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The delayed effect of mustard gas on housekeeping gene expression in lung biopsy of chemical injuries

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ABSTRACT

Objective: Sulfur mustard (SM) was used as a chemical weapon in Iraq-Iran war. Exposed people have major complications in important organs such as pulmonary system. Some studies have shown that SM could affect the expression of endogenous genes and non-housekeeping genes, time dependently. To understand the accurate molecular mechanism of the delayed effect of SM, the identification of the gene expression pattern in these patients is essential. Hence, we have evaluated mRNA expression of four common housekeeping genes (ACTIN, PGK1, β2m, GAPDH) in SM-exposed and non-exposed (control) formalin-fixed, paraffin-embedded (FFPE) human lung tissues.

Method: Paraffin block of lung biopsy of SM-exposed people (11 cases) and people without exposure to SM as control group (9 cases) have been selected. The mRNA expression of four endogenous control genes has been evaluated by qRT-PCR. The stability value of each gene was calculated by different methods.

Result: It was found that ACTIN mRNA has the highest expression (30.26 ± 2.87) and PGK1 has the lowest standard deviation (SD) (30.885 ± 2.215) between pooled groups. The best correlation was between ACTIN and PGK1 expressions. The M value has shown that ACTIN and then PGK1 are the most stable housekeeping genes among. The results obtained from the GeNorm and NormFinder have indicated that the pair ACTIN- PGK1 is the most suitable choice for endogenous control genes.

Conclusion: ACTIN and PGK1 genes are stable in studied lung tissues and are the better than two other housekeeping genes. In addition, mustard gas does not affect their expression in long term.

1. Introduction

Sulfur mustard (SM) is known as a chemical weapon and has been used by Iraq military forces several times in Iraq-Iran war during 1980–1988 [1]. SM is an alkylating agent which alters the DNA structure and may cause formation of intra- or interstrand crosslinks which finally induce double strand breaks in DNA [2]. Onset of the signs and the symptoms of SM exposure is 30 min to 6 weeks after exposure. But, exposed people suffer from delayed toxic effects of SM for even more than 20 years after exposure [3]. The main problem of the chemical injured people is ocular and pulmonary complications as well as skin tissue involvements [4]. Despite numerous studies, the molecular mechanism of SM in chronic phase and curative treatment for its long-term respiratory complications has not been found yet [2,5]. Therefore, a molecular study to identify the precise mechanism of tissue damage is necessary to promote the diagnosis and treatment procedures.

Some studies have shown that SM has effect on gene expression in vitro and in vivo conditions. Steinritz et al. [6] showed that the GAPDH protein (Glyceraldehyde-3-Phosphate Dehydrogenase), a usual housekeeping gene, is up regulated when human keratinocyte cell line is exposed to SM. Also Vallet et al. [7] demonstrated that SM has a time dependent effect on gene expression in hairless mice. They showed that expression of IL-6, IL-1b, MIP-1aR and Cxcl2 genes have upregulated as early as 6 h from exposure to after sufficient time for wound repair (over 14 days). However, K1 mRNA level increased only 21 days after SM challenge.

Because any alteration in gene expression could be a potential source for clinical problems of SM exposed people, it is necessary to

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evaluate genes expression in these patients. To this end, molecular techniques such as quantitative real-time PCR (qPCR) are being used by researchers. qPCR is known as a gold-standard assay for measuring the gene expression and is used for confirmation of microarray data [8]. This method is commonly performed to achieve the pattern of target genes expression due to its high sensitivity, specificity and broad quantification range [9]. The internal controls are essential to ensure the accuracy and reliability of qPCR results and also to normalize the target gene with an endogenous gene. Hence, housekeeping genes are indispensable to molecular studies. They should have stable expression in all tissue and cells of organism and do not affected by the external signal or cell cycle stages [10].

