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Alterations in Mitochondrial and Inflammasome Homeostasis by 2-Chloroethyl Ethyl Sulfide and Their Mitigation by Curcumin: An in Vitro Study

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ABSTRACT

The mitochondrion has a substantial role in innate immunity and inflammasome signaling pathways. Sulfur mustard (SM) induces toxicity in cytoplasmic organelles. We aimed to evaluate the potential therapeutic effect of curcumin on the toxicity of SM analog through measuring gene expression levels of mitochondrial dynamics followed by induction of the inflammasome signaling pathway.

After the treatment of pulmonary epithelial cell line (A549) by 2-chloroethyl ethyl sulfide (CEES) (2500 mM) for 48h, the transcriptional activity of mitochondrial fission and fusion genes such as *dynamin-related protein 1 (Drp1), mitochondrial fission 1 protein (Fis1), mitofusin-1 (Mfn1), mitofusin-2 (Mfn2), and Dominant optic atrophy (Opa1)* and inflammasome pathway genes including *absent in melanoma 2 (AIM2), NLR family containing protein 3 (NLRP3),* and *Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)* was measured. Furthermore, the inhibitory effect of curcumin (160 mM) concurrent with SM analog on the expression level of mitochondria and inflammasome genes was investigated.

CEES was able to over-express the fission, fusion (*Drp1* ~ 8, *Fis1* 4.5, *Mfn2* 15, and *Opa1* 16-fold) and inflammasome genes (AIM*2*, *NLRP3*, 8 and 6-fold, respectively), whereas *Mfn1* was significantly decreased (0.5-fold) and a not statistically significant decrease was observed in the *ASC* gene. Curcumin could modulate the effect of CEES, mitigate the expression of fission, fusion, and inflammasome genes exceedingly. However, a major increase in the repairer fusion gene (*Mfn1*, 6-fold) and complete suppression of the *ASC* gene were the outcomes of using the curcumin.

In conclusion, we suggest curcumin alleviates the disturbance of mitochondrial dynamics and downregulates the inflammasome genes exposed to the CEES.

Keywords: 2-chloroethyl ethyl sulfide; Curcumin; Inflammasomes; Mitochondrial genes

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INTRODUCTION

Sulfur mustard (SM) or in the military terms as HS (Hun Stuff, crude mustard gas), HD (purified mustard gas), and H (single capital letter) is a kind of agent that causes the blistering of the skin and mucous membranes on contact.[1] Due to its toxic effects on eyes, lungs, and skin as well as the relative ease of synthesis, SM has remained a potential chemical threat to the present day.[2] The pathophysiology of SM poisoning is highly complex, involves DNA and protein alkylation, altered gene expression, and energy crisis, and dose-dependently leads to cell cycle arrest and finally apoptosis or necrosis.[3] One of the mechanisms of SM toxicity is increasing reactive oxygen species (ROS). The production of ROS has been proposed to result from the electrophilic property with the depletion of cellular detoxifying thiol levels including glutathione.[1,2,4]

Mitochondria have emerged as organelles that are of significance in innate immunity and inflammation-mediated diseases. Mitochondria exist as a dynamic coordinated network and maintain their healthy pool by constant fusion, fission, biogenesis, repair, and sequestration or degradation via mitophagy or mitophagy-independent mechanisms.[5,6] In particular, mitophagy, i.e. the selective elimination of dysfunctional mitochondria that are fundamental for maintaining a normal function, has a positive role in controlling oxidative stress and inflammation.[7] Recently, *Pal* et al, have reported that 2-chloroethyl ethyl sulfide (CEES, SM analog) can cause the accumulation of ROS in keratinocytes that trigger an alteration in the mitochondria function and induce inflammatory signaling pathways such as extracellular signal-regulated kinases (ERK1/2) or phosphatidylinositol 3-kinases/ Protein kinase B (PI3-K/Akt).[8] Furthermore, CEES could increase reactive nitrogen species (RNS) in macrophages and increase the gene expression level of some inflammatory mediators such as tumor necrosis factor-alpha (TNFα), interleukin-1alpha (IL-1α), intercellular Adhesion Molecule (ICAM), chemokine (C-X3-C motif) ligand 1 (CX3CL1), Chemokine (C-C motif) ligand 8 (CCL8), and C-X-C motif chemokine ligand 10 (CXCL10). Based on the mentioned results, the inhibitory compounds against ROS and RNS as well as the inflammatory mediators have been proposed for preventing SM damages.[8-10]

