International Immunopharmacology xxx (xxxx) xxxx

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Delayed effects of sulfur mustard on autophagy suppression in chemically-injured lung tissue

Marzieh Eghtedardoost[a,b], Tooba Ghazanfari[a,c,*], Alireza Sadeghipour[d], Zuhair Mohammad Hassan[b,*], Mostafa Ghanei[e], Saeid Ghavami[f,g,h,i]

[a] Immunoregulation Research Center, Shahed University, Tehran, Iran
[b] Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran 14115111, Iran
[c] Department of Immunology, Shahed University, Tehran 3319118651, Iran
[d] Pathology Department, RasoulAkram Medical Complex, Iran University of Medical Sciences, Tehran, Iran
[e] Chemical Injuries Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Science, Tehran, Iran
[f] Department of Human Anatomy and Cell Science, Rady College of Medicine, Max Rady Faculty of Health Sciences, University of Manitoba, Winnipeg, Canada
[g] Biology of Breathing Theme, Children Hospital Research Institute of Manitoba, University of Manitoba, Winnipeg, Canada
[h] Health Policy Research Center, Institute of Health, Shiraz University of Medical Sciences, Shiraz, Iran
[i] Research Institute of Oncology and Hematology, CancerCare Manitoba, University of Manitoba, Winnipeg, Canada

ARTICLE INFO

Keywords:
Microtubule-associated protein light chain 3-B (LC3-B)
Beclin-1
Autophagy
Mustard gas
Lung
Formalin-fixed paraffin-embedded (FFPE)

ABSTRACT

Background: Autophagy is an intracellular hemostasis mechanism, responding to extracellular or intracellular stresses. Sulfur mustard (SM) induces cellular stress. Iranian soldiers exposed to SM gas, during the Iraq-Iran war, suffer from delayed complications even 30 years after exposure. In this study, for exploring the SM effect on autophagy pathway, gene and protein expression of autophagy markers are evaluated in the lung of SM-exposed people.

Methods: 52 FFPE lung tissues of SM-exposed people and 33 lung paraffin blocks of non-exposed patients to SM were selected. *LC3* and *Beclin-1* mRNA expressions were evaluated by QRT-PCR. LC3-B protein and LC3II/LC3I proteins ratio were detected by Immunohistochemistry and immunoblotting method. The collected data were analyzed in SPSS, and P value ≤ 0.05 was considered significant.

Results: *LC3* gene expression in SM-exposed subjects (median CT value = 4.97) increased about 4 fold compared with the control group (median CT value = 0.46, P = 0.025). *Beclin-1* mRNA expression had not significant difference between two groups. After adjusting the confounding variables such as drug usage, LC3-B protein (P = 0.041) and LC3II/LC3I ratio (P = 0.044) were found significantly lower in the lung cells of SM-exposed group.

Conclusion: Upon exposure to SM gas, the lung cells are affected by acute cellular stress such as oxidative stress. The study results show that LC3 mRNA level increases in these patients, but, surprisingly, LC3-B protein via unknown mechanism has been down-regulated. N-acetyl cysteine and salbutamol drugs could induce the autophagy, and help to reduce the SM effects and improve the clinical condition of SM-injured patients.

1. Introduction

Macroautophagy (here after autophagy) is induced in response to various stimuli, including endogenous and exogenous stresses, such as amino acid or growth factor deprivation, endoplasmic reticulum stress, mitochondrial damage, pathogen infection and in response to various immune stimuli [1–6]. From yeast to mammalian cells, one way to eliminate cellular stress is degradation of damaged cytoplasmic molecules and organelles by autophagosome-lysosomes (autolysosome) that is known as autophagy pathway [7–9].

> 30 genes have been known for the regulation of autophagy in yeasts, 16 similar genes are found in humans [10–12]. Many mechanisms regulate autophagy machinery such as the mammalian target of rapamycin (mTOR) that down-regulates it, while PI3K hVPS34 induces it [13–16]. Beclin-1 (acting like *Atg6* gene in the yeast) contributes to III phosphatidylinositol 3-kinase complex for autophagy stating and

* Corresponding authors at: Immunoregulation Research Center and Dep of Immunology, Shahed University, Tehran, Iran (T. Ghazanfari).
E-mail addresses: Eghtedar.m@gmail.com (M. Eghtedardoost), ghazanfari@shahed.ac.ir (T. Ghazanfari), sadeghipour.ar@iums.ac.ir (A. Sadeghipour), hasan_zm@modares.ac.ir (Z.M. Hassan).

https://doi.org/10.1016/j.intimp.2019.105896
Received 15 July 2019; Received in revised form 26 August 2019; Accepted 6 September 2019

autophagosome formation. Also, microtubule-associated light chain 3 (LC3 in mammals, same as ATG8 of yeast) plays a part in the fusion step of autophagosome to lysosome and formation of autolysosome. LC3 has three isoforms in mammalian tissues (LC3A, LC3B, and LC3C) and shows two forms: a soluble form called LC3 I (molecular weight, 18 kDa) and a lipidated form called LC3 II (molecular weight, 16 kDa) [17,18]. Autophagy stimulator converts LC3-I to LC3-II. Therefore, phosphatidylethanolamine (PE)-conjugated form of LC3 (LC3-II) is correlated to autophagosome formation, and the ratio of LC3-II/LC3-I is an indicator of autophagy process [19–21].

The autophagy and immune system have close and mutual relations. Some immune-related signaling molecules could regulate autophagy such as pathogen-recognition receptor (PAMPs and DAMPs), IFN-γ, tumor necrosis factor-α (TNF-α), and many other cytokines [1,22]. In the human atherosclerotic vascular disease, it was shown that TNF-α in the smooth cells could up-regulate LC3 and Beclin-1 genes expression [23]. However, stimulation of autophagy by TNF-α is cell dependent. The evidence shows that autophagy regulates TNF-α secretion by macrophages and dendritic cells [1]. Also, it has been confirmed that Interleukin (IL)-1α and IL-1β induce autophagy in human macrophages. On the other hand, disruption of normal autophagy pathway has been involved in increased secretion of the pro-inflammatory cytokines such as IL-1α, IL-1β, and IL-18 [1]. Altogether, the interaction of autophagy and the immune system is complicated and tightly depends on the trigger of autophagy and the type of organs which the autophagy induced.

The role of autophagy in the pathogenesis of lung diseases is dual and dubious [24]. The degradation of mitochondria by autophagy mechanism worsens the pathogenesis of the chronic obstructive pulmonary disease (COPD) by activating cell death programs [25]. Well, Zhi-Hua Chen and collagenous reported that the ratio of LC3B-II/LC3B-I and the expression of some essential proteins of the autophagy pathway significantly increased in COPD lung [26]. However, in pulmonary hypertension, autophagy may have a protective effect by modulating cell proliferation and death. In other disorders, autophagy may contribute to the pathogenesis of idiopathic pulmonary fibrosis and cystic fibrosis [25].

