BCPT Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology, 2016, **118**, 77–82 Doi: 10.1111/bcpt.12441

Serum Metabolomic Profiling of Sulphur Mustard-Exposed Individuals Using ^{1}HNuclear Magnetic Resonance Spectroscopy

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(Received 27 January 2015; Accepted 30 June 2015)

Abstract: Sulphur mustard is an alkylating agent that reacts with different cellular components, causing acute and delayed complications that may remain for decades after exposure. This study aimed to identify differentially expressed metabolites between mustard-exposed individuals suffering from chronic complications compared with unexposed individuals as the control group. Serum samples were obtained from 15 mustard-exposed individuals and 15 apparently healthy unexposed individuals. Metabolomic profiling was performed using ^{1}H nuclear magnetic resonance spectroscopy, and analyses were carried out using Chenomex and MATLAB softwares. Metabolites were identified using Human Metabolome Database, and the main metabolic pathways were identified using MetaboAnalyst software. Chemometric analysis of serum samples identified 11 differentially expressed metabolites between mustard-exposed and unexposed groups. The main pathways that were influenced by sulphur mustard exposure were related to vitamin B_6 (down-regulation), bile acid (up-regulation) and tryptophan (down-regulation) metabolism. Metabolism of vitamin B_6, bile acids and tryptophan are the most severely impaired pathways in individuals suffering from chronic mustard-induced complications. These findings may find implications in the monitoring of exposed patients and identification of new therapeutic approaches.

Sulphur mustard is a chemical warfare agent with acute and chronic toxic effects on several body organs, mainly lungs, eyes and skin [1]. This agent was used several times in the Iraq–Iran (1983–1988) war by Iraqi military forces against Iranian soldiers and both Iranian and Iraqi civilians. It has been estimated that around 100,000 Iranian individuals have been exposed to sulphur mustard during the mentioned war [2]. The use of sulphur mustard was not only against Iranian veterans and civilians, as the Iraqi forces utilized it in air raids against the Kurdish residential areas of Halabja in Iraq during 1988. These applications of sulphur mustard as a chemical warfare agent during the Iraq–Iran war have been documented by the United Nations Security Council [3]. Currently, around 50,000 individuals are suffering from the above-mentioned chronic complications and seek effective therapeutic measures [2]. Chronic complications of sulphur mustard are frequent even 20 years after exposure. According to a survey on 34,000 veterans of the Iraq–Iran war, the prevalence of chronic pulmonary, ocular and cutaneous complications is 42.5%, 39.3% and 24.5%, respectively [4]. These chronic complications adversely affect several personal and social activities of patients, causing a severe impairment of quality of life [5,6]. In spite of some advances [7–15], therapeutic options available for the management of chronic complications due to sulphur mustard are limited. This limitation is, at least in part, due to the lack of a thorough understanding on the molecular mechanism of mustard toxicity and the metabolic pathways that are mostly impaired by sulphur mustard.

Metabolomics is a relatively new branch of omics that can simultaneously determine all variations in metabolites after exposure to a xenobiotic. Metabolomics is especially used for diagnostic purposes owing to the ability of this technology to identify a unique fingerprint that is representative of a given external stimulus. Metabolomics is usually performed via either a mass spectrometry (MS) or nuclear magnetic resonance (NMR) approach. MS-based techniques usually require sample pre-separation by gas or liquid chromatography. On the other hand, NMR spectroscopy is the only detection technique that does not require pre-separation of the analytes. Using NMR, all small molecules can be measured simultaneously and the sample can be recovered for further analyses. In addition, NMR-based methods benefit from high analytical reproducibility and simplicity of sample separation [16].

Identification of differentially expressed metabolites in mustard-injured veterans may provide useful information on the pathomechanisms of mustard toxicity and help developing effective therapies. Hence, the present case–control study aimed at metabolomic profiling of sulphur mustard-exposed veterans in comparison with unexposed individuals using ^{1}HNMR spectroscopy.

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Methods

Sample collection. Fifteen millilitres of fasting blood was obtained from 15 male war veterans with documented exposure to sulphur mustard who were referring to the Baqiyatallah Hospital (Tehran, Iran)

for treatment of chronic complications due to mustard exposure. Individuals had a mean age of 49 years, and all had a history of single exposure to sulphur mustard more than 20 years ago (mean duration from exposure: 30 years). Individuals were suffering from chronic pulmonary complications due to sulphur mustard and had symptoms such as air trapping, bronchiectasis, mosaic parenchymal attenuation, irregular and dilated major airways, bronchial wall thickening and interlobular septal wall thickening. None of the individuals had malignancy. The severity of pulmonary symptoms was mild-to-moderate according to spirometry. Apart from pulmonary complications, patients were suffering from chronic cutaneous complications of sulphur mustard including pruritus and pigmentation disorders. Blood samples (15 mL) were also obtained from 15 unexposed male individuals of the same age and gender who were recruited as the control group. All individuals were fasting for 12 hr before blood sampling. Serum was separated and stored at −80°C until analysis. The study was approved by the Ethics Committee of the Baqiyatallah University of Medical Sciences, and written informed consent was obtained from the participants.