Given that sulfur mustard could alter the DNA structure and gene expression, in this study, which is a preliminary segment, we have tried to evaluate mRNA expression level of different housekeeping genes in lung tissue of chemical injuries after 25–30 years after SM exposure. The studied housekeeping genes include beta actin(β-actin) is a major part of the contractile apparatus, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the main enzymes of glycolysis pathway,beta-2-microglobulin (β2m)) is an extracellular part of MHC I complex, and phosphoglycerate kinase 1 (PGK1) catalyzes reversible transfer of a phosphate group from 1,3-bisphosphoglycerate. Finally, the most stable endogenous gene in SM exposed people has been chosen by comparing the threshold cycle (CT) mean, dispersion of studied genes expression, and calculating the gene expression stability measure (M value).

2. Material and method

2.1. Ethical statement

This study is part of a comprehensive research which has been approved in Immunoregulation Research Center, Shahed University and Research Ethics Committees of Shahed University and Medical Faculty of Trabiat Modares University.

2.2. Sample collection

This study has been performed on paraffin blocks of lung biopsy. All samples have been collected from archived blocks of department of pathology. These blocks have been obtained by surgical resection at general hospitals in Tehran, Iran during 2005–2011. The samples were used with a code without any name or other individual characteristic. The exposed group was lung biopsy of people with documented mustard gas exposure in Iraq-Iran war (1980–1988), which had delayed pulmonary complications (n = 11). SM exposed people were excluded if they had systemic or local diseases affecting on the study like acute and chronic infection, autoimmune disease, history of other toxic gases exposure and job pollutions. The control group (n = 9) was lung biopsy of patients that they had undergone surgery for diagnosis of their pulmonary diseases. The main inclusion criteria for control group was; to have normal histology block. These patients should not had history of exposure to mustard gas or other toxic gases and occupational pollutions, chronic disorders such as systemic or pulmonary inflammation diseases, systemic or local acute and chronic infection, autoimmune disease, and asthma. The hematoxylin and eosin (H & E) stain slides of control group evaluated by a pathologist. Blocks with normal histology were confirmed and were chosen for study. Both groups were men with age 30–60 years and were not smoker and had no history of addiction to opiates and alcohol. More data about samples is shown in Table 1.

Table 1
Histopathological characteristics of the exposed and control groups.

	Exposed (n = 11)	Control (n = 9)
Age (y)	42.7(33–61)	51.1(24–67)
Sex	Male	Male
Diagnosis		
Constructive bronchiolitis	7	0
Chronic bronchitis	2	0
Bronchiectasis	1	1
Anthracosis	0	2
Benign tumor	1	1
Malignant tumor	0	4
Sequestration chronic inflammation	0	1

2.3. RNA isolation and DNase treatment

Total RNA was extracted from collected tissue specimens by RNeasy FFPE Kit (Qiagene- Germany) and the manufacturer's protocol was carried out with a little modify. Firstly, four 10 μm sections were cut from each specimen and were deparaffinized in xylene (Merk-Germany) at 56 °C for 2 × 30 min with 400 rpm agitation. The residual xylene was washed by ethanol 96% (Merk-sigma). After drying in the air, the cell membrane was disrupted by heating at 56 °C for 3 h with proteinase K. In the following, proteinase K was inactivated with incubation of the pervious step supernatant at 80 °C for 15 min. DNase treatment was done by mixing of DNase Booster and DNase I and its time was increased to 20 min. Then, RBC and RPE buffers were added exactly according to the manufacturer's protocol, respectively. The extracted RNA was washed from RNeasy® MinElute® Spin Columns by RNase free water. The concentration and purity of RNA were measured via the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The quality of RNA sample was determined according to OD 260/280 ratio and the sample was discarded when OD 260/280 ratio was less than 1.8. The common method for quality measurement of the extracted RNA is determination of agilent RNA integrity number (RIN). But, it has been shown that the RIN values from degraded FFPE samples are not a sensitive measure of RNA quality. Instead, the investigators have found that the mean RNA fragment size is useful as a determinant of RNA quality for the extracted RNA from FFPE tissues [11]. Therefore we have used the percentage of RNA fragments with more than 200 nucleotides (DV200) to evaluate the quality of RNA. DV200 metric analysis was performed by Macrogen Co. (Korea) and the RNA samples with DV200 > 30% were accepted.