Sulfur mustard (SM) is known as an alkylating agent. In the acute phase of exposure, it could cause mild conjunctivitis in the eye and sneezing and cough in the pulmonary system [27]. In the delayed phase of SM exposure, the victims may suffer from blindness or obstruction of upper airways, chronic bronchitis, and bronchiolitis in the lung [28]. This chemical weapon was used several times by the Iraq army against Iranian civilians and soldiers in the Iraq-Iran war (1980–1988), resulting in > 45,000 victims of delayed complications of SM [29].

Although many studies have been conducted on the effects of SM gas, the molecular mechanism of SM effects at the cellular level is still unclear, especially in the delayed phase. Therefore, there is no therapeutic clinical approach to lung injuries caused by SM [30]. Studies indicate that the victims suffer from pulmonary complications, commonly chronic obstructive pulmonary disease, [31] and obliterate bronchiolitis [32].

Based on what was discussed, autophagy has a critical role in response to cellular stress. Thus, we designed the current study to evaluate the essential markers of autophagy such as mRNA and protein expression of LC3 and Beclin-1 in formalin-fixed paraffin-embedded (FFPE) lung tissues of SM-injured patients and compared them with FFPE lung tissues of patients non-exposed to SM.

2. Materials and Methods

2.1. Ethics Statement

The ethical issue of our study was approved by the Research Ethics Committee of Shahed University (41/198215) and Medical Faculty of Tarbiat Modares University (52/1547).

Table 1
The main characteristics of the SM-exposed and control groups.

	SM-exposed (n = 52)	Control(n = 33)
Sex	52 M 0 F	27 M 6 F
Age (year)	44.65 ± 10.19	47.78 ± 15.73
Smoking	10 (19.2%)	9(27.2%)
Pathology Diagnosis		
Respiratory bronchiolitis	5	1
Constrictive bronchiolitis	26	1
Chronic bronchiolitis	15	1
Bronchiectasis	2	0
Anthracosis	1	2
Benign tumor	1	1
Malignant-tumor	0	14
Bulla	0	3
Interstitial lung disease	0	4
Idiopathic pulmonary fibrosis	0	2
Pulmonary congestion	1	0
Severe acute inflammation with abscess formation	1	0
Trauma	0	1
Spontaneous pneumothorax	0	3
Drug Usage		
Fluticasone	23 (44.23%)	1 (3.03%)
Salbutamol	7 (13.46%)	6 (18.18%)
N-Acetyl cysteine	12 (23.07%)	2 (6.06%)
Atrovent	1 (1.9%)	2 (6.06%)
Prednisolone	5 (9.61%)	3 (9.09%)
The severity of pulmonary complications in the exposed group*		
Normal	14	
Mild	20	
Moderate-severe	7	

*The Severity of pulmonary involvement in the exposed group were classified by clinical symptoms such as lung sounds and spirometry documents, according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [63].

2.2. Tissue specimens

The lung paraffin blocks were provided by the pathology department of general hospitals in Tehran City, Iran (2005–2011). They were used as samples. The inclusion and exclusion criteria were described in our previous study [33], briefly, having documented exposure to sulfur mustard and chronic respiratory complication were important inclusion criteria for SM-exposed group and control group was chosen when their lung biopsies have normal area and not exposure history to mustard or other toxic gas. The age and addiction conditions of samples in the exposed and control group were matched. Table 1 presents the detailed characteristics of the samples.

2.3. Total RNA extraction and quantitative real-time reverse transcription-PCR

RNA extraction process from FFPE tissue has been explained in our previous study [34]. Briefly, high quality and quantity total RNA was extracted by modification in the manufacturer's protocol of RNeasy FFPE Kit (Qiagen- Germany). The Agilent RNA integrity number (RIN) show the quality of this extracted RNA, but this value is not sensitive for degraded RNA in FFPE samples. The mean RNA fragment size determines RNA quality in FFPE tissues [35]. The percentage of RNA fragments > 200 nucleotides was calculated (Macrogen, Korea) as the indicator of the quality of RNA, DV200 metric, and DV200 > 30% were accepted.

The high purity extracted RNA (280/260 ratio above 1.9) was transcribed to cDNA by high capacity cDNA reverse transcription kit (ABI-USA). The primers of *β-actin* and phosphoglycerate kinase 1 (*PGK1*) as two reference genes and *LC3* and *Beclin-1* as target genes

Table 2
The sequence of the primers.

Gene	Forward (3 > 5)	Reverse (5 > 3)	Amplicon length (bp)	Efficiency (%)	GenBank, accession number
Actin	CGTCTTCCCCTCCATCGTG	GGTGAGGATGCCTCTCTTGCTC	111	95.29	NM_001101.3
PGK	GGCATACCTGCTGGCTGGATG	ACAGGACCATTCCACACAATCTGC	104	96.06	NM_000291.3
LC3	CGAACAAAGAGTAGAAGATGTCCGAC	GCTGCTTCTCACCCTTGTATCG	92	98.47	NM_022818.4
BECN1	CAGCCAGGATGATGTCCACAG	CCCAGTGACCTTCAGTCTTCG	107	92.88	NM_003766.3

were designed manually with amplicon length < 125 bp and exon-exon junction manner to detect mRNA expression of the studied genes. The amplification efficiency of each primer was calculated using the slope of the regression line in the standard curve by ABI StepOne 2.3 software (ABI-USA). The standard curve of each gene was drowned using a serial dilution of the PCR product of the same genes. Table 2 presents the sequence of primers.

We used Gene Runner 5.0.1 and online oligo calculator software to estimate thermodynamic state and secondary structure of the primers [36]. The primers were produced commercially (TAG Copenhagen-Denmark). The RT-qPCR was performed with SYBR Green I dye on the Applied Biosystems StepOnePlus™ Real-Time PCR System with a holding stage at 95 °C for 15 min and amplifying stages of 1) 95 °C for 30 s and 2) 60 °C for 60 s (40 cycles). All assays were done three times. The Pfaffl equation was used to analyze the relative quantification of RT-qPCR [37].

2.4. Immunohistochemistry

The lung paraffin blocks of samples were supplied from the pathology department of tertiary hospitals. After cutting 3-μm sections from each sample, the immunohistochemistry standard protocol was carried out on samples [38]. After deparaffinization and rehydration, antigen retrieval step was done with heat and pressure created by autoclave (Prestige, England) in Tris-EDTA epitope retrieval buffer (pH 9). Endogenous peroxidase blocking was done by 3% H_2O_2 in methanol; protein blocker serum-free solution (Dako- Denmark) was used for protein blocking. Anti-human LC3 (NanoTools- USA, clone 5F10, 1:50) was used for detection of LC3. The Envision Detection System (Dako-Denmark) was used as detection kit. After hematoxylin counterstaining, the slides were mounted. For LC3, human normal kidney section was used as the positive control. For negative control slides, the primary antibody was not used.

