Trauma Mon. 2017 November; 22(6):e39192. doi: 10.5812/traumamon.39192.

Published online 2016 October 8. **Research Article**

Relationship Between Serum Bilirubin Concentration and Inflammatory Cytokines in Victims Exposed to Sulfur Mustard

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Received 2016 May 17; **Revised** 2016 August 31; **Accepted** 2016 September 25.

Abstract

Background: Despite observed post Sulfur Mustard (SM) exposure hemolysis, serum bilirubin concentration does not significantly increase in SM-exposed casualties. The concentration of serum bilirubin can be related to serum levels of inflammatory cytokines.
Objectives: In this study, the relationship between the serum levels of Interleukin (IL)-1α, IL-1β, IL-1Ra, Tumor Necrotizing Factor (TNF)-α and IL-6 with serum bilirubin concentration was investigated.
Methods: Overall, 368 individuals, who were exposed to SM in 1986, and 127 non-exposed control participants were studied in the context of hematological factors, serum bilirubin and inflammatory cytokines. Total serum bilirubin and direct bilirubin were analyzed using an enzymatic method, while the inflammatory cytokines were evaluated by enzyme linked immunosorbent assay (ELISA).
Results: The concentration of inflammatory cytokines in the exposed group was significantly different compared to the control group, however the serum concentrations of total and direct bilirubin did not differ between the two groups. Among cytokines, other than the relationship between the IL-6 concentrations and bilirubin level, there were no significant correlations between the levels of other cytokines with direct and total bilirubin.
Conclusions: Considering the lack of correlation between bilirubin concentrations and levels of inflammatory cytokines other than IL-6, we should identify other confounding factors for lack of increase in bilirubin in SM casualties, despite the observed hemolysis.

Keywords: Mustard Gas, Bilirubin, Inflammatory Cytokines, Iran

1. Background

Sulfur Mustard (SM) is an alkylating agent, which causes skin and lung blisters, and reacts with most biological molecules of the human body (1). The long-term effects of SM exposure manifest in several health-related problems later in life, however the pathophysiology of SM-induced complications is not clear and requires further conclusive investigations (2). The most damaging symptoms reported in casualties were related to the lungs (3), eyes (4), skin (5) and hematologic system (6). Malignant effects related to autoimmunity have also been reported in earlier publications (7).

Based on our previous studies, the serum pro-inflammatory cytokine (TNF, IL-1α, IL-1β and IL-1Ra) levels were significantly lower in the SM-exposed group than in the unexposed controls (8) and we also found significantly different hematological parameters (Hematocrit (HTC), Mean Cell Volume (MCV), Red Blood Count (RBC), White Blood Count (WBC), Platelet Count (PLT) and Polymorphonuclear Leukocyte (PMN)) between the SM-exposed group and unexposed controls (6). In another study, we investigated the association between total serum bilirubinemia and intensity of SM exposure. Despite the existence of hemolysis long-term after SM exposure in cases of more severe poisoning of SM, a clinically

significant increase in bilirubin concentration has not been found (9), which is an outcome that needs further illumination.

Indirect bilirubin is not only one of the liver function tests but is also a product of hemolysis. It conjugates in the liver and is excreted from hepatocytes to the bile canaliculi. Many factors affect bilirubin excretion and the competence of excretion is variable. Excretion is caused by the hemolysis of Red Blood Cells (RBCs) and is done by the liver. Although, in other studies alterations in the concentration of inflammatory cytokines have been reported, nevertheless, there have been limited investigations to show a correlation between some of these cytokines and bilirubin excretion (10, 11).

2. Objectives

In order to identify the probable reasons for lack of increase in bilirubin levels, despite the existence of hemolysis, the relationship between inflammatory cytokines, including Interleukin (IL)-1α, IL-1β, IL-1Ra, Tumor Necrotizing Factor (TNF)-α and IL-6 with bilirubin concentration was investigated in this study.