Furthermore, the mitochondrial network has a pivotal role in the initiation and regulation of the inflammasome signaling pathway and has a close mutual relationship with this signal transduction pathway.[11] Inflammasome involves an array of processes in response to the tissue damage resulting from oxidative stress or other causes and triggers repairs such as the subsequent extracellular matrix remodeling and fibrosis. By far, the best-studied and the most characterized inflammasome is NLRP3 inflammasome, which consists of NLRP3, ASC, and caspase-1.[12] Upon activation, NLRP3 protein recruits the adapter ASC protein, which recruits the procaspase-1 and results in its cleavage and activation. Its activation leads to the caspase-1-dependent secretion of proinflammatory cytokines like interleukin-1β (IL-1β) and IL-18 as well as an inflammatory form of the cell death termed pyroptosis.[13,14] In contrast, NLRP3 activators can induce the destabilization of mitochondrial membranes, de-ubiquitination of NLRP3, linear ubiquitination of ASC, which can cause the externalization or release of mitochondrial-derived molecules such as cardiolipin and mitochondrial DNA. These molecules bind to NLRP3, which is translocated on the mitochondria surface and activates the NLRP3 inflammasome.[15,16]

Besides, there is no efficient antidote against SM injuries. Curcumin, which is a natural compound extracted from *Curcuma longa* L., has numerous physiological properties such as anti-cancer, anti-microbial, anti-inflammatory, and anti-oxidant actions.[17] Curcumin affects the free radicals by several different mechanisms. It can scavenge different forms of free radicals such as ROS and RNS and can modulate the activity of glutathione, catalase, and superoxide dismutase enzymes that are active in the neutralization of free radicals. Moreover, it can inhibit ROS-generating enzymes such as lipoxygenase/ cyclooxygenase and xanthine hydrogenase/oxidase. In addition, curcumin is a lipophilic compound, which makes it an efficient scavenger of peroxyl radicals. Therefore, curcumin is also considered as a chain-breaking antioxidant like vitamin E.[18] Moreover, curcumin has immunomodulatory effects and can regulate some main inflammatory pathways such as

nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB), c-Jun NH2-terminal kinase (JNK) JNK- mitogen-activated protein kinase (MAPK), ERK1/2, PI3-K/Akt, p38, and especially inflammasome pathways.[14,19]

For the first time, the present study aimed at investigating the effects of CEES on the mitochondrial dynamics, including the expression levels of fission or fusion genes, and simultaneously evaluating their crosstalk with the inflammasome pathway. Then, the study sought to survey the potential role of curcumin in alleviating the molecular damages caused by CEES.

MATERIALS AND METHODS

Ethical Considerations

The project was performed considering ethical issues and obtaining a license from the local Ethics Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran (IR.BMSU.REC.2999).

Chemicals

The 2-chloroethyl ethyl sulfide (CEES, Sigma, 242640) was prepared in 1M ethanol and stored at -20°C. As CEES is a toxic and vesicating agent, CEES-containing samples were handled with gloves, and all works with this dangerous substance were performed in the hood. In addition, all items that came into contact with CEES were treated as solid biohazardous wastes so all CEES-containing solutions were decontaminated before disposal; using bleach. Curcumin (Merck, 820354) was dissolved in 1 M in corn oil.

Cell Culture

The experiments were carried out in the pulmonary epithelial cell line. The cell was obtained from the National Cell Bank of Iran (A549, Pasteur Institute, Tehran, Iran) and grown in a Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS).

Viability Assay

The pulmonary epithelial cells were seeded on 96-well plates, which allowed filling approximately 75% of the wells. Then, the cells were treated with CEES at different concentrations (500, 1000, 2500, 3500, and 5000 as well as0mM as control) and cultured at 37°C in a humidified atmosphere containing 5% CO2 according to the supplier protocol for 48 h. Then, cell death was measured using the MTT assay. Briefly, the treatment-containing medium was removed, and MTT was added to each well of the plates. After 4 h, the purple formazan product was dissolved by adding dimethyl sulfoxide (DMSO). The absorbance of the obtained solutions was measured at 492 nm using a microplate reader (Bio-Rad, USA).