Our pilot study, which its results are not presented here, revealed that despite DNase treatment was done in RNA isolation step, genomic DNA contamination has remained yet. Therefore extra DNase treatment (fermentase protocol) was carried out on eluted RNA. For this aim, 1 μl buffer and 1 μl DNase was mixed for each sample. Then 1 μg extracted RNA was added and incubated at 37 °C for 45 min and 1 μl EDTA was added. After that, incubation was done at 65 °C for 10 min.

2.4. Reverse transcription

Reverse transcription of the pure RNA was performed by a high capacity cDNA reverse transcription kit (ABI-USA). According to the manufacturer's instructions, to prepare 2X reverse transcription master mix, 2 μl 10X RT buffer, 0.8 μl 25X dNTP Mix (100 mM), 2 μl 10X RT random primers and 1 μl MultiScribe™ Reverse Transcriptase were mixed together. For each sample, 10 μl master mix was added to 1 μg extracted RNA (~10 μl of RNA) and final volume per reaction was increased to 20 μl by nuclease-free H_2O. The reactions took place at 25 °C for 10 min, followed by 37 °C for 120 min, and 85 °C for 10 min in a thermal cycler T100 (BioRad, USA).

2.5. Real time PCR TEST

The mRNA expression of four endogenous control genes; ACTB (NM_001101.3), β2m(NM-004048), GAPDH (NM-002046) and PGK1(NM_000291.3), were determined by comparing their expression in different samples. Their primers were manually designed according

to NCBI mRNA sequences except the GAPDH primer. It was used from Giulietti and collagenous study [12]. All primers span exon-exon junction. Thermodynamic state and secondary structure of primers were determined by Gene Runner 5.0.1 software and online oligo calculator software [13]. The primers were synthesized commercially (TAG copenhagen- Denmark). Real-time PCR was then performed with SYBR Green I dye on the Applied Biosystems StepOnePlus™ Real-Time PCR System- USA.

We used power sybr green 1 master mix (ABI-USA) for Real time PCR reagent. For each well, 10 µl ready to use master mix 2X, 7 µl RNase free H2o, 1 pmol forward primer (~0.5 µl), 1 pmol reverse primer (~0.5 µl), and 2 µl 1:3 dilute cDNA of each sample as the template were mixed. An amplification program was applied according to the following steps: 1) 10 min of denaturation at 95 °C, 2) 40 cycles of real-time PCR with 2-step amplification including 15 s at 95 °C for denaturation and 60 s at 60 °C for annealing and polymerase elongation, 3) adding a melt curve stage to the end of the run with slow heating started at 60 °C with a rate of 0.3 °C per second up to 95 °C with continuous measurement of fluorescence. All samples were performed in triplicate.

The real time PCR efficiency of the primers was evaluated using 1:10 dilution of PCR product of each gene. The sequences of the primers were reported at Table 2.

2.6. Statistical analysis

Threshold cycle (CT) value of each sample was determined by ABI Step One 2.3 software (ABI-USA). Mean, standard deviation (SD), and coefficient of variation (CV) were computed. The test of normality was done and then the one-way analysis of variance (ANOVA) test was performed to compare the mean value of CT in control and exposed group considering the p value ≤ 0.05 as significant. Correlation between housekeeping gene expressions was calculated by the Pearson correlation analysis.