3. Methods

3.1. Study Design and Participant

A historical cohort study was conducted in Sardasht, the northwest of Iran, approximately 20 years following the exposure to SM (sardasht-Iran cohort study). The methodology details have been previously described in our previous paper (12). Briefly, male subjects from the two adjacent cities of Sardasht (n = 372, SM exposed group) and Rabat (n = 128, unaffected civilians with no known exposure to SM as the control group) were selected randomly. Exposed participants were classified to two subgroups, hospitalized (n = 169) and non-hospitalized (n = 203) based on the hospitalization and severity of the clinical problems at the time of exposure. The control group was matched with the exposed group in terms of age and gender. There were no statistical differences in Body Mass Index (BMI), smoking and marital status between the two groups (2). Inclusion criteria were male gender, exposure to SM based on medical records (for exposed group), age range between 20 and 60 years, no systemic immunosuppressive medication, and provision of informed consent. Exclusion criteria included current treatment with systemic immunosuppressive drugs, history of systemic disease before exposure based on medical records, having an acute infectious disease at the time of sampling, and disinclination to continue participation.

3.2. Ethical Considerations

The study was approved by the ethical committee of board of research ethics of Janbazan medical and engineering research center (JMERC), the board of research of the ministry of health and medical education, and Shahed university. Written informed consent was obtained from all participants ultimately selected for inclusion in the study.

3.3. Collection of Samples

In 2007, blood samples were taken from cephalic veins, following at least 12 hours of fasting. The blood samples were mixed with Ethylenediaminetetraacetic acid (EDTA) anticoagulant for complete blood count (CBC), and the CBC was analyzed using a hematology automatic cell counter (Sysmex, Kx21N; Japan). The main components studied were red blood count (RBC), Hemoglobin (Hb), Hematocrit (HCT) and RBC indices including mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC). In order to examine other components, the blood samples were centrifuged at 1500 g for 10 minutes and the serum was kept at -80°C. The total and direct bilirubin was analyzed by an enzymatic method (Pars Azmoon Kit, Iran) using an auto analyzer (Selectra E, Japan).

3.4. Cytokines Measurement

Human IL-1α, IL-1β, IL-1Ra, TNF-α, and IL-6 DuoSet ® enzyme linked immunosorbent assay (ELISA) development kits (R&D Systems) were used to measure the serum levels of IL-1α, IL-1β, IL-1Ra, TNF-α, and IL-6. According to the kits' protocol, a sandwich ELISA method was used, in which the primary antibody was mouse anti-human and secondary antibody was biotinylated goat anti-human (8, 13).

3.5. Statistical Analysis

Data were presented as mean $\pm$ standard deviation (SD) or median (first and third quartiles/ Q1, Q3). Independent sample t-test and Mann-Whitney tests were used to compare the data between the exposed and control groups. Correlation between cytokines and liver enzymes was calculated by the Spearman rank correlation. The data were analyzed using statistical package for social science (SPSS Inc., Chicago, IL) software version 16.0 at a significance threshold of 5% ($P < 0.05$). A descriptive analysis was first performed to identify the main trends in the data.

4. Results

Blood samples of 372 SM-exposed individuals and 128 controls were analyzed for hematological parameters in our previous study (6) and their results are summarized

in Table 1. The parameters related to HCT and RBC indices, especially MCV, showed significant differences between exposed and controls. Also, among the exposed groups, significant increased HCT was seen in the hospitalized group as compared with the control (P = 0.001). As can been shown clearly, the volumes of RBCs were increased. Increasing of these parameters can be considered secondary to hemolysis and overproduction of bone marrow. Immature RBCs (reticulocytes) are large in size and found in peripheral blood due to hemolysis.

Table 2 shows the total and direct bilirubin concentrations in both control and exposed groups. Despite the differences in some hematological parameters, no significant difference was observed.

Serum levels of cytokines were determined using ELISA (8, 13). Overall, IL-1α, IL-1β, IL-1Ra, IL-6 and TNF-α levels were significantly different between exposed and control groups, so that the level of these cytokines in the exposed group (Hospitalized (H) and Non-Hospitalized (NH)) was significantly lower than the control group (Table 3). There was no statistically significant difference between H and NH in the exposed group, regarding the serum levels of IL-1α, IL-6 and TNF-α. However, serum levels of IL-1β and IL-1Ra were significantly higher in H than NH exposed group.