The Effect of CEES on Mitochondria and Inflammasome Related Genes Expression Level

By considering 2500 mM CEES as the sublethal dose,[14] the cells were treated with it for 48 h. Subsequently, the cells were harvested and washed twice with PBS (centrifuge, 10 min/1800 rpm, 4°C). After the mRNA extraction, the mitochondria and inflammasome-related gene expression levels were evaluated.

The Modulatory Effect of Curcumin

To examine the modulatory effect of curcumin, different concentrations of curcumin (20, 40, 80, and 160 mM) as modulator compounds were simultaneously treated with 2500 mM of CEES for 48 h. Then, the cells were harvested and washed twice with PBS (centrifuge, 10 min/1800 rpm, 4°C). After the mRNA extraction, the gene expression levels were evaluated.

RNA Isolation and cDNA Preparation

The pulmonary epithelial cells were harvested after desired treatment as mentioned above, the total RNA was extracted and cDNA was synthesized; using the SuperScript III reverse transcriptase (GeneAll, Korea). The concentration and purity of RNA were assessed; using a NanoDrop ND-1000 Spectrophotometer.

Quantitative Real-time PCR

Quantitative real-time PCR was carried out with Mini-8 Real-time PCR system (Coyote Bioteck-Korea) using SYBR green master mix reagents (GeneAll, Korea) with specific primers (Table 1). A total reaction volume of 25 µL was obtained by mixing 2 µL of cDNA template that corresponded to 50 ng of the total RNA, 12.5 µL of SYBR Green PCR Master Mix (1.5 mmoL/MgCl2), 1 µL of the forward primer (10 pmol/L), 1 µL of the reverse primer (10 pmoL/L), and 8.5 µL of ddH2O. PCR condition was initially denaturized at 95ºC for 5 minutes and was followed by 37 amplification cycles, which consisted of

denaturation at 94ºC for 30 seconds, annealing at a suitable temperature for 30 seconds, and extension at 72ºC for 30 seconds. Threshold cycle values were normalized by the GAPDH expression. After calculating the CT of samples, the changes in the gene expression were calculated using the relative method, the formula ΔΔCT, and the relative quantification (RQ)=$2^{(-\Delta\Delta CT)}$ (Table 1).

Data Analysis and Statistics

The non-parametric Wilcoxon signed-rank test was performed using SPSS software (version21.0, IBM Corp.) and all charts created by GraphPad Prism 8.0 software (San Diego, CA, USA). A $p<0.05$ was considered to be statistically significant. All experiments were performed 3 times, and the results were expressed as the mean±standard error of the mean (SEM).

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

Designation		Sequence	Ann. Temp (ºC)	Band length (bp)
Mfn1	Forward	5′-GGCATCTGTGGCCGAGTT-3′	60	59
	Reverse	′5′-ATTATGCTAAGTCTCCGCTCCAA-3′		
Mfn2	Forward	5′-GCTCGGAGGCACATGAAAGT-3′	60	62
	Reverse	5’-ATCACGGTGCTCTTCCCATT-3′		
Opa1	Forward	5′-GTGCTGCCCGCCTAGAAA-3′5′-	61	58
	Reverse	TGACAGGCACCCGTACTCAGT-3′		
Drp1	Forward	5′-TGGGCGCCGACATCA-3′	58	53
	Reverse	GCTCTGCGTTCCCACTACGA		
Fis1	Forward	5′-TACGTCCGCGGGTTGCT-3′	60	55
	Reverse	5’-CCAGTTCCTTGGCCTGGTT-3’		
NLRP3	Forward	5'- AAGGGCCATGGACTATTTCC-3'	57	101
	Reverse	5'- GACTCCACCCGATGACAGTT-3'		
ASC	Forward	5’-AACCCAAGCAAGATGCG-3'	60	82
	Reverse	5'- TTAGGGCCTGGAGGAGCAAG-3'		
AIM2	Forward	5’-AGCAAGATATTATCGGCACAG-3’	55	185
	Reverse	5′-TGACAGGCACCCGTACTCAGT-3′		
GAPDH	Forward	5'- TCGACAGTCAGCCGCATCTTCTTT-3'	62	98
	Reverse	5'- ACCAAATCCGTTGACTCCGACCTT-3'		