Co-author pathologist (blinded to sample data) evaluated staining under a light microscope. The number of cytoplasmic and nucleus LC3 + puncta/cell was counted, too. The slides were assessed according to intensity and amount. The intensity of staining was scored as follows: negative (0); weak (1); moderate (2); strong (3). The percentage of LC3 positive cells was graded into five categories: 0 (≤5%), 1 (6–25%), 2 (26–50%), 3 (51–75%) and 4 (> 75%). Quantity and quality of LC3 stained cells were calculated by multiplying the intensity score at the percentage of positive cells. Throughout each slide, 10 randomly chosen oil immersion field were evaluated at ×1000 for LC3 by Labomed light microscope (LX500, USA).

2.5. Protein extraction and immunoblotting

Protein extraction from FFPE tissue was derived from Kawashima et al. described method [39], with a little modification. Four 10-μm FFPE sections were cut and de-paraffinized with 1.5 mL xylene (twice) and rehydrated with a serial dilution of ethanol. The pellets were re-suspended in extraction buffer (300 mM Tris-HCl, sodium dodecyl sulfate [SDS] 2%, 0.5 M 2-mercaptoethanol) and homogenized by homogenizer stirrer (WiseStir- Mexico) for 15 s with high power. After that, they were incubated at 90 °C for 90 min. At the final step, after 14,000*g* centrifugation for 20 min at 4 °C, the supernatant solution would have the extracted protein. The soluble protein was precipitated by acetone method [40]. The obtained protein pellet was re-suspended in re-suspension buffer (1.5 M Tris base, 20% Glycerol, 0.002 M SDS, 0.06 M urea). The protein concentration was measured by 2-D Quant kit (GE-USA).

The extracted protein was stained with Coomassie Brilliant Blue R 250 (Merk-Germany), and 100 μg of each total protein sample was loaded to each well of SDS-PAGE 20%. Electrophoresis was done by BioRad Mini-PROTEAN II system with a standard protocol [41]. After that, the separated protein was transferred onto PVDF (Polyvinylidene Fluoride, BioRad, USA) membrane by BioRad Criterion Blotter. The transferring time was 90 min, 30 mA. After blocking of the membrane with 2% BSA (Bovine Serum Albumin, Sigma, Germany), the membrane exposed to anti-LC3 antibody (1:300, NanoTool-USA) and anti-α-Tubulin antibody (1:800, Sigma, Germany) overnight at 4 °C with low shaking. The anti-mouse IgG (1:7000, Sigma, Germany) conjugated with horseradish peroxidase *(*HRP*)* was applied as the secondary antibody. For visualization of the protein bands, ECL detection kit (Kardan, Iran) was used. The bands appeared on X-ray film were analyzed by Image J software (Wayne Rasband [retired from NIH]) and the LC3-I, LC3-II proteins were normalized to α-Tubulin. In the end, the ratio of LC3-II/LC3-I was determined for each sample.

2.6. Statistical analysis

Statistical analyses were performed in SPSS 22 (SPSS Inc., Chicago Ill). The normality of data was evaluated by the Kolmogorov-Smirnov test. The difference between the control and exposed groups was considered significant at $P \leq 0.05$.

For real-time PCR test, the cycle threshold (CT) value was determined by ABI StepOne 2.3 software (ABI-USA) and median, and standard deviation (SD) were computed. The Spearman correlation was used for determination of correlation among gene expression or protein expression and clinical documents (for example, forced expiratory value in one score [FEV1], forced vital capacity [FVC], etc.). The correlation between gene or protein expression with the clinical symptom, pathological finding, and drug usage were done by Mann-Whitney test. The comparing between severity of pulmonary complications with *Beclin1* and *LC3* genes expression were estimated by the Kruskal-Wallis test. The comparison of LC3B between two groups was done with the Mann-Whitney test. Adjustment of this test for extracting the confounding variable -drug-using- effect were done with two way ANOVA. At the first step, LC3B transformed to rank. Then in two way ANOVA, the rank of LC3B was considered as the dependent variable, study group and using medicine as the independents.

3. Results

3.1. The concentration and quality of the extracted mRNA

After extraction of mRNA from FFPE tissues, their concentration was measured with NanoDrop 2000. The samples discarded when OD 260/280 ratio was < 1.8. The mean ± SD of mRNA concentration for the exposed and control groups were 292.8 ± 140.24 ng/μL and 322.52 ± 298.8 ng/μL, respectively. The DV 200 metric analysis showed that the %fragmented RNA < 200 bp was 51.7 ± 3.66% for

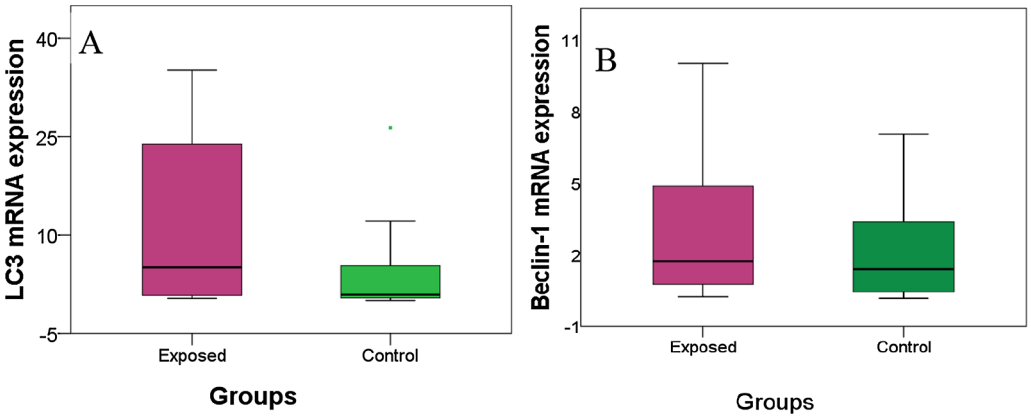

Fig. 1. The mRNA expression of microtubule-associated light chain 3 (*LC3*) (A), *Beclin-1* (B) in lung tissue of SM-exposed and control subjects. RT-qPCR valued mRNA expression of 85 samples (52 SM-exposed and 33 control). The expression of *LC3* is upregulated in SM-exposed lung while Beclin-1 showed no significant difference between the SM-exposed and control groups.

the exposed group, and 50.95 ± 10.76% for the control group. The samples with DV200 > 30% were chosen for further studies.

