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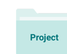

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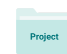

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Immunopharmacology and Immunotoxicology

http://informahealthcare.com/ipi
ISSN: 0892-3973 (print), 1532-2513 (electronic)
Immunopharmacol Immunotoxicol, 2013; 35(3): 419–425
© 2013 Informa Healthcare USA, Inc.
DOI: 10.3109/08923973.2013.797993

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RESEARCH ARTICLE

Assessing the relationship of paraoxonase-1 Q192R polymorphisms and the severity of lung disease in SM-exposed patients

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Abstract

Late respiratory complications in patients suffering from pulmonary lesions due to sulfur mustard (SM) gas are asthma, chronic obstructive pulmonary disease and bronchiectasis. Recently PON1 antioxidant activity draws attention as the enzyme which prevents the oxidation of lipoproteins during oxidative stress. In this study we aimed to investigate PON1 192 polymorphisms and paraoxonase and arylesterase activity in the serum of SM-exposed lung disease patients. Also, we examined the detection of PON1 and apoA1 proteins in BAL fluid. 101 male patients were included who were categorized to three groups of mild, moderate and severe suffering from pulmonary lesions due to SM. Significant reduction in paraoxonase activity [Healthy: 412.46 ± 89.1 U/L, Severe: 89.66 ± 20.7 U/L] ($p < 0.0001$) and arylesterase activity [Healthy: 25826.4 ± 4425.23 U/L, Severe: 16760.43 ± 3814.9 U/L] ($p < 0.0001$) with increase in severity of disease was demonstrated statistically. With respect to the distribution of the PON1 polymorphism, the RR genotype was more frequent in severe patients [37.2%] than healthy group [10%] ($p < 0.05$) and no significant regression was found between genotype and PON1 activity. On the other hand, the results of PON1 and apoA1 detection illustrated that only apoA1 protein was found in BAL fluid. According to our findings it seems that increase in the stress oxidative in chemical injured veterans with pulmonary complications comes with reduction in PON1 enzyme activity and appearance of RR genotype rises up with the increase in disease severity. Since a significant correlation between enzyme activity and genotype was not observed altering these two variables with each other requires more studies.

Keywords

ApoA1, genotype, lung, paraoxonase, sulfur mustard gas

History

Received 22 January 2013
Revised 14 April 2013
Accepted 17 April 2013
Published online 14 May 2013

Introduction

Sulfur mustard gas (SM) is a chemical warfare agent classified as a weapon of mass destruction. Mustard gas is one of the first chemical weapons used against troops during World War I[1] and recently used as a vesicant warfare agent in the Iran–Iraq War (1981–1989) against Iranian[2]. Many organs such as skin, lung, respiratory tract and eyes as well as the gastrointestinal, endocrine and hematopoietic system are affected by SM exposure[3]. Lung injury is a common health problem after inhalation of SM which leads to chronic bronchitis and interstitial lung diseases[4]. At present time, there are more than 40 000 persons suffering from pulmonary lesions due to mustard gas in Iran[5].

In SM-exposed patients main late respiratory problems are chronic obstructive pulmonary disease, bronchiectasis and asthma[6]. Today bronchiolitis obliterans (BO) is known as a main respiratory clinical complication in these patients[7,8].

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Although, at present the exact mechanisms of SM-induced delayed respiratory complications are not fully understood, it appears to be different from responsible mechanisms of its acute complication. It has been suggested that SM-induced pulmonary complications are a neutrophil and/or lymphocyte disorder[9]. Furthermore, it has been shown that the pathogenesis of BO is closely related to release of some inflammatory mediators from many different cell types[10]. This condition induced increased production of reactive oxygen and nitrogen species (ROS and RNS), resulting in oxidative stress which finally plays an important role in the pathogenesis of many different lung diseases such as asthma and chronic obstructive pulmonary disease (COPD)[11,12].