^{1}HNMR spectroscopy. One-dimensional ^{1}HNMR spectra were acquired on a Bruker DRX-400 NMR spectrometer operating at 400.13 MHz at 298 k. For each serum sample, the free induction decay (FID) was weighted by an exponential function with a 0.3-Hz line-broadening factor prior to Fourier transformation (FT). Water pre-saturation pulse sequence (D-90-t1-90-tm-90-acquired FID) with relaxation delays 5 sec and flip angle 90°, the broad protein resonances and water signals were suppressed by a combination of pre-saturation and the Carr–Purcell–Meiboom–Gill (CPMG) (90-(t-180-tn-acquisition) (τ = 200, n = 100) pulse sequence. Phase and baseline correction were made for each spectrum, and a spectral width of 8992.806 Hz and an acquisition time of 0.911 sec were used [17].

Data reduction. CPMG-NMR spectra were segmented into regions of 0.01 ppm width using Chenomx 6.4 software. Pseudo-variables were generated by integrating the spectral data into discrete regions about the width of spectral peaks associated with metabolites. The variables were calculated from the integrated area under the curve of each of these regions (referred to as 'bins' or 'buckets'). The water signal (δ 4.44–5.2) was removed from all spectra to eliminate variation in water suppression efficiency. The area for each segmented region was calculated and the integral values resulted in an intensity distribution and description of the whole spectrum with 1000 variables prior to PR analysis. The spectrum was normalized by setting it to the total region of each spectrum and then exported to an Excel file. The normal intensity of the samples was then used for analysis.

Partial linear square. Partial linear square (PLS) is a supervised method that uses multivariate regression techniques to extract the information that can predict the class membership (Y) using linear combination of original variables (X). PLS was performed after orthogonal signal correction (OSC) using the Y matrix including 0 for normal and 1 for abnormal for all the data set [18]. PLS was performed with and without OSC and results were obtained with more than 95% confidence. The chemical shifts of the differentially expressed metabolites were identified.

Orthogonal signal correction filters were developed to remove unwanted variation from spectral data matrix [19]: X consists of ^{1}HNMR data of exposed war veteran samples and Y represents ^{1}HNMR data of unexposed individuals. OSC subtracts from X, factors that account for as much as possible of the variance in X and are orthogonal to Y. It is important to avoid overfitting after OSC treatment, in order to prevent poor predictive performance; hence, precise determination of the number of removed OSC factors is very important, and here, only one factor was removed.

Detection of metabolites. The NMR search link of the Human Metabolome Database (HMDB; available at www.hmdb.ca) was used to identify the differentially expressed metabolites using certain chemical shifts. It is a freely available electronic database containing detailed information about metabolites found in the human body [20].

Metabolic pathway analysis. The list of metabolites obtained through database search was uploaded on MetaboAnalyst 2.0 software (www.metaboanalyst.ca) for pathway analysis and visualization. These pathways were the ones recognized to be relevant by their *p*-value [21].

Results

^{1}HNMR spectra of mustard-exposed veterans superimposed with unexposed individuals showed a difference in the 1 to 4 chemical shift regions (fig. 1). The score plot showed a complete separation of metabolites between mustard-exposed and mustard-unexposed sets (fig. 2), and the levels (serum concentrations calculated from the signal intensities in the NMR spectra) of metabolites were obtained from the loading plot (fig. 3). The biplot displayed differentially expressed metabolites between the two groups. The outliers of the metabolites on the biplot showed metabolites with the highest degree of difference between the groups. Chemical shifts of the metabolites were detected from the Excel file and differentially expressed metabolites were identified using HMDB testing (table 1), as shown in the superimposed spectra (fig. 1). The main metabolites that were changed between exposed and unexposed groups were pyridoxamine, pyridoxine, indoleacetic acid, L-histidine, 5-hydroxy L-tryptophan, S-adenosylhomocysteine, taurocholic acid, 7-ketocholesterol, cholesteryl ester, omo-L-arginine and cholesterol. These metabolites were entered into the MetaboAnalyst software to identify the main metabolic pathways affected by sulphur mustard (fig. 4) and the statistical significance of alterations in each pathway (table 2; fig. 2). Vitamin B_6 ($p < 0.001$), bile acid ($p = 0.015$) and tryptophan ($p = 0.040$) metabolism were the most significantly influenced pathways in the mustard-exposed group compared with the unexposed group.