Statistical analyses were performed by IBM SPSS 22 (SPSS Inc, Chicago Ill). Stability of each gene was analyzed by Two different statistical algorithms designed for this purpose [14] using Microsoft Excel add-in, NormFinder (N1) and GeNorm, in a pool of sample group. NormFinder calculates the stability value according to the combined estimate of intra- and intergroup expression variations of the studied genes. GeNorm calculates the stability value (M value) by averaging the pairwise variation of a particular gene with respect to all other candidate reference genes. The lower the M value of a given gene, the more consistent its expression relative to other genes in a multiplex [15]. Thus, we compared the CV, the NormFinder, and the GeNorm approaches and ranked the candidate genes. For these analysis, the data was transformed by the formula $\eta^{(\text{min-CT})}$, where η is the efficiency of the primer, and min is the minimum of CT.

3. Results

3.1. Distribution expression of the endogenous genes

CT value of each gene of control and exposed samples was obtained by Real Time software automatically. Mean ± SD of CT in exposed and control groups are shown in Table 3. ACTIN and after that GAPDH, two popular reference genes, had the lowest expression in control (30.70 ± 3.13) and exposed (29.89 ± 2.73) lung tissues. But, GAPDH had the most variation among all genes (SD = 3.770).

The calculated coefficient of variation of each gene showed that GAPDH is very distributed in different samples of both control (CV = 10.1%) and exposed (CV = 14.2%) groups (Table 3). ACTIN and secondly PGK1 had the least expression change in different individuals. The distribution of reference gene expressions is presented in Fig. 1. One-way ANOVA test showed no significant difference between expression of genes in control and exposed groups.

3.2. Correlation between four reference genes

To measure the strength of a linear association between the expressions of these genes, we used correlation coefficient according to Pearson correlation analysis. The relationship between the two genes was determined. Results are shown in Table 4. The strongest correlation is between ACTIN and PGK1 ($r = 0.779$, $p < 0.0001$) in pooled control and exposed lung tissues. This relationship is shown in detail in Fig. 2. β_2m and PGK1 have the weakest correlation between pairwise genes ($r = 0.514$, $p < 0.0001$).

At the final step of determining the correlation between genes, we studied that whether the expression of a single gene can show the mean expression of the three other genes. Hence, the CT value of each gene was compared to the mean CT value of the three other genes. Correlation coefficient (r) was computed for control and exposed groups as well as pooled of these groups (Table 5).

The expression of β_2m had the highest coefficient of correlation ($r = 0.864$, $p = 0.003$) compare to the other genes in control group. In exposed group, ACTIN had the best correlation ($r = 0.822$, $p = 0.002$) with other genes. But in mix CT value of control and exposed groups, GAPDH had the highest correlation coefficient ($r = 0.833$, $p < 0.001$).

In the following, we calculated the mean difference between the CT of each gene and the mean CT of the other three genes. This computation allows us to show the fixed distance between the expression of each housekeeping gene and the mean expression of the other genes. Finally, GAPDH expression had the lowest difference. This means that GAPDH expression could show the mean expression of other genes. But, calculation of the accuracy (2 × SD) illustrated the diversion degree of each endogenous control gene expression from the remaining genes (Table 5). Consequently, ACTIN had the minimum deviation among the other endogenous genes.

3.3. Stability determination of four housekeeping genes

We characterized the expression stability of the studied genes. For this goal, we used geNorm and NormFinder softwares. Analysis of the raw CT data using geNorm indicated that ACTIN had the lowest M value (2.305) followed by PGK1 (2.417), β2M (2.825), and GAPDH (2.936). Therefore, ACTIN is the most stable gene among these four housekeeping genes and GAPDH had the most expression variation. GeNorm software could determine the suitable pair of genes as the reference gene for qRT-PCR. Fig. 3 illustrates the average expression stability value of remaining control genes. As a result, the pair ACTIN-PGK1 is determined as the best endogenous gene in control and exposed lung tissues compare to two other housekeeping genes.

Table 2
The sequences of the primes.