PON1; EC 3.1.8.1; a calcium-dependent serum enzyme belonging to the class of A-esterases is closely associated with the high-density lipoprotein complex and apo lipoprotein A-I (apoA1) in mammals, which protects both high-density lipoproteins (HDL) and low-density lipoproteins (LDL) from oxidation. PON1 is primarily synthesized in the liver and a part of it being secreted into the plasma where it is associated with HDL. PON1 is a multi-functional enzyme and one of its physiological functions is the metabolism of toxic

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oxidized lipids of LDL and HDL particles[13,14]. It is an endogenous free-radical scavenger in human body, so its role in various disease etiologies and prevention has been a subject of great interest[15,16].

So recent progress in PON1 research has focused on its role in the antioxidant system as well as detoxification (hydrolysis) of organophosphate (OPs), and provided notable evidence for the enzyme as a risk factor for the development of lung disease, hypercholesterolemia, diabetes, coronary heart disease (CHD) and cancer[13,17].

There are two PON1 polymorphisms in human which are very interesting, because they only differ in one amino acid but exhibit different catalytic properties. Polymorphism A (hPON1A) has a glutamine (Q) residue at amino acid 192, but polymorphism B (hPON1B) has an arginine (R) residue at this position. These two polymorphisms have similar arylesterase activities, but the R polymorphism has significantly higher paraoxonase activity[18,19] and 192 Q allele is more efficient at metabolizing oxidized HDL and LDL than 192R allele[20,21]. PON1 is seen in non-ciliated bronchiolar epithelial cell (clara cells) which maintain the epithelium of the distal conducting airways in human[20,22].

In this study, we investigated the existence of possible relationship between serum PON1 activity and the severity of lung disease in SM-exposed patients. To verify these findings, we also demonstrated the relation between Q192R polymorphism and disease severity.

Patients and methods

Patient groups

Male patients ($n = 101$) suffering from delayed respiratory complications due to the unique dose of SM exposure during the 1988–1995 Iran–Iraq war are grouped by symptoms of BO as the SM-injured. They were divided into three groups – mild ($n = 25$), moderate ($n = 43$) and severe ($n = 45$) – based on our spirometric and (HRCT) high-resolution computed tomography (General Electric Medical System, Milwaukee, WI) findings according to the American thoracic society(ATS) classification[23]. The expiratory images were assessed for the presence of air trapping and its lobar distribution. We evaluated the lung function of all patients by spirometry (by a HI-801 Chest M.I. Spirometer, Tokyo, Japan) and measured the forced expiratory volume in 1s (FEV1), forced vital capacity (FVC), FEV1/FVC ratio and TLC.

Control group

Healthy age-matched males ($n = 40$) were used as the control group. Patients and control subjects were excluded from the study if they had a positive history of a chronic disease (tuberculosis, diabetes, hypertension, heart disease, hepatic diseases, etc), resection of one or more lobes of the lungs, pneumonia and/or acute bronchitis, cigarette smoking or substance abuse. None of the patients or control subjects had a history of allergy or asthma. All patients and controls were in a stable condition and none of them had been administrated corticosteroids during the two-month period immediately preceding the study. This study was conducted in accordance with the ethic protocol approved by the ethic committee of Baqiyatallah Medical Sciences University (BMSU).

Sample collection

BAL fluid sampling was performed based on the standard procedure in according to Thomason et al.[7].

Serum and whole blood samples

10 ml fasting venous blood sample were collected in the morning (8–10 am). 5 ml of it was drawn into the tube containing K_2EDTA for genotype analysis. Since EDTA inhibits PON1 activity, EDTA containing tubes were not used for collecting all 10 ml serum samples[24]. So another 5 ml of blood sample was drawn into another tube and centrifuged at 1500 rpm (4 °C) for 10 min, then it was promptly frozen in aliquots and stored at −80 °C until the usage time.

Biochemical measurements

Serum levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) were determined by using an auto analyzer (BT 3000, Italy) at the same day of blood collection.

Enzyme assay

Paraoxonase and arylesterase activity were measured in the serum sample according to our published article[24].

DNA extraction and genotyping

DNA was extracted by the salting-out method[25] with slight modification. PON1 genotypes were determined following the polymerase chain reaction (PCR) according to the Adkins et al. method[19].