RESULTS

Determining the Sublethal Dose of CEES

After the treatment of pulmonary epithelial cells with various concentrations of CESS, the sublethal dose of this SM analog for the A549 cell line was determined as 2500 mM during 48 h exposure.[14]

SM Analog as the Stressor Compound

The control cells (not exposed to CEES) with the normal expected gene expression levels of inflammasome complex as well as mitochondrial fission/fusion genes were considered as the normal situation. Therefore, the alterations in gene expression levels were evaluated after exposure to CEES. As we speculated the mitochondria organelle was dramatically influenced by the destructive effects of CEES. After cell exposure to 2500 mM CEES during 48h, all fission genes that contributed to the mitochondria fragmentation indicated an increase in the expression level as was expected. Thus, a significant increase in the expression level occurred in *drp1* (~ 8-fold, $p<0.01$)

and *Fis1* (4.5-fold, $p<0.01$) (Figure 1A). Contrary to expectations, the fusion genes that were involved in the mitochondria repair also showed an increase in the expression level. In this manner, there was a considerable increase in the expression level of *Mfn2* (15- fold, $p<0.01$) and *Opa1* (16-fold, $p<0.01$). Unlike, the expression level of *Mfn1* was significantly decreased (0.5-fold, $p<0.01$) (Figure 1B).

Moreover, inflammasome genes showed an increase in the expression levels as was expected. The *AIM2* and *NLRP3* genes indicated a remarkable increase (8 and 6-fold, respectively $p<0.01$), whereas a minor non-significant decrease was observed in the *ASC* gene ($p=0.3$) (Figure 1C).