3.2. The different effect of SM gas on LC3 and Beclin-1 genes expression

RT-qPCR evaluated *LC3* and *Beclin-1* genes expression. Fig. 1 shows the median value of L*C3* expression as 4.97 in the exposed group and 0.46 in the control group. The Mann-Whitney analysis shows this difference was significant (P = 0.025). But *Beclin-1* expression showed no significant difference between the two groups. Median values of *Beclin-1* were 1.85 and 1.43 in the exposed and control groups, respectively (P = 0.323). Fig. 1 shows the mRNA expression of these two genes.

There was not any significant correlation between *LC3* and *Beclin-1* gene expression in control and SM exposed patients group. Moreover, in the exposed group, *Beclin-1* and FEV1/FVC ratio had a direct correlation (r = 0.714, P = 0.047). Also, no correlations were found between the expression of these two genes and the clinical signs and symptoms such as cough, sputum, and dyspnea as well as with pathological findings.

3.3. The SM gas down-regulate the LC3-B protein in immunohistochemistry test

For detection and localization of LC3-B in lung samples, we used specific antibody for this protein. Distribution of brown patches indicates LC3-B localization in the lung cells. We calculated the presence of positive cells and intensity of LC3-B protein expression in each sample. The results demonstrated that the median value of the LC3-B protein was 25 in the exposed lung and 62.5 in the control group. This difference was non-significant (P = 0.164). Fig. 2 shows the LC3-B Immunohistochemistry test.

Some of the exposed and control patients used salbutamol, fluticasone, N-acetyl cysteine, and prednisolone (Table 1). When we deleted the effect of some influencers such as “drug use” in both group, the results changed, and the difference between LC3-B protein of the SM-exposed and control group got significant (P = 0.041). In the exposed group, among the above drugs, salbutamol and N-acetyl cysteine had the most substantial effect. The results show that LC3-B protein expression was 25 in non-consumers of salbutamol and 125 in the drug consumers in the exposed group (P = 0.017). Salbutamol increased LC3-B protein in the lung of people exposed to SM. Also, the same result obtained when N-acetyl cysteine studied; 0 and 100 LC3-B protein values were observed in non-consumers and consumers of NAC in the exposed lung, respectively (P = 0.034). In the control group, we did not obtain this finding, and these two drugs did not affect LC3 protein expression at the lung of the control group (P = 0.8).

In the lung of SM exposed individuals, LC3 protein had a direct correlation with chronic bronchitis pathology (P = 0.029). Also, this protein was associated with malignancy diagnosis (Squamous-cell carcinoma (SCC) in the control group (P = 0.05).

3.4. The SM gas reduce LC3-II/LC3-I in western blotting test

After protein extraction from FFPE lung tissues, 100 μg was used for the detection of LC3 mount and determination of LC3-II/LC3-I ratio. The α-tubulin protein was consider as normalized control. The LC3-I, LC3-II and α-tubulin immune blot bands were quantified by Image-j software. After normalization with α-tubulin, median values of LC3-I, LC3-II, and LC3-II/LC3-I in the control group were 0.80, 0.31, and 0.52, respectively. These parameters for the SM exposed group were 0.70, 0.18, and 0.16, respectively. The statistical analysis shows that these differences were not significant (P = 0.533, P = 0.423, and P = 0.238, respectively). Fig. 3 shows the immunoblot of LC3 protein.

LC3-II protein and LC3-II/LC3-I ratio had inverse correlation with FEV1 spirometry parameter (r = −0.828, P = 0.000; r = −0.751, P = 0.001, respectively) in the exposed group. Also, the LC3-II/LC3-I ratio was higher in the exposed group who had chronic bronchitis pathology (P = 0.02). In the control group, LC3-II protein in malignancy cases was higher than that in the other pathologic conditions (P = 0.05).

4. Discussion

SM causes many complications in different organs include chronic bronchitis and pulmonary fibrosis in the respiratory system [43]; hyperpigmentation, dry skin and multiple cherryangiomas in the skin [44] and bulbar conjunctiva and limbal tissue abnormalities in the eyes [45]. Lung and pulmonary system are the major organs affected by the SM agent. Lung involvement extends from acute to chronic (long term and delayed phase) [46]. Many studies have been conducted in animal models and on human samples for determination of the delayed mechanism of SM gas many years after exposure. Emad and colleagues have shown an inflammation state in the lung of SM-exposed patients. They analyzed the cytokines such as IL-8, IL-1*β*, IL-6, TNF-*α*, IL-12, TGF-β, insulin-like growth factor-1, and epidermal growth factor in bronchoalveolar lavage fluids. They reported that these cytokines and chemokines were higher in SM-exposed patients with pulmonary fibrosis ratio compared to the healthy controls [47]. They also mentioned that IL-8, IL-1β, IL-6, TNF-α, IL-12 cytokines in the patients with bronchiectasis caused by SM gas were higher than that in people non-exposed to SM [48]. But other studies reject this hypothesis. Yaraee and colleagues reported the serum level of TNF, IL-1α, IL-1β, and IL-1Ra cytokines were significantly lower in the exposed group after 20 years than those in the controls [49]. Also, our previous study on FFPE lung samples of SM-exposed patients showed that mRNA expression of proinflammatory cytokines such as IL-1β, TNF-α and TNF-α receptor in the lung of SM-exposed people had no difference with non-SM-exposed patients with other pulmonary diseases [42]. Therefore, the