For the 192 polymorphism, sense primer 5′-TAT TGT TGC TGT GGG ACC TGA G-3′ and antisense primer 5′-CAC GCT AAA CCC AAA TAC ATC TC-3′ which encompass the 192 polymorphic region of the human PON1 gene were used.

The PCR reaction mixture contained 100 ng DNA template, 0.5 μM of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs and 1U Taq DNA polymerase. After denaturing the DNA for 5 min at 94 °C, the reaction mixture was subjected to 35 cycles of denaturation for 1 min at 95 °C, 1 min annealing at 60 °C and 1 min extension at 72 °C for the 192 genotype. The 99 bp PCR product was digested with 5U BspPI(AlwI) restriction endo nuclease (MBI Fermentas, Lithuania) over night at 55 °C and the digested products were separated by electrophoresis on 4% agarose gel and visualized using ethidium bromide. The R genotype contains a unique BspPI restriction site which results in 66 and 33 bp products and the Q genotype cannot be cut, allowing the 192 genotype to be determined[19].

Detection of apoA1 and PON1 protein in BAL by Western blot

BAL fluid (broncho alveolar lavage) protein was desalted and concentrated using the trichloroacetic acid (TCA) procedure according to Jiang et al.[26] with slight modification. Briefly 60% TCA was added to the BAL fluid bringing to the final optimized concentration of 12%w/v TCA. The mixture was

vortexed and incubated overnight on ice and centrifuged at 12 000 *g*, 4 °C for 15 min. The supernatant was removed and pellet was dispersed in ice-cold acetone. Samples were vortexed and incubated at 20 °C for 30 min and then centrifuged as above. The acetone-containing supernatant was removed and the pellet was air-dried. Then the pellet was suspended in sample buffer(Ripa)consisting of Tris 20 mM, NaCl 150 mM, EDTA 1 mM, Triton X-100 and SDS 1%, pH 7.5[27]. Protein concentration in the recovered samples was determined using a modified Bradford method[28].

Samples, each containing 20 µg proteins, were separated on 10% SDS–PAGE with 100 V of power conditions for 70 min at room temperature (RT). The proteins in the gels were blotted on to nitro cellulose membranes using multiphor II novablotunit (Amersham Biosciences, Amersham, UK) for 1 h at 25°C. Non-specific protein-binding sites on the membranes were blocked using TBST (Tris-buffer solution with 0.05% Tween 20) containing 1% non-fat dry milk for 60 min at RT. Membranes were subsequently exposed to rabbit polyclonal PON1 antibody (1:500) (J-24): sc-133919, rabbit polyclonal apoA1 antibody (1:1000) (FL-267): sc-30089 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After washing three times with Tris buffered saline Tween-20 (TBST), the membranes were incubated with HRP-conjugated anti-goat IgG (0.04 mg/ml) for 60 min at RT. Then membranes were washed three times with TBST and incubated with 3,3,5,5 tetramethylbenzidine (TMB) liquid substrate system (Sigma, Dorset, UK) until protein bands appeared. For PON1 protein we also used ECL method. In this procedure, the blots were visualized using ECL detection reagents (Sigma, CPS-1-120, Dorset, UK). Subsequently, the films were scanned with a densitometer (GS-800, Bio-Rad, Hertfordshire, UK). To insure that a uniform amount of protein was loaded on to the gels, the membranes were stained for total protein with Fast Green (FCF, Sigma-Aldrich, F7252, Dorset, UK).

Statistical analysis

In order to examine the correlation between enzyme activity as a dependent variable and disease severity and genotype as independents leaner regression was accomplished. Due to the exclusion of genotype in this relation, ANOVA test was applied between disease severity and PON1 enzyme activity and the Tukey test was used as *post hoc*, and finally chi square test has been run between disease severity and genotype as qualitative variables.

