500 mM concentrations). Moreover, 2500mM CEES could cause an approximately 3.6-fold change in the *NLRP1* gene 48h after the treatment. It seems that the low doses of CEES in a short time were not able to cause the damage that was detected by cell sensors, or maybe the injuries were under the threshold to up-regulate the inflammasome genes. However, CEES could up-regulate the *NLRP1* gene at higher doses and/or in a long time. DNA damage is considered an important aspect of SM toxicity and damage. Among the DAMPs, DNA can be alkylated on its guanine directly by SM or its analogs, as a result of which eventually DAMP will be produced. The mentioned damage to dsDNA can be sensed by NLRP1 and AIM2 molecules.¹⁸ In addition, CEES can destruct creatine proteins such as keratin 9, actin, and annexin A2 that are identified by NLRP1 and activate the inflammasome pathway.¹⁹ Moreover, oxidative stress includes direct depletion of cellular thiols such as GSH, alkylation, or oxidation of DNA strands, and lipid peroxidation is the other SM toxicity mechanism that leads to the inflammasome pathway activation.²⁰

Meanwhile, the *caspase-1(Casp1)* gene started up-regulation by the lowest dose of CEES (500 mM) at the shortest time (12h) and continued to a vigorous overexpression (up to 38-fold) by increasing CEES concentration. Caspase 1 was synthesized as an inactive zymosan form and activated by inflammasome components such as NLRP1 and NLRP3. NLRP1, a NOD-like receptor protein with a CARD domain can directly act as a mediator for the maturation of caspase. Whereas NLRP3 has not CARD domain and can activate the caspase via the CARD domain of ASC protein. Subsequently, the activated caspase was able to convert pro-IL-1 β and Pro-IL-18 to their activated forms. So, the present researchers contemplate that the up-regulated NLRP1 is directly bound to caspase via

the CARD domain and can cause the activation of caspase1. However, the exact transcription factor responsible for *caspase 1* gene expression was not exactly identified. Some studies suggest and candidate NF- κ B as the transcription factor for the *caspase 1* gene.^{21,22} The present study found that *NF- κ B* gene expression level was stable and not influenced by any CEES concentrations at various times, whilst the *caspase* gene was overexpressed. Normally, NF- κ B was expressed and maintained as a dimeric form with I κ B as its inhibitor component in the cytosol. I κ B kinase as another enzyme can phosphorylate and dissociate the I κ B from NF- κ B. Upon activation, the NF- κ B was transported to the nucleus and regulated some inflammatory genes such as *caspase 1* and *IL-1 β* .^{23,24} In this study, expression levels of *caspase 1* and *IL-1 β* genes started to increase at different concentrations of CEES at 12h post-CEES exposure and reached the highest level (38 and 19- fold, respectively) in 2500 mM of CEES at 48 h after treatment. The stable expression of *NF- κ B* gene suggests that the CEES stress could activate the I κ B kinase, which in return was able to dissociate NF- κ B-I κ B and NF- κ B translocate to the nucleus, and can up-regulate the caspase1 and *IL-1 β* genes.

Curcumin is used as a treatment substance in many diseases such as cancer and chronic inflammation. Many studies have shown its pharmacological effects. Curcumin has antioxidant, immunomodulatory, and anti-inflammatory effects. The mentioned various effects depend on the structure of its molecules, which can bind to a variety of biomolecules in a non-covalent, hydrophobic, and hydrogenated manner.²⁵

In the present study, 20, 40, and 80 mM curcumin down-regulated the expression of *NLRP1*, *caspase 1*, and *IL-1* genes, and curcumin normalized the expression of the genes at 160 mM. The accumulated data demonstrated that curcumin via both direct interactions with NF- κ B and I κ B blocked the activation of NF- κ B and modulated the NF- κ B network. Moreover, curcumin competed with the binding of NF- κ B to the κ B site in the promoter.^{11,26} In addition, previous studies showed that curcumin is a silencer of inflammasome genes such as *NLRP3*, *ASC*, and *caspase*.²⁷⁻³⁰ For the first time, the present study revealed that inflammasome genes were up-regulated by damages caused by SM. The researchers concluded that curcumin as an anti-inflammatory agent may have beneficial immunomodulatory effects following CEES

exposure. However, the data about the expression level of inflammasome pathway genes needs to be validated by proteomics methods. It seems that after confirming the obtained results in the laboratory animal model and determining the delivery route, curcumin can be evaluated as a potentially effective drug candidate in reducing the harmful effects in victims exposed to mustard gas.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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