Table 4 demonstrates the correlations between total and direct bilirubin with inflammatory cytokines. The results of further analysis showed a correlation between bilirubin concentration and the serum level of IL-6 in the exposed group, which was significantly different between the two groups.

5. Discussion

In this study, significant differences were found between SM exposed and the control groups in most of hematological parameters and inflammatory cytokines. However, there was no statistically significant difference between the two groups in direct and total bilirubin levels. Furthermore, among cytokines, other than the relationship between the IL-6 concentrations and direct bilirubin level in the exposed group, there were no significant correlations between the levels of other cytokines with direct and total bilirubin. Also, the linear relationship between cytokines and bilirubin had no particular relevance.

One possible explanation for the observed clinical results may be due to long term side-effects of poisoning, reported by other investigators, which include severe respiratory complications and the resulting oxygenation disorder that may ensue (5, 14, 15). Therefore, it is expected for the victims to develop polycythemia, to compensate for the hypoxemia, but according to previous studies, this type of manifestation, which can be caused by destruction of RBCs, was not noted. Furthermore, MCV and HCT in the exposed group were significantly higher compared to the control group. It could be hypothesized that hemolysis causes activation of bone marrow to produce a greater amount of reticulocytes with a larger volume than that of RBC. The reticulocytes enter the peripheral blood and consequently, there are increases in the volume of RBC (MCV) and in the volume percentage of RBCs compared to the HCT. Destruction of RBCs (hemolysis) causes bilirubin production, which has to be excreted by the liver; on the other hand, the capacity of the liver to discharge, is limited and in case of overproduction of bilirubin, its concentration increases in the blood. We expected indirect bilirubin to increase due to hemolysis. The fact that it was not increased can be attributed to over excretion of bile. Meanwhile, despite the hemolysis in the exposed group, the concentration of bilirubin was not increased.

Ikeda et al. showed that perfusion of IL-6 and TNF-α caused a decrease in contraction of small biliary canaliculi and subsequent decrease in bile flow, which, in turn, caused a rise in blood bilirubin concentration in a rat model (11). With respect to the results of this study, we can conclude that the decrease in inflammatory cytokines in our patients might be a result of increased contraction of the biliary canaliculi and bile flow. Consequently, despite the overproduction of bilirubin, its concentration does not increase in blood. The positive correlation between IL-6 and direct bilirubin in the exposed group confirms this hypothesis, but it does not correlate with the relationship between other cytokines and bilirubin.

In another study by Chen et al. on patients with ovarian hyper stimulation syndrome, it was observed that in individuals with abnormal liver function tests, the concentration of IL-6 is higher, and the possibility of IL-6 role in abnormal liver function tests has been postulated (10). Considering the role of IL-6 in abnormal liver function tests in this study, it may be concluded that the decrease in the concentration of this cytokine could be related to the increase in biliary excretion and improvement in liver function. Regarding this relationship, a particularly feasible conclusion seems to be that the concentration of direct bilirubin is significantly related to the concentration of IL-6.

Ott et al. in their study concluded that semi purified cytokine preparation having IL-1 but not TNF, caused a significant depression in bile in the rat isolated perfused liver (16). Moreover, Jones et al. stated that TNF-α causes increase in bilirubin and jaundice (17). The results of the aforementioned studies confirm the role of IL-1 and TNF-α inflammatory cytokines in the excretion of bilirubin. These in vitro studies were performed under controlled circumstances, whereas our study was accomplished in phys-

Table 1. Hematological Parameters in Sulfur Mustard -Exposed and Control Groups[a]