Results

Chest HRCT findings

In our patients, bronchiectasis, air trapping, mosaic parenchymal attenuation and bronchial wall thickening were seen by using chest HRCT. Air trapping was defined as the presence of a radiolucent region of the lungs on expiratory images. The volume of air trapping was measured by comparing end-inspiratory and expiratory images at similar anatomic level and at each of the three levels. These findings as well as previous studies approved the presence of BO in all the cases[8].

According to lung functions (FEV1, FVC, FEV1/FVC, TLC), the patients were classified into three groups: mild, moderate, severe. There were significant decline in above spirometry parameters in all the categorized stages as shown in Table 1.

Biochemical findings

Their lipid profiles and pulmonary function tests were shown in Table 1. The results of lipid profiles showed that there was not any significant difference in TG and cholesterol amounts between all four groups. But there was significant difference between HDL of healthy group and the three patient groups, also there were significant differences between mild and moderate as well as mild and severe groups. There were significant differences in LDL amount between healthy and severe, as well as mild and moderate, also moderate and severe groups.

PON1 and arylesterase activity

PON1 assay showed that by increasing disease severity the enzyme activity decreased and this was significant in three stages in compare with control group, as shown in Figure 1

Table 1. Clinical characteristics, spirometric and hematologic findings of exposed patients and control subjects.

Variables	Healthy	Mild	Moderate	Severe	*p*
Age (year)	43.1 ± 1.4	45.8 ± 1.1	44.4 ± 2.2	46.4 ± 1.2	NS
Weight (kg)	84.5 ± 1.3	78.9 ± .9	79.1 ± 1.1	76.4 ± 1	NS
Lipid profile:					
Total cholesterol (mg/dl)	182.7 ± 4.5	166 ± 5.7	175 ± 5	168.5 ± 4.5	NS
Triglyceride (mg/dl)	133.7 ± 6.7	129.5 ± 7.8	143.6 ± 7	118.7 ± 6.8	NS
HDL cholesterol (mg/dl)	***VS other groups 49.4 ± 4.6	44.1 ± 4.2	*VS mild 40.4 ± 3.63	***VS mild 38.3 ± 3.8	
LDL cholesterol (mg/dl)	121 ± 8.6	127.8 ± 8.4	*VS mild 117.9 ± 5.6	*VS healthy ***VS moderate 131.1 ± 11.7	
Lung function tests:					
FEV1 (%)		82.8 ± 6.4	70.8 ± 6.76	42.1 ± 5.52***	
FVC (%)		77.3 ± 5.9	69.3 ± 6.6	52 ± 4.8***	
FEV1/FVC		111.8 ± 6.52	102.2 ± 8.1	80.6 ± 5.61***	
TLC (%)		107.9 ± 7.96	94.5 ± 5.67	78.6 ± 7.59***	

NS: Non-Significant; VS: Versus. Data are mean ± SEM. Difference between the groups were calculated by Tukey *post hoc* test and marked by: ***($p < 0.0001$) [Note: in lung function test the *** indicates a significance among 3 groups of mild, moderate and severe].
*($p < 0.05$).

((Paraoxsonase activity) [Healthy: 412.46 ± 89.1 U/L, mild: 291.83 ± 89.5 U/L, Moderate: 213.79 ± 47.97 U/L, severe: 89.66 ± 20.7 U/L] ($p < 0.0001$)).

There is significant decrease in the arylesterase activity in three patient groups when compared with the healthy group, but there was no significant difference between mild and moderate as well as moderate and severe groups (Figure 2) ((Arylestrase activity):[Healthy: 25 826.4 ± 4425.23 U/L, Mild: 20 081.04 ± 3383.54 U/L, Moderate: 17 961.31 ± 4420.2 U/L, severe: 16 760.43 ± 3814.9 U/L] ($p < 0.0001$)).

PON1 genotype (Q192R) and BO disease

PON1 Q192R genotypes significantly differed between patient and control groups (Figure 3). Chi-Square survey among the genotype frequency and disease severity showed that there is significant correlation between the severity of the disease and genotype frequency.

Our results showed that with increasing disease severity the RR genotype frequency increases in spite of the reducing of the QQ genotype frequency at the same time, while significant changes were not observed in QR genotype between groups.