	Forward (3 > 5)	**Reverse** (3 > 5)	**Amplicon length (bp)**
Actin	CGTCTTCCCCTCCATCGTG	GGTGAGGATGCCTCTCTTGCTC	111
B2m	GGCTATCCAGCGTACTCCAAAG	ACCCAGACACATAGCAATTCAGG	92
GAPDH	TCACCACCATGGAGAAGGC	GCTAAGCAGTTGGTGGTGCA	168
PGK	GGCATACCTGCTGGCTGGATG	ACAGGACCATTCCACACAATCTGC	104

Table 3
Mean ± SD of CT and CV (coefficient of variation × 100) in the housekeeping genes expression.

Housekeeping genes	Control (n = 11)		Exposed (n = 9)		Pooled (n = 20)	
	Mean ± SD	CV%	Mean ± SD	CV%	Mean ± SD	CV%
ACTIN	30.70 ± 3.13	10.2	29.89 ± 2.73	9.1	30.26 ± 2.87	9.5
β_2M	31.667 ± 2.52	8.0	30.582 ± 3.67	12.0	31.070 ± 3.17	10.2
PGK1	31.408 ± 2.16	6.9	30.457 ± 2.26	7.4	30.885 ± 2.21	7.2
GAPDH	31.253 ± 3.15	10.1	29.930 ± 4.26	14.2	30.526 ± 3.77	12.4

The data are presented as mean ± standard deviation (SD) of CT value and percent of coefficient of variation of each gene is calculated by (SD/mean) × 100 in different samples of control and exposed groups. In pooled column, mean ± SD and CV% of two groups is shown. ACTIN: β-actin gene, β $_2$M: β_2 microglobulin gene, PGK1: Phosphoglycerate kinase 1gene, GAPDH: glyceraldehyde-3-phosphate dehydrogenase gene. Control group means non exposed people with mustard gas (n = 9), and exposed group is patients with mustard gas exposure (n = 11).

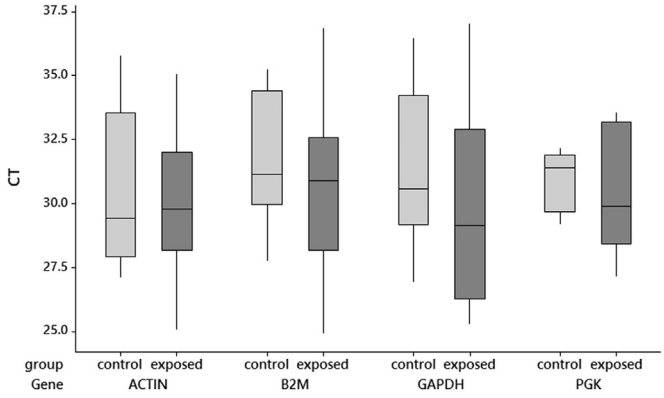

Fig. 1. Distribution of housekeeping gene expressions in the control and exposed groups. High expression dispersion of some genes such as GAPDH is clear.

Table 4
Correlation between the studied housekeeping genes.

	β_2M	PGK1	GAPDH
ACTIN	0.711	0.779	0.637
β $_2$M		0.514	0.586
PGK1			0.703

Pearson correlation coefficient, *r value*, between two housekeeping genes is shown.

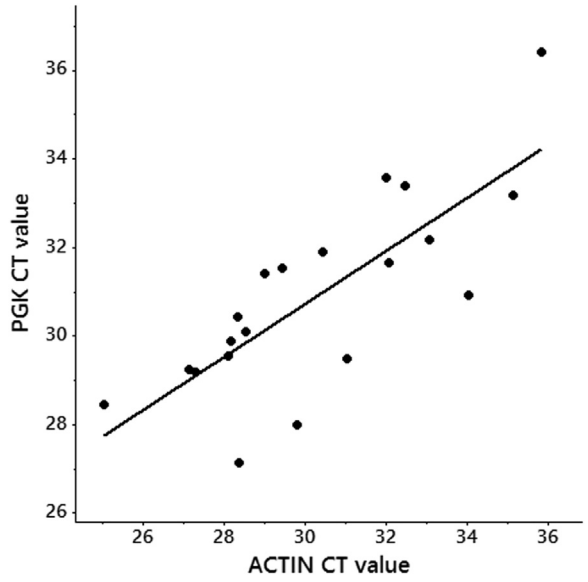

Fig. 2. Correlation between expression of ACTIN and PGK1 genes in lung tissues.