	Control	Exposed					
	(N = 128)	All (N = 372)		Non-Hospitalized (N = 203)		Hospitalized (N = 169)	
	Mean ± SD	Mean ± SD	P Value[b]	Mean ± SD	P Value[c]	Mean ± SD	P Value[d]
RBC	5.21 ± 0.51	5.18 ± 0.49	0.597	5.11 ± 0.45	0.074	5.27 ± 0.52	0.349
HB	15.7 ± 1.48	15.90 ± 1.31	0.161	15.79 ± 1.30	0.592	16.03 ± 1.31	0.043
HCT	44.1 ± 3.67	45.09 ± 3.46	0.007	44.77 ± 3.37	0.092	45.57 ± 3.52	0.001
MCH	30.25 ± 3.14	30.81 ± 2.39	0.07	30.90 ± 2.11	0.041	30.69 ± 2.68	0.195
MCV	84.75 ± 8.26	87.10 ± 5.95	0.004	87.75 ± 4.76	< 0.001	86.31 ± 7.06	0.081
MCHC	35.53 ± 1.34	35.23 ± 1.30	0.025	35.21 ± 1.21	0.022	35.26 ± 1.40	0.094

Abbreviation: SM: sulfur mustard.
[a] Hematological parameters were assessed and a comparison was undertaken between the exposed and control groups (6). Data are presented as Mean ± SD.
[b] between exposed and control groups (t-test).
[c] between exposed non-hospitalized and control groups (t-test).
[d] between exposed hospitalized and control groups (t-test).

Table 2. Serum Level of Total and Direct Bilirubin in Sulfur Mustard-Exposed and Control Groups[a]

	Control	Exposed					
	(N = 128)	All (N = 372)		Non-Hospitalized (N = 203)		Hospitalized (N = 169)	
(mg/dL)	Mean ± SD	Mean ± SD	P Value[b]	Mean ± SD	P Value[c]	Mean ± SD	P Value[d]
Bilirubin Total	0.93 ± 0.39	0.92 ± 0.36	0.792	0.87 ± 0.31	0.148	0.97 ± 0.41	0.341
Bilirubin Direct	0.22 ± 0.07	0.22 ± 0.10	0.556	0.21 ± 0.08	0.077	0.23 ± 0.13	0.682

[a] The serum levels of total and direct bilirubin were assessed, and a comparison was undertaken between the control and exposed groups. Data are presented by Mean ± SD.
[b] between exposed and control groups (t-test).
[c] between exposed non-hospitalized and control groups (t-test).
[d] between exposed hospitalized and control groups (t-test).

iological state of affairs of humans and would have been affected by other confounding factors. Despite these limitations, we believe that the strengths of this study, which make its findings particularly significant, are its cohort nature and the large number of individuals examined in each group.

In conclusion, despite the significant decrease in cytokines concentration in SM-exposed casualties and testimonies certifying the relationship between cytokines and the concentration of bilirubin in some studies, no significant relationship was found in this study. Therefore, further investigations should be performed to identify the reason for the lack of increase in bilirubin. Also, additional research is needed to illuminate the interesting relationship among other biochemical and immunological parameters to clarify the mechanisms involved in order to better understand the clinical implications of this finding.

Acknowledgments

This study was carried out by the Immunoregulation research center of Shahed university and Janbazan medical and engineering research center (JMERC). We would like to thank all the participants, who took part in this investigation.

Footnotes

Competing Interests: The authors declare that they had no competing interests.

Funding/Support: This research was supported financially by the Iranian Foundation of Martyrs and Veterans Affairs and Ministry of Health and Medical Education.

Table 3. Serum Levels of Inflammatory Cytokines in Sulfur Mustard-Exposed and Control Groups[a,b,c,d,e]