PON1 192 polymorphism frequency was found to be 52.5% for QQ, 37.5% for QR and 10% for RR in the healthy group and 40% for QQ, 44% for QR and 16% for RR in mild diseases group, 24.2% for QQ, 51.5% for QR and 24.2% for RR in the moderate group and 20.9% for QQ, 41.9% for QR and 37.2% for RR in severe group (Figure 3).

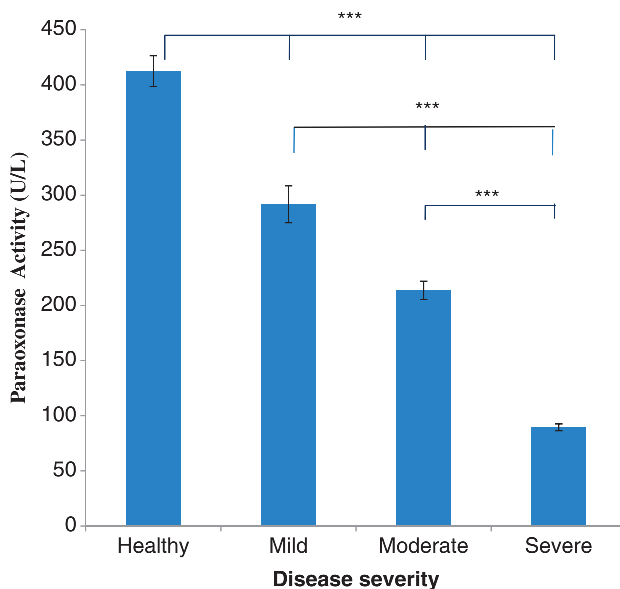

Figure 1. Serum Paraoxanase activity of PON1 in healthy and patients with different severity of lung disease. PON1 assay showed that by increase in disease severity the enzyme activity decreased and this was significant in three stages in compare with control group [Data are mean ± SEM. Difference between the groups were calculated by Tukey *post hoc* test, *** ($p < 0.0001$)].

Detection of PON1 and apoA1 in BAL fluid

To detect the presence of apoA1 and PON1 protein in BAL fluid samples, Western blot analysis was performed. When the membrane fractions of BAL fluid were Western blotted employing polyclonal anti apoA1 and PON1 antibodies, protein bands with the molecular weight of 55–60 kDa apoA1 were observed (Figure 4), but there is no evidence of PON1 protein. For PON1 protein we also used the ECL method to be sure that the negative result is true.

Discussion

In the present study, we examined the relationship between the serum PON1 level and the severity of lung disease in SM-exposed patients. To verify these findings, we longitudinally investigated the relation between Q192R polymorphisms and the severity of the complication.

The latest reports have shown that BO is the main chronic pulmonary complication among Iranian SM-exposed patients which poisoned with this inhalant during Iran–Iraq conflict[7,8].

Consistent with these findings, Ghanei et al.[4] reported that oxidative stress due to excessive production of reactive substances and depletion of endogenous antioxidants plays an important role in the pathogenesis of this delayed pulmonary sequel in these veterans[11,12,29,30]. Also, Shohrati and Jafari have proposed and confirmed the changes of these parameters on these veterans in their studies[31,32].