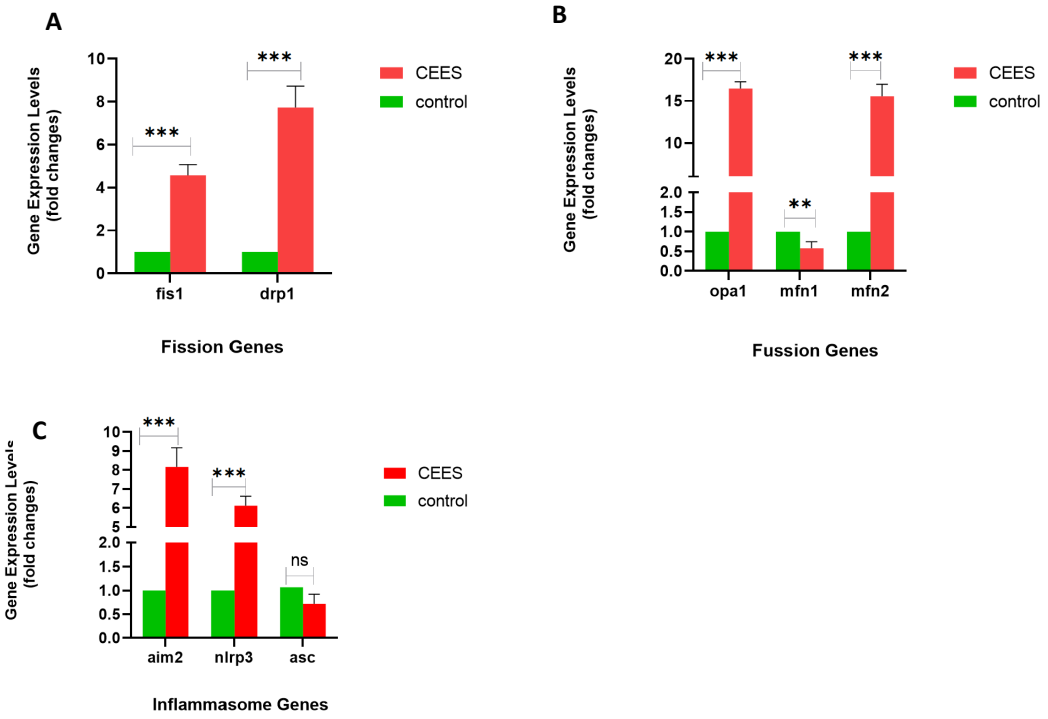

Figure1. Effects of 2-chloroethyl ethyl sulfide (CEES) exposure on the expression level of the mitochondrial fission (a), fusion (b), and inflammasome (c) genes. The graphs depict the fold change of fission, fusion, and inflammasome genes. Both mitochondrial fission (*fis1 and drp1*) and fusion genes (*opa1*and *mfn2*) showed over-expression, and the *mfn2* gene significantly decreased following the exposure to 2500 mM CEES after 48h. Moreover, the expression level of inflammasome genes (*Aim2* and *NLRP3*) vigorously increased upon CEES exposure. *Asc* gene did not alter by CEES. Data were expressed as means (SD, n=3). Error bars represent the standard deviation of each experimental group, respectively. ns: not significant, ***$p<0.01$.

The Inhibitory Role of Curcumin in Reducing the Mitochondrial Injuries and Inflammasome Upregulation

Next, the inhibitory effect of curcumin on the alteration of mitochondrial fission/fusion genes as well as the inflammasome pathway due to CEES exposure was studied. Hence, the pulmonary epithelial cell was simultaneously treated with 160 mM curcumin and 2500 mM of CESS. Curcumin has an inhibitory role in reducing mitochondrial injuries due to CEES and normalized fission gene expression levels, i.e., *Fis1* and *Drp1* (Figure 2A). Although the reduction in the mentioned two genes can be considered very high and fall below the control level, they showed no significant reduction in comparison to the control level ($p=0.9$ and 0.09, respectively). Likewise, this normalization extended to fusion genes such as *Opa1* and *Mfn2*. Adversely, *Mfn1* that was reduced by CEES showed an over-expression by curcumin and reached a ~ 6-fold increase ($p<0.01$) (Figure 2B).

Furthermore, although the *aim2* gene expression in the inflammasome genes was reduced4-fold by curcumin, its expression level did not return to the normal level (p<0.01). The *Nlrp3* gene was normalized by curcumin. However, curcumin caused a massive reduction in the expression level of the *Asc* gene, which reduced to a level less than the control level (p<0.01) (Figure 2C).