Cytokines (pg/mL)	Study Groups		N	Median	Q1 - Q3	Mean ± SEM	P Value	P Value[b]
IL-1 α	Control		127	1.889	0.540 - 3.812	2.570 ± 0.277		
	Exposed	H	168	0.808	0.274 - 2.162	2.007 ± 0.293	< 0.001[c]	0.270
		NH	200	0.808	0.010 - 1.618	1.940 ± 0.319	< 0.001[d]	
		All exposed	368	0.808	0.274 - 1.889	1.971 ± 0.219	< 0.001[e]	
IL-1 β	Control		127	1.915	1.513 - 2.406	3.635 ± 0.718		
	Exposed	H	168	1.803	1.449 - 2.209	4 ± 0.668	0.281	0.002
		NH	200	1.647	1.329 - 2.007	2.940 ± 0.543	< 0.001	
		All exposed	368	1.726	1.384 - 2.102	3.426 ± 0.425	0.004	
IL-1 Ra	Control		127	33.010	21.590 - 52.200	53.027 ± 6.201		
	Exposed	H	168	30.640	18.930 - 49.020	50.033 ± 5.428	0.261	0.042
		NH	200	24.375	17.375 - 41.602	38.721 ± 3.379	0.002	
		All exposed	368	26.940	18.208 - 44.980	43.901 ± 3.097	0.015	
IL-6	Control		127	1.533	0.530 - 3.162	13.630 ± 5.574		
	Exposed	H	168	0.848	0.033 - 3.130	8.579 ± 3.819	0.039	0.117
		NH	200	0.526	0 - 1.943	3.771 ± 1.096	< 0.001	
		All exposed	368	0.604	0 - 2.562	5.973 ± 1.849	0.001	
TNF-α	Control		127	25.835	0.533 - 46.710	38.791 ± 5.505		
	Exposed	H	168	12.768	0 - 24.490	27.265 ± 4.155	0.014	0.253
		NH	200	9.385	0 - 21.737	22.613 ± 3.294	0.001	
		All exposed	368	11.113	0 - 23.125	24.732 ± 2.607	0.001	

Abbreviations: IL, Interleukin; TNF-α, Tumor necrosis factor-α.
[a] The serum level of cytokines was assessed using the ELISA method in all of the participants, including control and exposed groups (8, 13). A comparison was undertaken between the exposed groups with control group. Data are presented as median and first and third quartiles (Q1 - Q3).
[b] Comparison of hospitalized and non-hospitalized with control group (Mann-Whitney).
[c] Comparison of exposed hospitalized with control group (Mann-Whitney).
[d] Comparison of exposed non-hospitalized with control group (Mann-Whitney).
[e] Comparison of all exposed with control group (Mann-Whitney).

Table 4. Correlation Between Serum Bilirubin and Inflammatory Cytokines Between Sulfur Mustard - Exposed and Control Groups[a]

	Control		Exposed					
	(N = 127)		All (N = 368)		Non-Hospitalized (N = 200)		Hospitalized (N = 168)	
Cytokines (pg/mL)	Bilirubin Total (mg/dL)	Bilirubin Direct (mg/dL)	Bilirubin Total (mg/dL)	Bilirubin Direct (mg/dL)	Bilirubin Total (mg/dL)	Bilirubin Direct (mg/dL)	Bilirubin Total (mg/dL)	Bilirubin Direct (mg/dL)
IL-1α								
r	0.080	0.073	0.080	0.052	0.073	0.074	0.073	0.009
P Value	0.372	0.417	0.125	0.318	0.301	0.295	0.344	0.909
IL-1β								
r	0.034	0.001	0.012	0.092	0.031	0.122	- 0.030	0.038
P Value	0.703	0.988	0.813	0.077	0.666	0.085	0.703	0.623
IL-1Ra								
r	0.127	0.011	- 0.082	- 0.069	- 0.121	- 0.078	- 0.059	- 0.076
P Value	0.156	0.901	0.118	0.184	0.088	0.273	0.443	0.324
IL-6								
r	0.150	0.164	0.065	0.104	0.024	0.111	0.087	0.079
P Value	0.092	0.066	0.215	0.047	0.736	0.120	0.259	0.308
TNF-α								
r	0.106	0.019	0.033	0.059	0.023	0.061	0.037	0.044
P Value	0.234	0.831	0.532	0.261	0.749	0.392	0.634	0.569

Abbreviations: r, Spearman's rank correlations coefficient; p, p value two tailed test; IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-1Ra, interleukin-1 receptor antagonist; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; SM, sulfur mustard.
[a] Correlations were undertaken between IL-1α, IL-1β, IL-1Ra, IL-6 and TNF-α with total and direct bilirubin using Spearman's rank correlations coefficient.

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