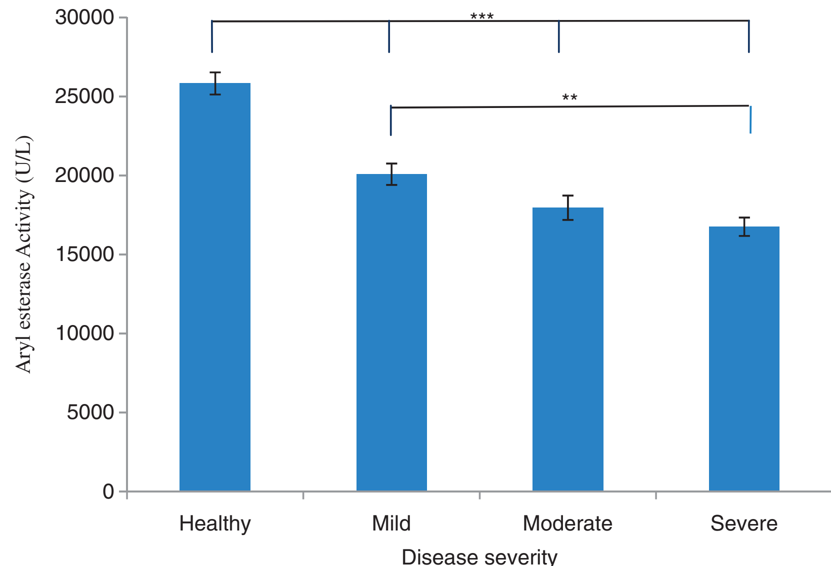

Figure 2. Serum arylestrase activity of PON1 in healthy group and patients with different severity of lung disease. There is a significant decrease in arylesterase activity in comparison between healthy and patient group. Other side, there is a significant difference between mild and severe groups [Data are mean ± SEM. Difference between the groups were calculated by Tukey *post hoc* test, *** ($p < 0.0001$), ** ($p < 0.01$)].

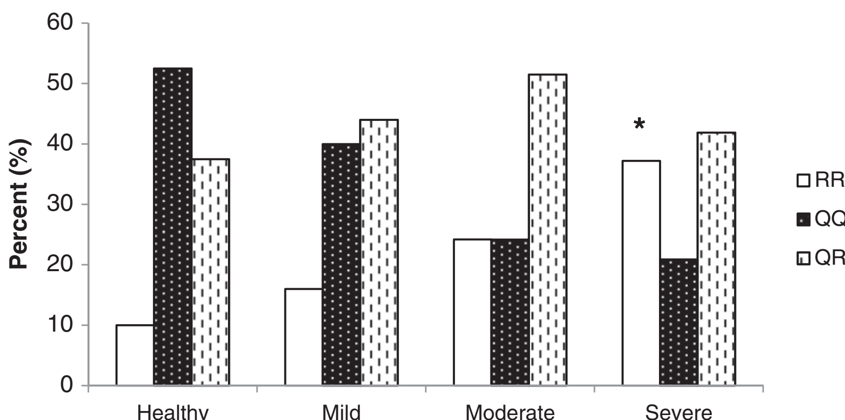

Figure 3. The frequency of genotypes in healthy and patient groups. PON1 Q192R genotypes significantly differed between patient and control groups. There is a significant correlation between the severity of disease and genotype frequency, illustrating that by increase in disease severity the RR genotype rises up even though the reduction of the QQ genotype at the same time, while significant changes were not observed in QR genotype between groups. ($p < 0.05$*).

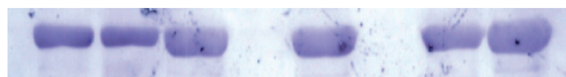

Figure 4. Western blot analysis of ApoA1. Protein bands with molecular weight of 55–60 kDa (apoA1) were observed in a sample gathered from two patients that did not exist in the healthy group.

In 2008, Uzun et al. studied on 26 active sarcoidosis subjects and concluded that oxidative stress increases in sarcoidosis might be due to both increase in lipid peroxidation and decrease in antioxidant status (PON1) and the relationship between oxidative status and the activation of the disease should be discussed by comparing the previously known activation criteria[33–35].

In our study we identified that decrease PON1 has a correlation with the severity of COPD or bronchio oblitrain symptoms in sulfur mustard-exposed patients, therefore it might be identified as a potential new therapeutic target as well as a potential diagnostic tool for following up the effect of therapy in these patients.

Furthermore, Lgyesi et al. found that in human asthmatic patients, serum PON1 activity was reduced at exacerbation, but increased parallel with improving asthma symptoms. PON1 gene polymorphisms did not influence the susceptibility to the disease. Their observations suggest that an altered PON1 activity might be involved in the pathogenesis of asthma and serum PON1 level might be used for following up the effect of therapy[36].