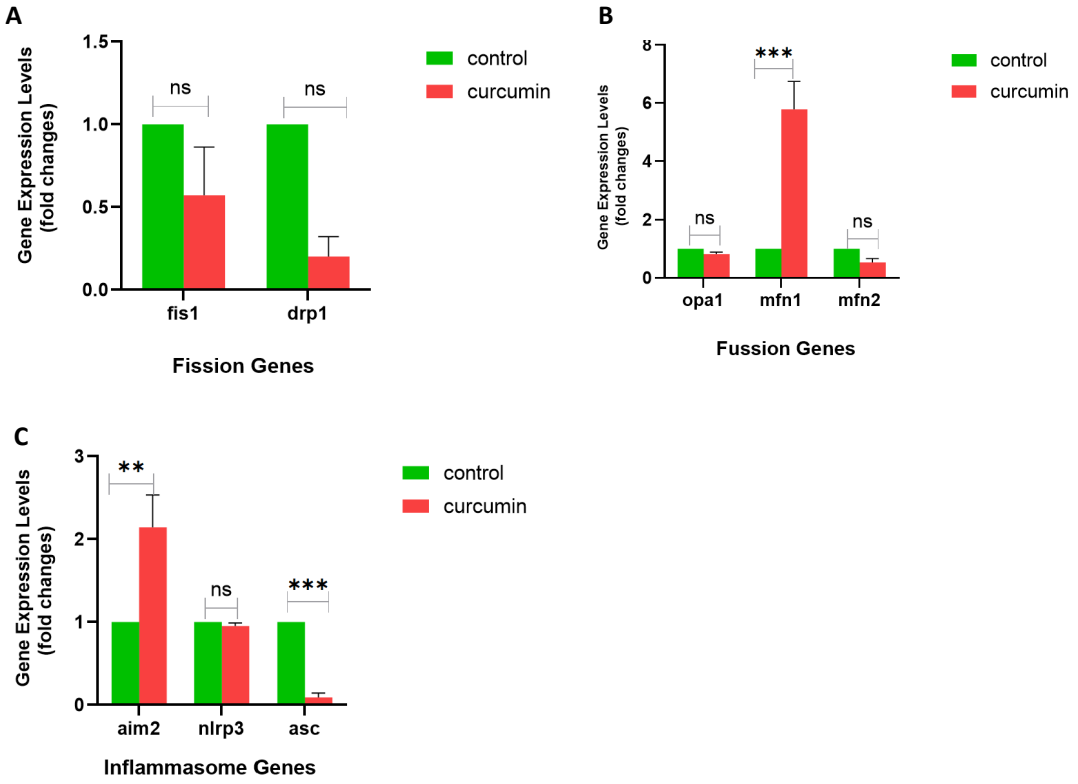

Figure 2. Effects of curcumin treatment (160 mM) on the expression level of the mitochondrial fission (a), fusion (b), and inflammasome (c) genes in 2-chloroethyl ethyl sulfide (CEES)-exposed cells. The graphs depict the fold change of fission/fusion and inflammation genes. Curcumin was able to normalize the fission genes (*fis1* and *drp1*) as well as fusion genes (*opa1* and *mfn2*). *Mfn1* as the repairer gene showed an over-expression (p=0.0007). *Nlrp3* as a cell damage sensor was considered to be normalized, and the *ASC* gene was fully suppressed by the curcumin treatment (p=0.001). In inflammasome genes, although the *Aim2* gene was highly reduced upon curcumin treatment (p=0.003), it did not return to a normal level. The vertical error bars represent the standard deviation of each experimental group, respectively. Data were expressed as means (SD, n=3). ns: not significant, **p<0.01, ***p<0.001.

DISCUSSION

Normally, mitochondria are not static structures and undergo frequent changes in their morphology from a long to fragmented shape by the mitochondrial fission/fusion mechanism.[20] The fission or fragmentation process is highly achieved by the phosphorylation of dynamic-related protein 1 (DRP1) and human fission 1 (FIS1) protein, which promotes the recruitment of DRP1 from the cytosol to the mitochondria surface.[21] In addition, the mitochondria fusion process is an adaptive stress-resolving mechanism that involves exchanging the damaged mitochondrial DNA, lipids, or proteins with healthy ones. In the fission/fusion process, three large guanidine triphosphatases (GTPases) including

mitochondria mitofusin 1 and 2 (Mfn 1 and 2) and optic atrophy 1 (OPA1) are of particular interest in the mitochondria morphologic maintenance.[22]

The present investigation sought to examine the CEES-induced injuries to the mitochondrial fission/fusion mechanism and whether curcumin could protect against the CEES exposure in the pulmonary epithelial cells. The obtained results indicated that CEES could induce an upregulation in the mitochondria fission genes, i.e. *Drp1* (8-fold) and *Fis1* (4- fold), in the pulmonary epithelial cell line. Therefore, CEES could induce mitochondrial fragmentation and network damage by the up-regulation of *Fis1* and *Drp1* genes. Moreover, CEES could upregulate the fusion genes *Mfn2* and *Opa1*, which were involved in the mitochondria morphologic maintenance. Zhao et al,[23] showed T-2 toxin by oxidative stress, a dose-dependent decrease in the protein expression of mitochondrial fusion (OPA1, Mfn1, and Mfn2), increased fission (Drp1 and Fis1) protein, and activated cellular apoptosis in normal human liver cells. Furthermore, GES-1 cell in case of exposure to ochratoxin A showed a disruption in the balance of the mitochondrial fusion/fission with a decrease in the expression of fusion proteins Mfn1 and Mfn2.[24] Other studies have indicated that chemicals such as heavy metals including lead (Pb) or copper (Cu) and benzo (a) pyrene-7, 8-dihydrodiol-9,10-epoxide (BPDE) can induce an imbalance in mitochondrial fusion/fission dynamics.[25,26] In the present study, despite our expectations that fusion genes would remain stable or not increase during the SM stress, SM could up-regulate the expression levels of fusion genes such as *Opa1and Mfn2* (15 and 16 fold, respectively). CEES, simultaneously, by an up-regulation in both fission and fusion genes such as *fis1*, *drp1, Mfn2*, and *Opa1* caused a vigorous disruption in the mitochondrial fission/fusion process, which could lead to mitochondrial fragmentation. It seems that CEES by the oxidative stress and an increase in the expression level of fission genes can damage mitochondria.[8] In response to the mentioned injury, the fusion genes simultaneously started an over-expression to minimize the mitochondrial damages. Hoffmann et al,[27] studied the effect of long-term exposure to cigarette smoke in alveolar epithelial cells and found an up-regulation in both mitochondrial fission (*Fis1* and *Drp1*) and fusion (*Mfn1, Mfn2*, and *Opa1*) genes. Moreover, Kosmider[15] reported the mitochondrial dysfunction and decreased expression level of fusion and fission genes in human primary alveolar type II cells and detected low levels of *Mfn1*, *Opa1*, *Fis1*, and *Drp1*. In our study, the *Drp1* gene was upregulated and contrarily the *Mfn1* gene had a minor reduction following the CEES exposure. Aravamudan et al,[28] found that cigarette smoked (CS) airway smooth muscle cells showed an increase and a decrease in the expression level of *Drp1* and *Mfn2* genes, respectively. They concluded that *Mfn2* contributed to sustaining mitochondrial networks (fusion) under a normal condition, whereas the role of *Drp1*, which promoted fragmentation, was minor. With the CS exposure, *Mfn2* expression and function were reduced, whereas *Drp1*-mediates mitochondrial fragmentation increased. These processes were mediated and modulated by ROS and were relevant to airway inflammation by a range of signaling pathways.