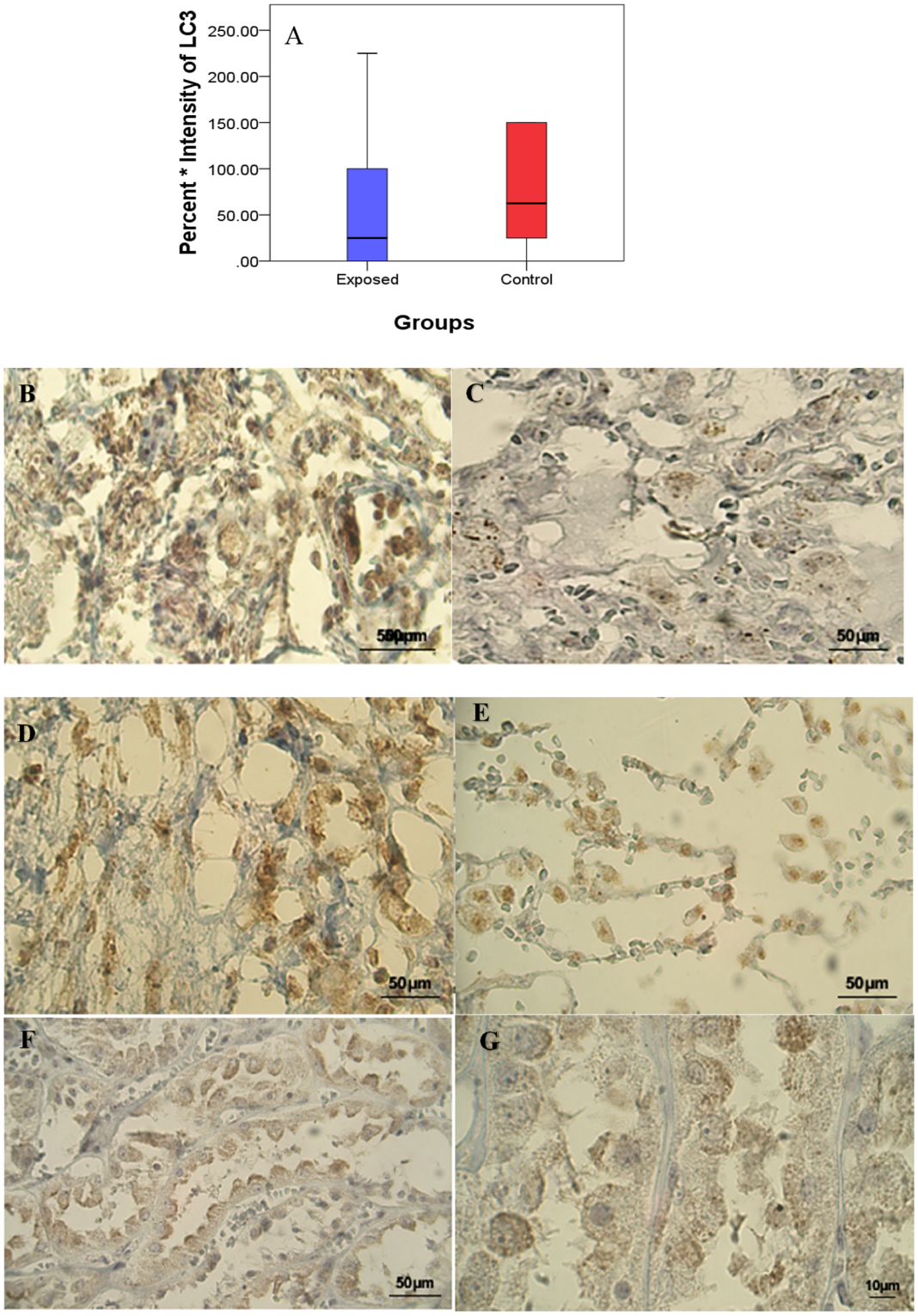

Fig. 2. Protein expression of LC3-B (A) detected by an antibody against this antigen in lung section in the exposed and control groups. LC3-B protein in the exposed lung non-significantly decreased. B and C show LC3-B positive cells in the control group lung and D and E show the distribution of LC3-B positive cell in SM-exposed lung. LC3-positive puncta are clear in D and E. F and G show positive control for LC3 molecule, human normal kidney tissue.

inflammation could not be the main reason for the observed complications in the pulmonary system of SM-exposed people many years after the exposure. Some research has shown that autophagy pathway has a protective effect in infectious, autoimmune, and inflammatory diseases [22], and autophagy regulates production and secretion of inflammatory cytokines and can inhibit inflammation initiation [1]. In the COPD disease have shown autophagy was inhibited in their macrophages that contribute to the excessive inflammatory response in airway [50].

On the other hand, Malaviya et al. reported that 6 h after the exposure of rats with SM gas, the LC3 expression transitionally increased in the rats' lungs. This expression was not observed 24 h after exposure,

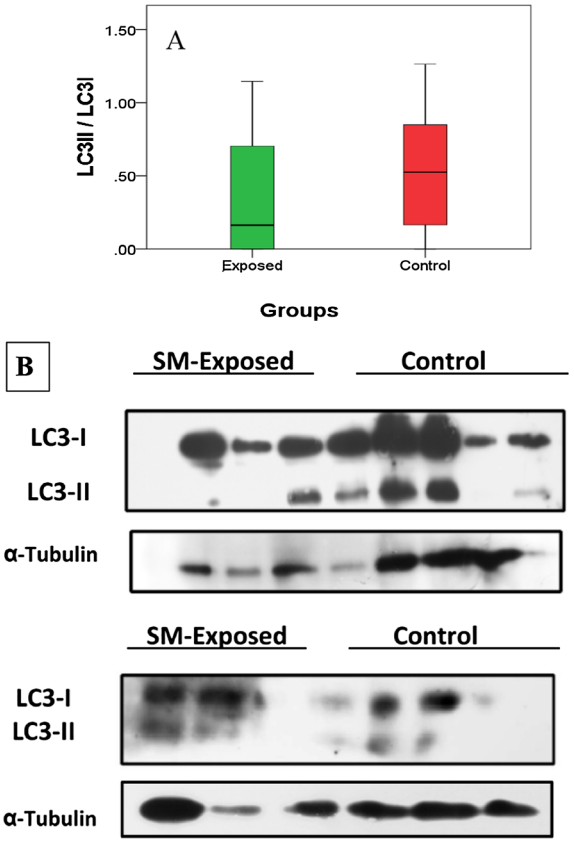

Fig. 3. The result of LC3 immunoblotting illustrate. (A) The boxplot curve of the median value of the LC3-II/LC3-I ratio after normalization by α-tubulin as the control protein. (B) The bands of LC3-I, LC3-II, and α- tubulin in different patients. LC3-II expression is higher than LC3-I in control group in compare with SM-exposed group. In first image, the first band) is a sample of low quality protein extracted from FFPE lung.

and there were no differences between LC3 expression of the exposed and non-exposed rats with SM gas [51]. Thus, SM gas exposure could induce autophagy in lung cells, and autophagy could regulate inflammation cascade.

Another inducer of autophagy pathway is oxidative stress and formation of intracellular reactive oxygen species [7]. Taravati et al. reported that antioxidant paraoxonase 1 activity decreased significantly in veterans who exposed to SM 20–25 years ago [52]. Also, Shohrati et al. reported serum level of glutathione in patients with a history of exposure to a single high dose of SM gas decreased and malondialdehyde activities in these patients increased compared to healthy nonsmoking individuals with no history of exposure to SM [53]. Another role of autophagy is the clearance of apoptotic cell to prevent inflammation or reduction of inflammation in efferocytosis mechanism [54]. Mosayebzadeh et al. compared apoptotic features and caspase-3 expression in FFPE lung of the SM-exposed people and control subjects and reported apoptotic cells were significantly higher in the lungs of the SM-exposed group > 20 years after exposure [55]. All above documents consider that autophagy pathway should occur for resolving many different stresses in the SM-exposed lung cells.