Table 5
Coefficients of correlation between each gene and the mean CT of the remaining three genes.

		Coefficients of correlation			MD C + E	AC C + E
		Control	Exposed	Pooled (total)		
ACTIN	Pearson Correlation	0.813	0.822	0.810	−0.57	3.42
	Sig. (2-tailed)	0.008	0.002	0.000		
β $_2$M	Pearson Correlation	0.864	0.581	0.680	0.51	4.76
	Sig. (2-tailed)	0.003	0.061	0.001		
PGK1	Pearson Correlation	0.742	0.759	0.761	0.27	3.7
	Sig. (2-tailed)	0.022	0.007	0.000		
GAPDH	Pearson Correlation	0.688	0.729	0.833	−0.21	5.28
	Sig. (2-tailed)	0.040	0.011	0.000		

The illustrated data are the coefficients of correlation (r) of CT value of each housekeeping gene compared to the CT value mean of other genes in control, exposed groups and pooled of two groups. MD: mean difference, AC: accuracy (2 × SD), C + E: control + exposed group.

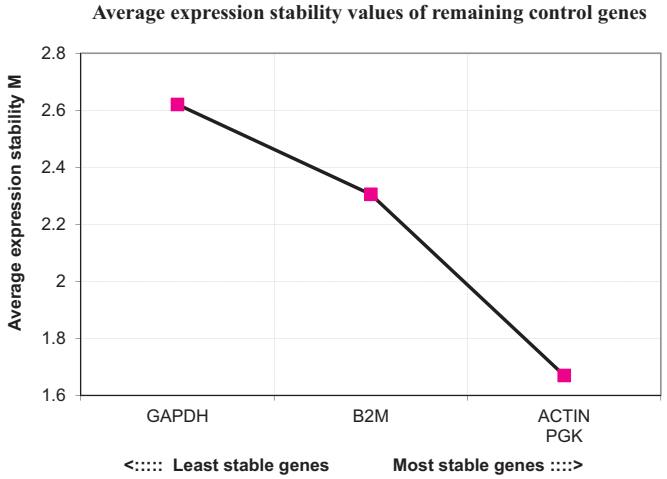

Fig. 3. Average expression stability M was show and ACTIN, PGK1 are most stable genes, GAPDH is least stable gene.

NormFinder software is another tool for determination of the expression stability of genes. This algorithm also confirms the results obtained from geNorm. According to NormFinder output, the best gene in terms of stability is ACTIN with the stability value of 0.248, and the best combination of two genes is ACTIN and PGK1 with the stability value of 0.207.

4. Discussion

In this study, we tried to evaluate mRNA expression rate of four common housekeeping genes in formalin-fixed, paraffin-embedded (FFPE) lung tissues of people who were exposed to mustard gas, and compared the results with non-exposed (control) samples. We found that ACTIN mRNA was the most frequent expressed gene in both control and exposed groups, whereas PGK1 has the least standard deviation and discrepancy among other genes in different individuals of one group. The best correlation was between ACTIN and PGK1 expressions. In addition, when one of these gene's expression had increase or decrease the other one had the same trend. This is important for normalization of a target gene by these two housekeeping genes.