Nowadays, mitochondria have emerged as organelles, which are important in innate immunity and have a close mutual relevance with the inflammasome signaling pathway. It seems that damage-associated molecular patterns (DAMPs) such as cardiolipin, cytochrome c, mtDNA, and mROS may initiate the inflammasome pathway.[15,16] According to the results of the present study, the NLRP3 inflammasome pathway is activated upon the CEES exposure. The upregulation in inflammasome genes, namely *Aim2* (∽8 fold) and *Nlrp3* (∽6 fold) and a minor non-significant down-regulation in the *Asc* gene were observed. *Sabnam*[8] showed that keratinocytes exposed to CEES could release mtDNA and mROS into the cytoplasm and activate the inflammatory signaling pathway. Zhong[29] demonstrated that the mitochondrial damage caused a release or externalization of molecules that were directly binding to NLRP3 and activated the inflammasome pathways. Sangjun et al,[30] evidenced that the aberrant mitochondrial elongation caused by the knockdown of the *Drp1* gene led to a marked increase in the NLRP3-dependent caspase-1 activation and the interleukin-1-beta secretion in mouse bone marrow-derived macrophages. In another study, treatment with the mitochondrial complex I inhibitor rotenone not only led to the loss of the mitochondrial membrane potential and the increased ROS production but also enhanced the NLRP3-dependent IL-1β secretion.[31]

Curcumin has various pharmacological properties such as anti-oxidant and anti-inflammatory properties.

Accumulated data showed that curcumin could be effective in treating mitochondrial injuries.[32] The present experiment indicated that the concurrent administration of curcumin with CEES was able to down-regulate and/or normalize upregulated genes such as mitofission and mitofusin genes. It seems that curcumin via their antioxidant mechanism inhibited mitochondria injuries due to the CEES exposure. Meanwhile; curcumin upregulated the *Mfn1* repairer gene. Moreover, the present study indicated that the expression of inflammasome genes (*Aim2*, *Nlrp3*, and *Asc*) was seriously inhibited by curcumin. The suppressive effects of curcumin and its mechanisms in the NLRP3 inflammasome activation have been widely studied.[33]

To sum up, we indicated that CEES could induce mitochondrial damage and upregulation in both fission and fusion genes (*Drp1, Fis1, OPA1*, and *MFN 2*). Moreover, the mitochondrial damage was sensed by the inflammasome pathway, and the dysregulation occurred in inflammasome sensor genes such as *NLRP3*, *Aim2*, and *ASC*. Furthermore, curcumin was able to alleviate the mitochondrial damages and downregulate the inflammasome genes. However, additional *in vitro* and *in vivo* studies are required to survey the therapeutic usage of curcumin after mitochondrial injuries.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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