The present study shows that the mRNA level of LC3 molecule from the autophagy pathway in SM-exposed lungs significantly increased more than four-fold compared with that in the control group. But LC3 protein decreased in these samples. We infer that the lung cells are trying to increase autophagosome formation for reduction of SM gas stress, but an unknown mechanism reduces LC3 protein expression and inhibits the beneficial intracellular clearance. This agent regulates autophagy at the protein level and may inhibit autophagosome vacuole formation or reduce the half-life of LC3-B protein. The determination of this inhibitory mechanism needs further molecular studies. Also, we found that some drugs such as N-acetyl cysteine (NAC) and salbutamol would affect LC3 protein expression, and this protein increased in SM-exposed patients who had received these drugs. Pajonk and colleagues reported NAC could inhibit 26S proteasome function which blocked radiation induced NF-KB and affect on following genes expression [56]. Also the reduction of glutathione could active mammalian target of rapamycin (mTOR) and cause oxidative stress in cells. So, supplementation of NAC, as a precursor of glutathione, replenishes intracellular glutathione and inhibits the mTOR signaling and then induces autophagy. Afterward, autophagy could reduce oxidative stress and inhibit damages to the cells [57]. The improvement of symptoms and clinical conditions of SM-exposed patients which consume NAC compared to the placebo group [58] may be due to this effect of NAC. Also, salbutamol as β_2 adrenergic receptor agonist is known as an inducer of autophagy [59]; however, the mechanism of salbutamol action in the autophagy pathway has not been determined. Hence, NAC and salbutamol drugs attempt to recover the distorted condition of SM-exposed lung cells via increasing LC3 protein of the autophagy pathway.

In our study, LC3 mRNA was high, but an expression of Beclin-1 mRNA, another gene in the autophagy pathway, has not changed. Some studies show that autophagy has not a unique pathway and when Beclin-1 gene is knocked out by small interfering RNA (siRNA), LC3-associated phagocytosis (LAP) of *Burkholderia pseudomallei* in murine macrophage (RAW 264.7) cells is observed after rapamycin treatment but not after starvation condition. Therefore, autophagy is independent of Beclin-1 in rapamycin induction [60]. This finding confirms the observed rapamycin dependent autophagy pathway in SM-exposed people. The cells under stress condition select the best way for survival and resolve the stressor. Our result may be due to the use of an alternative pathway of autophagy in lung cells exposed to SM. Also, Adibzadeh et al. reported that the serum level of Vitamin D3 in the exposed people to SM diminished compared to non-exposed individuals [61]. As Vitamin D3 is an inducer of autophagy [62], administration of this vitamin along with NAC may synergistically induce autophagy.

This study was designed on archived FFPE lung of patients and obtaining the match sample to defined criteria was very problematic, also usage of less than human biological sample is an important ethical issue. So, we designed at least tests for detection of autophagy and we could not carry out additional tests on these samples such as IHC of P62 protein or prepare transmission electron microscopy (TEM) images.

Finally, we suggest that further studies should investigate the lung cells (type I or II pneumocytes, alveolar macrophage, etc.), which go through autophagy. This result helps us to determine the precise molecular mechanism of long-term and delayed effects of SM gas and is beneficial for decision making about the clinical approach for SM-injured people.

5. Conclusion

The evidence suggests that autophagy pathway should be stimulated for reduction of cellular stress in lung tissue of SM-exposed people. Thus, the therapeutic approaches that support autophagy clearance mechanism are useful for SM-injured people.

Declaration of Competing Interest

The authors report no conflict of interest in this study.

Acknowledgements

Immunoregulation Research Center of Shahed University has carried out this study and acknowledged the spiritual and financial supports of Shahed University and Janbazan Medical and Engineering Research Center (JMERC), Iranian Foundation of Martyr and Veterans

Affairs and the Ministry of Health and Medical Education, Tehran, Iran. We would like to kindly thank all the participants who took part in this study.