GAPDH mRNA had the lowest mean difference among the other three housekeeping genes in pooled results of control and exposed lung tissues. This means that GAPDH expression was slightly different from the average expression of other genes and its expression was almost equal to the mean expression of the remained three endogenous control genes. However, this endogenous gene had the lowest accuracy and the most variation in different patients. M value which refers to the expression stability rate of a gene showed that ACTIN and secondly PGK1 are the most stable housekeeping genes among the other, while GAPDH had the least M value which characterized it as a non-stable gene. GeNorm determined that the pair ACTIN- PGK1 is the best choice for endogenous control gene among these housekeeping genes and NormFinder confirmed these results. This study had a secondary result. We studied on stability of GUSB (glucuronidase, beta) but pilot result show, in exposed people may be expression of a new transcript of this control gene in the lung. Of course this claim needs more study.

A suitable housekeeping gene should has a high stable expression and external conditions does not affect its mRNA value [16]. Liu et al. studied mRNA expression of seven endogenous control genes in non-small cell lung cancer (NSCLC) tissues, namely, GAPDH, ABL1, β2m, HPRT1, PGK1, PPIA, and RPLP0 [17]. They found that GAPDH is a stable internal control gene, while β2m and PGK1 are not suitable housekeeping genes. Regarding their results, we decided to evaluate the expression of GAPDH, PGK1, and β2m genes on human lung tissue. Moreover, we selected ACTIN gene because it is usually used as an internal control in molecular techniques and is known as an ideal reference gene for qRT-PCR analysis [18]. In spite of the fact that Glare et al. reported that b-Actin and GAPDH housekeeping genes are variable in asthmatic patients and not suitable for normalizing mRNA levels [19], in our study ACTIN and PGK1 were stable in examined lung tissues. This discrepancy between the obtained results might be caused by the difference in the disease type and the human race reviewed. Therefore, it is strongly recommended that the expression of different endogenous genes to be reviewed before they are used in quantitative mRNA assays in a study.

The numerous studies on people who exposed to sulfur mustard have shown that this toxic gas could affect genes expression of different pathways in long term. For instance, metallothionein-1A mRNA has up-regulated in patients who had exposed to sulfur mustard [20]. Moreover, the mRNA level of CuZn Superoxide dismutase and Mn Superoxide dismutase has up-regulated in SM-injured patients compared to control groups [21]. Nevertheless, in some cases no change has been observed in gene expression of mustard gas injuries in comparison with control people in long term. For example, TLR4 gene expression in lung tissue of chemical injuries remained the same as non-exposed people [22]. On the other hand, in a study of acute effect of SM on cell culture it has been shown that GAPDH is up-regulated at the protein level [6]. Previous studies have shown that this gas has an impact on endogenous genes in acute phase and on non-housekeeping genes at delayed phase. Our results showed that the sulfur mustard does not significantly affect the studied housekeeping genes expression in long term. This difference between results may be due to different repair system in housekeeping and ordinary genes. Of course, the document is insufficient for this hypothesis. This idea should be investigated in continuous studies such as the animal model and evaluated step by step from acute phase to chronic and delayed phases to find the SM effect on conserved and functional genes and to figure out their response to this toxicant.

The study on human tissue has many limitation, especially lung tissue, because of obtain the section of lung is very invasive and dangerous for patient, whether exposed or control individual. So, there were severely limits in number of samples. Hence, University and Research Ethics Committees allow minimum sample size that was confirmed by statistics formula. But there are two important points in our study. Firstly, we used archival FFPE tissues and did not take fresh lung biopsy from patients. The ethical notice is that a human study does not necessarily require a fresh sample and archival biopsy samples can be used for this purpose. Secondly, although the patients of the control group did not have similar diseases, nevertheless the stability of housekeeping genes was not affected and their mRNA expression remained constant.

Finally, to find the accurate molecular mechanism of SM effect on the gene expression in long term, we suggest a whole genomic study on a large population of SM gas victims.

5. Conclusion

We evaluated expression deviation and stability of four common housekeeping genes in lung tissues of exposed and non-exposed to mustard gas people. We found that ACTIN and PGK1 are the best reference genes among studied genes. The determined stable housekeeping genes could be used in molecular studies on expression of different genes in mustard gas exposed people.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.04.012.

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