Statistical comparison of differentially expressed metabolites was performed using PLS discriminant analysis (PLS-DA) algorithms defined in the MetaboAnalyst 2.0 software. A *p*-value of <0.05 was considered as statistically significant.

Discussion

In the present study, we used a chemometric method to obtain a separation pattern using ^{1}HNMR spectroscopy between 15 male veterans exposed to sulphur mustard and the same number of age-matched males not exposed to sulphur mustard. Score plot of OSC-PLS showed a clear separation between the two groups and eleven differentially expressed metabolites were detected. Using the HMDB, the differentially expressed metabolites were identified and respective main metabolite pathways were recognized.

Vitamin B_6 metabolism was found to be the main pathway affected by sulphur mustard exposure. Vitamin B_6 has many

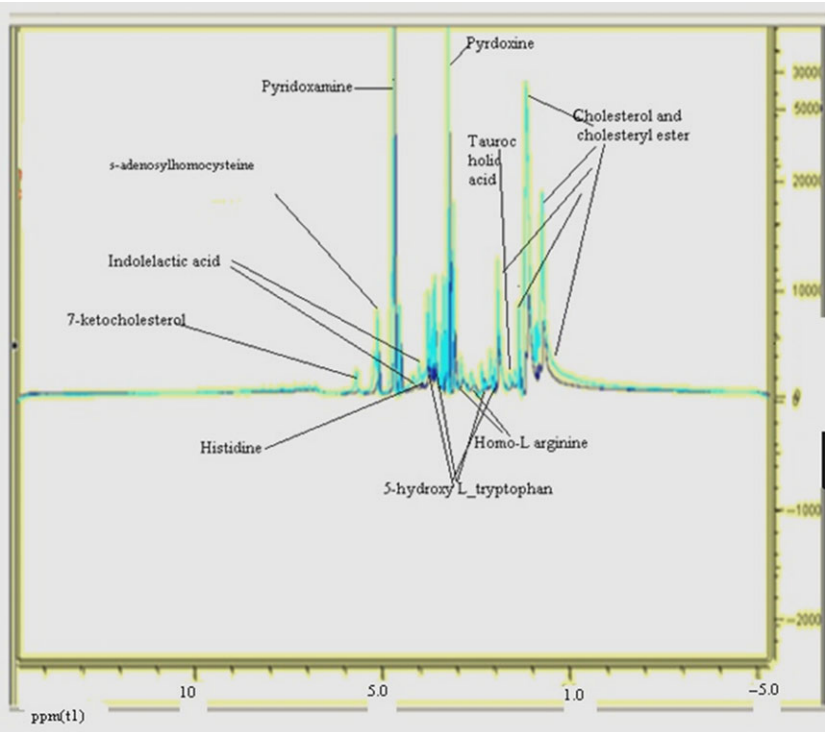

Fig. 1. Superimposed ^{1}HNMR spectra of sulphur mustard-exposed veterans and unexposed individuals showing different metabolites.

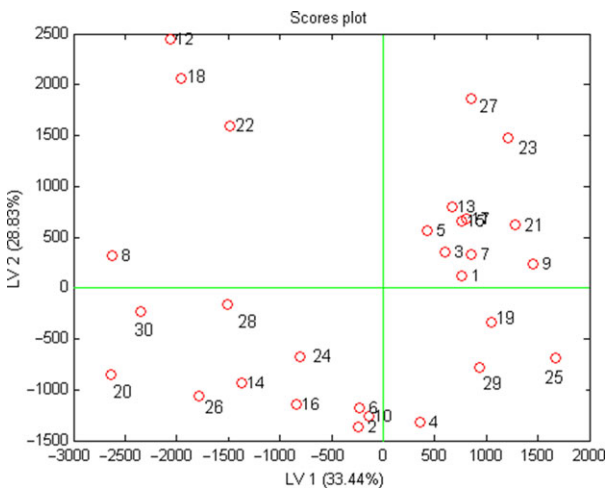

Fig. 2. Scores plot of OSC-PLS in sulphur mustard-exposed war veterans and unexposed individuals. Odd numbers show serum samples of war veterans exposed to sulphur mustard and even number show unexposed samples.