References

[1] J. Harris, Autophagy and cytokines, Cytokine 56 (2) (2011) 140–144.
[2] P. Mokarram, M. Ahmadi, S. Shojaei, S. Ghavami, Autophagy and cancer research in Iran, Autophagy 1–4 (2019).
[3] P. Mehrbod, S.R. Ande, J. Alizadeh, S. Rahimizadeh, A. Shariati, H. Malek, et al., The roles of apoptosis, autophagy and unfolded protein response in arbovirus, influenza virus, and HIV infections, Virulence 10 (1) (2019) 376–413.
[4] J. Alizadeh, S. Shojaei, A. Sepanjnia, M. Hashemi, E. Eftekharpour, S. Ghavami, Simultaneous detection of autophagy and epithelial to mesenchymal transition in the non-small cell lung cancer cells, Methods Mol. Biol. 1854 (2019) 87–103.
[5] B. Yeganeh, S. Ghavami, A.L. Kroeker, T.H. Mahood, G.L. Stelmack, T. Klonisch, et al., Suppression of influenza A virus replication in human lung epithelial cells by noncytotoxic concentrations bafilomycin A1, Am. J. Physiol. Lung Cell Mol. Physiol. 308 (3) (2015) L270–L286.
[6] B. Yeganeh, A. Rezaei Moghadam, J. Alizadeh, E. Wiechec, S.M. Alavian, M. Hashemi, et al., Hepatitis B and C virus-induced hepatitis: apoptosis, autophagy, and unfolded protein response, World J. Gastroenterol. 21 (47) (2015) 13225–13239.
[7] C. He, D.J. Klionsky, Regulation mechanisms and signaling pathways of autophagy, Ann. Rev. Genet. 43 (2009) 67–93.
[8] A.R. Moghadam, S.C. da Silva Rosa, E. Samiei, J. Alizadeh, J. Field, P. Kawalec, et al., Autophagy modulates temozolomide-induced cell death in alveolar Rhabdomyosarcoma cells, Cell Death Discov. 4 (2018) 52.
[9] M.A. Moosavi, M. Sharifi, S.M. Ghafary, Z. Mohammadalipour, A. Khataee, M. Rahmati, et al., Photodynamic N-TiO2 nanoparticle treatment induces controlled ROS-mediated autophagy and terminal differentiation of leukemia cells, Sci. Rep. 6 (2016) 34413.
[10] J. Wang, X.-L. Pan, L.-J. Ding, D.-Y. Liu, L. Da-Peng, T. Jin, Aberrant expression of Beclin-1 and LC3 correlates with poor prognosis of human hypopharyngeal squamous cell carcinoma, PloS One 8 (7) (2013) e69038-e.
[11] D.J. Klionsky, K. Abdelmohsen, A. Abe, M.J. Abedin, H. Abeliovich, A. Acevedo Arozena, et al., Guidelines for the use and interpretation of assays for monitoring autophagy, Autophagy. 12 (1) (2016) 1–222.
[12] A.A. Zeki, B. Yeganeh, N.J. Kenyon, M. Post, S. Ghavami, Autophagy in airway diseases: a new frontier in human asthma? Allergy 71 (1) (2016) 5–14.
[13] I. Mitroulis, I. Kourtzelis, K. Kambas, S. Rafail, A. Chrysanthopoulou, M. Speletas, et al., Regulation of the autophagic machinery in human neutrophils, Eur. J. Immunol. 40 (5) (2010) 1461–1472.
[14] M. Iranpour, A.R. Moghadam, M. Yazdi, S.R. Ande, J. Alizadeh, E. Wiechec, et al., Apoptosis, autophagy and unfolded protein response pathways in Arbovirus replication and pathogenesis, Expert Rev. Mol. Med. 18 (2016) e1.
[15] S. Ghavami, R.H. Cunnington, S. Gupta, B. Yeganeh, K.L. Filomeno, D.H. Freed, et al., Autophagy is a regulator of TGF-beta1-induced fibrogenesis in primary human atrial myofibroblasts, Cell Death Dis. 6 (2015) e1696.
[16] J. Alizadeh, A. Glogowska, J. Thliveris, F. Kalantari, S. Shojaei, S. Hombach-Klonisch, et al., Autophagy modulates transforming growth factor beta 1 induced epithelial to mesenchymal transition in non-small cell lung cancer cells, Biochim. Biophys. Acta Mol. Cell Res. 1865 (5) (2018) 749–768.
[17] J. Mintern, J. Villadangos, Autophagy and Mechanisms of Effective Immunity, 2012, 3(60).
[18] S. Hombach-Klonisch, M. Mehrpour, S. Shojaei, C. Harlos, M. Pitz, A. Hamai, et al., Glioblastoma and chemoresistance to alkylating agents: involvement of apoptosis, autophagy, and unfolded protein response, Pharmacol. Ther. 184 (2018) 13–41.
[19] N. Mizushima, T. Yoshimori, How to interpret LC3 immunoblotting, Autophagy 3 (6) (2007) 542–545.
[20] S. Ghavami, S. Gupta, E. Ambrose, M. Hnatowich, D.H. Freed, I.M. Dixon, Autophagy and heart disease: implications for cardiac ischemia-reperfusion damage, Curr. Mol. Med. 14 (5) (2014) 616–629.
[21] P. Mokarram, M. Albokashy, M. Zarghooni, M.A. Moosavi, Z. Sepehri, Q.M. Chen, et al., New frontiers in the treatment of colorectal cancer: autophagy and the unfolded protein response as promising targets, Autophagy 13 (5) (2017) 781–819.
[22] B. Levine, N. Mizushima, H.W. Virgin, Autophagy in immunity and inflammation, Nature 469 (7330) (2011) 323–335.
[23] G. Jia, G. Cheng, D.M. Gangahar, D.K. Agrawal, Insulin-like growth factor-1 and TNF-alpha regulate autophagy through c-jun N-terminal kinase and Akt pathways in human atherosclerotic vascular smooth cells, Immunol. Cell Biol. 84 (5) (2006) 448–454.
[24] S. Ghavami, B. Yeganeh, A.A. Zeki, S. Shojaei, N.J. Kenyon, S. Ott, et al., Autophagy and the unfolded protein response promote profibrotic effects of TGF-beta1 in human lung fibroblasts, Am. J. Physiol. Lung Cell Mol. Physiol. 314 (3) (2018) L493–L504.
[25] S.W. Ryter, A.M. Choi, Autophagy in lung disease pathogenesis and therapeutics, Redox Biol. 4 (2015) 215–225.
[26] Z.H. Chen, H.P. Kim, F.C. Sciurba, S.J. Lee, C. Feghali-Bostwick, D.B. Stolz, et al., Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease, PloS One 3 (10) (2008) e3316.
[27] M. Ghanei, I. Adibi, Clinical review of mustard lung, Iran. J. Med. Sci. (IJMS) 32 (2) (2007) 58–65.
[28] S. Mansour Razavi, P. Salamati, M. Saghafinia, M. Abdollahi, A review on delayed toxic effects of sulfur mustard in Iranian veterans, DARU, J. Pharm. Sci. 20 (1) (2012) 51.
[29] M. Ghanei, Z. Poursaleh, A.A. Harandi, S.E. Emadi, S.N. Emadi, Acute and chronic effects of sulfur mustard on the skin: a comprehensive review, Cutaneous Ocular Toxicol. 29 (4) (2010) 269–277.
[30] S.M. Razavi, P. Salamati, A.A. Harandi, M. Ghanei, Prevention and treatment of respiratory consequences induced by sulfur mustard in Iranian casualties, Int. J. Preventive Med. 4 (4) (2013) 383–389.
[31] M. Shahrzad, DAaMT, Lari, COPD Due to Sulfur Mustard (Mustard Lung). Chronic Obstructive Pulmonary Disease – Current Concepts and Practice, 2012.
[32] M. Ghanei, M. Mokhtari, M.M. Mohammad, J. Aslani, Bronchiolitis obliterans following exposure to sulfur mustard: chest high resolution computed tomography, Eur. J. Radiol. 52 (2) (2004) 164–169.
[33] M. Eghtedardoost, Z.M. Hassan, T. Ghazanfari, A. Sadeghipour, M. Ghanei, Evaluation of mRNA expression levels of TNFα, TNFR1 and IL1β in lung tissue 20 years after sulfur-mustard exposure, Iran J. Allergy Asthma Immunol. 17 (4) (2018) 379–387.
[34] M. Eghtedardoost, Z.M. Hassan, N. Askari, A. Sadeghipour, M.M. Naghizadeh, S. Ghafarpour, et al., The delayed effect of mustard gas on housekeeping gene expression in lung biopsy of chemical injuries, Biochem. Biophys. Reports 11 (2017) 27–32.
[35] O. Eikrem, C. Beisland, K. Hjelle, A. Flatberg, A. Scherer, L. Landolt, et al., Transcriptome sequencing (RNAseq) enables utilization of formalin-fixed, paraffin-embedded biopsies with clear cell renal cell carcinoma for exploration of disease biology and biomarker development, PLoS One 11 (2) (2016) e0149743.
[36] www.biotools.nubic.northwestern.edu/OligoCalc.
[37] M.W. Pfaffi, A new mathematical model for relative quantification in real time RT-PCR, Nucl. Acids Res. 29 (9) (2001) 1–6.
[38] M.T. Rosenfeldt, C. Nixon, E. Liu, L.Y. Mah, K.M. Ryan, Analysis of macroautophagy by immunohistochemistry, Autophagy 8 (6) (2012) 963–969.
[39] Y. Kawashima, Y. Kodera, A. Singh, M. Matsumoto, H. Matsumoto, Efficient extraction of proteins from formalin-fixed paraffin-embedded tissues requires higher concentration of tris(hydroxymethyl)aminomethane, Clin. Proteomics (2014;11(1):) 4-.
[40] D.B. Friedman, S. Hoving, Westermeier R, Methods Enzymol. (2009).
[41] T. Mahmood, P.-C. Yang, Western blot: technique, theory, and trouble shooting, N. Am. J. Med. Sci. 4 (9) (2012) 429–434.
[42] M. Eghtedardoost, Z. Hassan, T. Ghazanfari, A. Sadeghipour, M. Ghanei, Evaluation of mRNA Expression Levels of TNFα, TNFR1 and IL1β in Lung Tissue 20 Years after Sulfur-mustard Exposure, Iran J. Allergy Asthma Immunol. 17 (4) (2018) 379–387.
[43] M. Balali-Mood, S. Mousavi, B. Balali-Mood, Chronic health effects of sulphur mustard exposure with special reference to Iranian veterans, Emerg. Health Threats J. 1 (2008) e7-e.
[44] M. Hefazi, M. Maleki, M. Mahmoudi, A. Tabatabaee, M. Balali-Mood, Delayed complications of sulfur mustard poisoning in the skin and the immune system of Iranian veterans 16–20 years after exposure, Int. J. Dermatol. 45 (9) (2006) 1025–1031.
[45] H. Ghasemi, A. Mostafaie, R. Yaraee, Z.M. Hassan, A. Rezaei, M. Mahmoudi, et al., Association of serum immunoglobulins levels and eye injuries in sulfur mustard exposed: Sardasht-Iran Cohort Study, Int. Immunopharmacol. 17 (3) (2013) 944–951.
[46] M. Ghanei, A.A. Harandi, The respiratory toxicities of mustard gas, Iran J. Med. Sci. 35 (4) (2010).
[47] A. Emad, Y. Emad, Levels of cytokine in bronchoalveolar lavage (BAL) fluid in patients with pulmonary fibrosis due to sulfur mustard gas inhalation, J. Interferon Cytokine Res.: Official J. Int. Soc. Interferon Cytokine Res. 27 (1) (2007) 38–43.
[48] A. Emad, Y. Emad, CD4/CD8 ratio and cytokine levels of the BAL fluid in patients with bronchiectasis caused by sulfur mustard gas inhalation, J. Inflamm. 4 (2007) 2.
[49] R. Yaraee, T. Ghazanfari, M. Ebtekar, S.K. Ardestani, A. Rezaei, A. Kariminia, et al., Alterations in serum levels of inflammatory cytokines (TNF, IL-1alpha, IL-1beta and IL-1Ra) 20 years after sulfur mustard exposure: Sardasht-Iran cohort study, Int. Immunopharmacol. 9 (13–14) (2009) 1466–1470.
[50] N. Vij, P. Chandramani-Shivalingappa, C. Van Westphal, R. Hole, M. Bodas, Cigarette smoke-induced autophagy impairment accelerates lung aging, COPD-emphysema exacerbations and pathogenesis, Am. J. Physiol. Cell Physiol. 314 (1) (2018) C73–C87.
[51] R. Malaviya, V.R. Sunil, J. Cervelli, D.R. Anderson, W.W. Holmes, M.L. Conti, et al., Inflammatory effects of inhaled sulfur mustard in rat lung, Toxicol. Appl. Pharmacol. 248 (2) (2010) 89–99.
[52] A. Taravati, S.K. Ardestani, M.R. Soroush, S. Faghihzadeh, T. Ghazanfari, F. Jalilvand, et al., Serum albumin and paraoxonase activity in Iranian veterans 20 years after sulfur mustard exposure, Immunopharmacol. Immunotoxicol. 34 (4) (2012) 706–713.
[53] M. Shohrati, M. Ghanei, N. Shamspour, F. Babaei, M.N. Abadi, M. Jafari, et al., Glutathione and malondialdehyde levels in late pulmonary complications of sulfur mustard intoxication, Lung 188 (1) (2010) 77–83.
[54] D.R. Green, T.H. Oguin, J. Martinez, The clearance of dying cells: table for two, Cell Death Differentiation 23 (2016) 915.
[55] M. Mosayebzadeh, T. Ghazanfari, A. Delshad, H. Akbari, Evaluation of apoptosis in the lung tissue of sulfur mustard-exposed individuals, Iran J. Allergy Asthma Immunol. 15 (4) (2016) 283–288.
[56] F. Pajonk, K. Riess, A. Sommer, W.H. McBride, N-acetyl-L-cysteine inhibits 26S proteasome function: implications for effects on NF-kappaB activation, Free Radical Biol. Med. 32 (6) (2002) 536–543.
[57] D. Shah, S.K. Nath, Glutathione: a possible link to autophagy in systemic lupus erythematosus, Am. J. Immunol. 10 (3) (2014) 114–115.