In 2008, Selsk et al. have seen that there was a potent oxidative stress in patients with pulmonary tuberculosis who participated in their study and there was a decreased PON1 activity[37].

Furthermore, in this study we found significant differences in the distribution of PON1 Q192R genotypes between subjects who were exposed to sulfur mustard gas with different lung disease severity and healthy non-exposed individuals. The result of our study indicates that PON1 192 RR genotype occurred with more frequency in patients with Severe symptoms but the PON1 192 QQ genotypes occurred with less frequency and this support the hypothesis that the PON1 Q192R gene may has a role in genetic susceptibility to lung disease in patients who were exposed to sulfur mustard gas.

In 2010, in consistent with our results Tekes et al. found a significant difference between the PON1 192 RR allele frequency in patients with COPD and control group[38].

In 2011, Aksoy-Sagirli et al. investigated the possible association of paraoxonase-1 Q192R polymorphisms with lung cancer (LC) risk in Turkish population and suggested that PON1 192RR polymorphsim is associated with an increased risk of LC[39]. Seo et al. suggested that the 192RR allele may be a new genetic risk factor for airway injury in current smokers in grain workers[40]. 1 QQ genotype would be more susceptible to lung cancer than those with the PON, But despite of our findings, Lee et al. in 2005 found people with the PON1 RR genotypes[41].

Eventually, performing statistic test of leaner regression between the quantitative parameter with the paraoxsonase activity as dependent variable and disease severity & genotype as independent variables excluded QQ and QR. This means that there is no significant correlation between genotype and enzyme activity, but there is a relationship between disease severity and enzyme activity. Performing ANOVA statistic test demonstrates the relation quality of disease severity and enzyme activity. On the other hand, in order to examine the relationship between disease severity and genotype chi-square test was applied that implies on the significant correlation between these two.

However, because of PON1 distribution in a variety of tissues and its role in the maintenance of tissue homeostasis, we examined the presence of PON1 protein in the BAL extract of SM-exposed patients. Our results did not confirm the presence of PON1 in spite of the presence of apoA1 protein in the BAL extract of these patients, as reported by Mehrany et al.[27].

In 2001, a study conducted by Rodrigo et al. has demonstrated the expression of paraoxonase-1 in different rat tissues like kidney, brain and lung[22]. In consistence with our results in 2011, Mackness et al. showed that there is no sign of PON1 mRNA existence in lung as opposed to their previous study[42], so they suggested that their mistake happened because of an error in their immunohistological assay (they did not omit capillary from lung tissue).

Conclusion

Many patients suffer from late respiratory complications due to mustard gas. In lung diseases the oxidative stress is

increased. More recently it has been shown that PON1protein has antioxidant activity. So, in this study we studied PON1 192 polymorphisms and paraoxonase activity in blood serum of 101 SM-exposed patients with lung disease and 40 healthy persons. Also, we determined the expression of the PON1 and $apoA_1$ proteins in their BAL fluid. Individual genotypes were determined by extracting their blood DNA. In this study a statistically significant reduction in PON1 activity with increase in severity of disease was demonstrated. With respect to distribution of the PON1 polymorphism coinciding with severity of disease, the RR genotype was more frequent in severe patients than in the healthy group. The evaluation of regression coefficient between genotype and PON1 activity was demonstrated and no significant correlation was found. Only apoA1 protein was found in their BAL fluid.

According to our findings, it seems that increase in the stress oxidative in chemically injured veterans with pulmonary complications comes with reduction in PON1 enzyme activity and appearance of RR genotype rises up with the increase in disease severity. Since a significant correlation between enzyme activity and genotype was not observed, altering these two variables with each other requires more studies.

Acknowledgements

The authors would like to thank Dr Manouchehr Eghbal (Ph.D), Associate Professor working in Bio-Medical Engineering Department in Iranian Research Organization for Science and Technology for proofreading this manuscript.

Declaration of interest

This study is a research which was done completely in Chemical Injury Research Center, Baqiyatallah University of Medical Sciences and all its expense were covered by this research center. We declare to have no conflicts of interest.

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