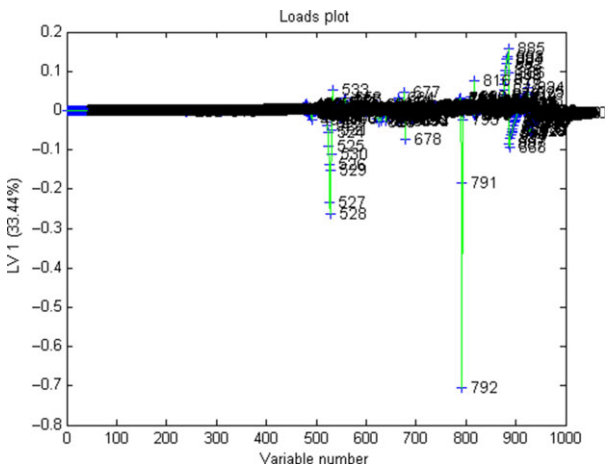

Fig. 3. Loading plot of OSC-PLS in sulphur mustard-exposed war veterans and unexposed individuals. Numbers depict differentially expressed metabolites. Metabolites below the *x*-axis show descending levels.

important functions in the metabolism of amino acids, glucose and lipids, synthesis of neurotransmitters, histamine and haemoglobin, gene expression, and homeostasis of sodium and potassium. Vitamin B_6 is also important for cardiovascular health owing to its role in decreasing the formation of homocysteine [22]. It inhibits the formation of advanced glycation end products which are associated with many human diseases, including vascular complications of diabetes [23]. The soluble receptor for advanced glycation end products (sRAGE) has anti-inflammatory properties, and deficiency of circulating sRAGE is associated with human diseases [24]. In lung diseases like COPD, plasma levels of sRAGE are positively correlated with forced expiratory volume and recent reports claim that they can be looked upon as biomarkers of emphysema. Pyridoxamine may interact with metal ions that are necessary for the formation of Amadori products and subsequent breakdown of glycated proteins [25]. Deficiency of serum vitamin B_6 has been reported in theophylline users and has also been associated with an increased risk of lung and colon cancers

Table 1.
Differentially expressed metabolites between sulphur mustard-exposed veterans and unexposed individuals.

Name of metabolites	Chemical shift	Multiplet	ppm	Inferred levels
Pyridoxamine	4.321	S34	4.311–4.33	↓
Pyridoxine	4.73	S1	4.72–4.75	↓
Indolelacetic acid	3.060	1dd	3.001–3.117	↓
L-Histidine	3.23	1dd	3.22–3.28	↑
5-Hydroxy L-tryptophan	3.40	10dd	3.36–3.45	↓
S-adenosylhomocysteine	2.03	2m	1.98–2.04	↓
Taurocholic acid	0.92	3d	0.90–0.93	↑
7-ketocholesterol	2.52	2dd	2.48–2.57	↑
Cholesteryl ester	2.31	1d	2.35–2.29	↑
Homo-L-arginine	3.74	1m	3.71–3.77	↓
Cholesterol	1.98	4m	1.91–2.07	↑

Metabolites identified by HDMB and their inferred levels from loading plot of OSC-PLS. Eleven metabolites were identified as different between the two groups.

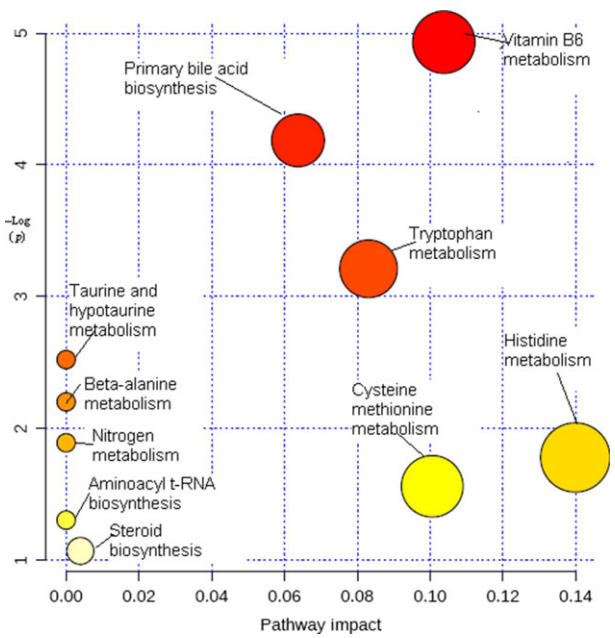

Fig. 4. Summary of pathway analysis. Higher and darker circles, closer to the *y*-axis, indicate pathways with greater alterations by sulphur musatrd exposure.

Table 2.
Detailed results from pathway analysis.