[58] M. Shohrati, J. Aslani, M. Eshraghi, F. Alaedini, M. Ghanei, Therapeutics effect of N-acetyl cysteine on mustard gas exposed patients: evaluating clinical aspect in patients with impaired pulmonary function test, Respiratory Med. 102 (3) (2008) 443–448.
[59] K. Nakahira, A.M.K. Choi, Autophagy: a potential therapeutic target in lung diseases, Am. J. Physiol. Lung Cell. Mol. Physiol. 305 (2) (2013) L93–L107.
[60] X. Li, M. Prescott, B. Adler, J.D. Boyce, R.J. Devenish, Beclin 1 is required for starvation-enhanced, but not rapamycin-enhanced, LC3-associated phagocytosis of Burkholderia pseudomallei in RAW 264.7 cells, Infection Immunity 81 (1) (2013) 271–277.
[61] M.M. Adibzadeh Seresgi, S. Ardestani Kaboudanian, S. Faghihzadeh, T. Ghazanfari, Vitamin D3 reduction in individuals exposed to sulfur mustard, %J Immunoregul. 1 (2) (2018) 93–100.
[62] S. Wu, J. Sun, D. Vitamin, Vitamin D receptor, and macroautophagy in inflammation and infection, Discovery Med. 11 (59) (2011) 325–335.
[63] S. Khateri, M. Ghanei, S. Keshavarz, M. Soroush, D. Haines, Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustard agent, J. Occup. Environ. Med. 45 (11) (2003) 1136–1143.