Name of pathway	Total	Hits	*p*-Value
Vitamin B_6 metabolism	32	2	<0.001
Primary bile acid biosynthesis	47	2	0.015
Tryptophan metabolism	79	2	0.040
Taurine and hypotaurine metabolism	20	1	0.080
β-Alanine metabolism	28	1	0.111
Nitrogen metabolism	39	1	0.151
Histidine metabolism	44	1	0.169
Cysteine and methionine metabolism	56	1	0.210
Aminoacyl-tRNA biosynthesis	75	1	0.272

Total is the total number of compounds in the pathway; the Hits is the actually matched number from the user-uploaded data; the *p* is the original *p*-value calculated from the enrichment analysis.

[26,27]. Impaired vitamin B_6 metabolism may also predispose to cutaneous diseases. A study in a mouse model has shown that deficiency of vitamin B_6 may decrease the levels of vitamin B_6-dependent enzymes and result in damaged synthesis of proline from ornithine which causes impaired collagen neogenesis [28,29]. Pyridoxamine also inhibits the development of retinopathy and neuropathy in streptozotocin-diabetic rats [30]. In the present study, we observed a decreased level of two vitamin B_6 metabolites, pyridoxamine and pyridoxine, in the serum of sulphur mustard-exposed war veterans. Owing to the above-mentioned roles of vitamin B_6, impaired metabolism of this vitamin may play a role in the pathophysiology of chronic mustard-induced complications.

Bile acid biosynthesis was the next important pathway affected by mustard exposure, represented by elevated levels of cholesterol and taurocholic acid. A study on sulphur mustard-exposed war veterans has shown higher serum cholesterol and low-density lipoprotein levels compared with unexposed individuals [31]. Bile acids and pepsin have been reported in the bronchoalveolar lavage fluid (BAL) of sulphur mustard-exposed veterans [32]. Gowdy and Festler [33] have reported that cholesterol and lipoproteins may play a role in lung disease. In addition, there is some evidence indicating that lipid-lowering agents such as statins, apolipoprotein mimetic peptides and liver X receptor agonists are effective in the treatment of lung diseases [34] and smoking-associated COPD. In a recent study in patients suffering from chronic mustard-induced COPD, atorvastatin was found to improve quality of life, but not spirometric parameters [35]. Taurocholic acid may also be involved in the pathophysiology of pulmonary diseases. Pulmonary damage and increased concentrations of albumin in BAL have been reported in the taurocholate-induced pancreatitis mouse model [36]. Considering these findings, modification of lipid metabolism through exercise, diet or pharmacotherapy may help improving the overall health status of mustard-exposed veterans.

Tryptophan metabolism was the next significant pathway affected by mustard exposure. Tryptophan can theoretically affect all three organs that are most extensively damaged by sulphur mustard, that is lungs, eyes and skin. The breakdown

of tryptophan is mainly in two directions, either in the serotonin pathway or in the kynurenine pathway, the latter leading to the formation of nicotinamide adenine dinucleotide. It is has been reported that the ratio of tryptophan to kynurenine in chronic pulmonary patients is low, and this ratio might serve as a useful measure of disease activity in chronic inflammatory parenchymal disease and idiopathic pulmonary fibrosis [36,37]. Tryptophan deficiency may also be involved in skin hyperpigmentation associated with Grave's disease [38].

The fourth pathway that was influenced in the mustard-exposed group, though not significantly, was related to β-alanine metabolism. In relation to pulmonary diseases, alanine and glutamine have been reported to increase considerably in pulmonary sepsis in rats [39]. In patients with lung cancer, elevated rates of glucose and alanine turnover have been reported, and this observation might be related to weight loss due to cancer [40]. In this context, reduced plasma concentrations of glutamine, glutamate and alanine were reported in eight underweight patients with emphysema and muscle wasting [41]. In another study, elevated levels of histamine (another altered metabolite in this study) were reported in the BAL fluid of individuals with pulmonary disease [42].

Conclusion

Of all the pathways studied, vitamin B_6 metabolism followed by bile acid biosynthesis and tryptophan metabolism appear to be the most seriously impaired. Whilst a direct association between altered levels of these metabolites and clinical symptoms of sulphur mustard-exposed individuals remains elusive, the present results generate hypothesis as to the metabolites that are most affected by sulphur mustard. The causal association between altered levels of vitamin B_6, bile acids and tryptophan and severity of symptoms in sulphur mustard-exposed individuals need to be confirmed in randomized controlled trials. To this end, future studies are warranted to test the efficacy of vitamin B_6 replenishment and lipid-lowering measures on the severity of chronic mustard-induced complications.

Acknowledgement

The authors wish to acknowledge the Baqiyatallah University of Medical Sciences for financial support of this project.

Conflict of Interests

The authors have no conflict of interests to declare.

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