Research Article:
Association of Nitric Oxide With Delayed Skin Problems After Sulfur Mustard Exposure: Part of Sardasht-Iran Cohort Study

Nayere Askari[1], Shohreh Jalaie[2], Athar Moin[3], Seyed Naser Emadi[4], Ali Khamesipour[5], Seyed Emad Emadi[6], Elham Faghihzadeh[3], Tooba Ghazanfari[3*]

1. Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Kerman, Iran.
2. Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.
3. Immunoregulation Research Center, Shahed University, Tehran, Iran.
4. Department of Dermatology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
5. Center for Research and Training in Skin Diseases and Leprosy, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
6. Mazandaran University of Medical Sciences, Sari, & Iranian Red Crescent Society, Tehran, Iran.

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ABSTRACT

Background: Exposure to Sulfur Mustard (SM) leads to short- and long-term adverse effects on various organs, including the skin. Despite several studies on long-term clinical manifestations of skin toxicity in SM-exposed individuals, the pathogenesis of SM-induced skin disorders is not fully understood.

Materials and Methods: As part of Sardasht-Iran Cohort Study (SICS), this study aimed to find out the possibility of any correlation between the serums level of Nitric Oxide (NO) and skin problems due to the long-term effect of SM as well as the kind of skin illness. In this historical cohort study, 372 male SM-exposed subjects and 128 age-matched unexposed controls were studied. Clinical evaluation was carried out for all participants, and their serum concentration of NO was measured.

Results: According to our results, the Mean±SD serum level of NO in the exposed group with skin disorders were significantly higher than that in the exposed group without skin disorders (1483.00±488.754µg/mL vs. 1364.50±487.887µg/mL; P=0.024). Also, among the exposed group, there was a significant elevation of serum NO associated with the type of lesion. For ezxample, specific lesions like mustard scar were associated with higher levels of NO compared to non-specific lesions like xerosis, itching, seborrheic dermatitis, etc. Also, a significant elevation in serum NO levels was found in the exposed subjects with pigmentation disorders (both hypo- and hyper-pigmentation) compared to the exposed participants without these skin problems (P<0.05). Finally, a significant difference in the serum level of NO was seen between the SM-exposed and the control subjects with no cherry angioma (P<0.05).

Conclusion: Our results show the highest serum level of NO in the exposed group with specific lesions and the lowest or normal level of NO in the unexposed group with no skin illness. The elevated serum levels of NO may be associated with the progression of some skin complications in the SM-exposed subjects. This finding serves as a basis for further research on the molecular mechanisms and pathways involved in the pathogenesis of skin disorders in SM-exposed patients.

*** Corresponding Author:**
Tooba Ghazanfari, PhD.
Address: *Immunoregulation Research Center, Shahed University, Tehran, Iran.*
Phone: *+98 (21) 66418216*
E-mail: *tghazanfari@yahoo.com*

Introduction

Sulfur Mustard (SM) is a blister agent with cytotoxic and mutagenic effects. The mechanism(s) of SM cytotoxicity has not yet been fully understood [1]. SM exposure leads to short- and long-term adverse effects on body organs, especially the skin, eyes, respiratory tract, and immune system [2]. The most common SM-induced chronic skin complications are mustard scars, xerosis, eczema, seborrheic dermatitis, cherry angioma, hyperpigmentation, and some rare skin cancers [2-5]. Despite various studies on the long-term clinical manifestations of skin toxicity in SM-exposed individuals, the pathogenesis of SM-induced skin disorders needs more investigations. The role of inflammatory mediators and certain hormones and molecules like Nitric Oxide (NO) in the pathogenesis of skin disorders in other diseases has been partly elucidated [6, 7].

NO is a ubiquitous free radical with a variety of biological functions that can influence the immune system, neurotransmission, and smooth muscle relaxation [8]. The skin is an enormous organ in the human body which consists of three main layers and various cells, including keratinocytes, Langerhans cells, melanocytes, dermal dendritic cells, fibroblast, endothelial cells, and migratory lymphocytes [9]. Keratinocytes, fibroblasts, melanocytes, Langerhans, and endothelial cells express Nitric Oxide Synthase (NOS) and are capable of releasing NO [10]. Nitric oxide may induce itching via neurogenic inflammation [11].

According to the most updated identification of the NOS pathway in diverse cell types in the skin, NO plays a significant role in the pathogenesis of several human skin diseases. Lots of research efforts have discovered that a defect in the NO pathway may contribute to several human skin diseases [8]. As part of Sardasht-Iran Cohort Study (SICS), we evaluated in this study the possible association between NO and SM-induced long-term skin complications in SM-exposed individuals.

Materials and Methods

Study design and participants

This study is part of SICS, and the details of its methodology were previously described [1]. Briefly, 368 male 20-60 years old subjects who had been exposed to SM more than 25 years ago and 126 age and sex-matched unexposed volunteers were included in the cohort. SICS was initiated in 2006. The clinical evaluation and sample collection were carried out in June 2007, and the experiments were completed in 6 months. All volunteer participants underwent clinical evaluation. Each volunteer was interviewed and physically examined by a dermatologist. SM-exposed participants showed various organ complications and were categorized into proper subgroups to understand easier any possible relationship between serum NO level and skin disorders. A total of 368 male subjects of this study were recruited out of surviving veterans of the Iraq-Iran war whose exposure to SM On June 28, 1987 (Sardasht. Iran) was documented in their wartime medical records [1, 2].

They had at least one cutaneous sign or symptom at the time of this study. The exposed group was categorized into two subgroups, mild and moderate to severe, depending on the severity of the problems at the exposure time. All skin conditions which had been related to any metabolic, internal, infectious, and adverse drug reactions were excluded from both case and control groups.

Eventually, the participants were divided into two main groups (1- exposed and 2- unexposed) with five subgroups: 1-1. SM-exposed group with specific lesions like mustard scar with permanent pigmentation, vascular and trophic changes which developed mainly at the sites of previous SM-induced bulla or infected ulcers; 1-2. SM-exposed group with nonspecific skin disorders, including seborrheic dermatitis, eczema, xerosis, acne, urticaria, hyperpigmentation, and other skin illness which are common in general population; 1-3. SM-exposed group with no skin disorder; 2-1. Control group (SM-unexposed) with skin disorder; 2-2. Control group (SM-unexposed) with no skin finding.

Nitrite assay

The serum nitrite level was determined by a method based on the Griess reaction using a Kayman product kit (USA). A total of 100µL of the sample was mixed with 100 µL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl enediamide dihydrochloride in water) and incubated at room temperature for 10 min followed by measuring the absorbance in a plate reader at 540nm (Stat-Fax 2100). Nitrite concentration in the samples was determined using a standard curve generated by different concentrations of sodium nitrite [12].

Statistical analysis

For nitric oxide analysis, the data were presented as median and first and third quartiles (Q1, Q3) and compared between the study groups using the Mann–Whit-

ney test. (P≤0.05) was considered as statistically significant. Analyses were performed in SPSS V. 13.

Results

According to our previous results, the serum levels of NO were not significantly different between the exposed and control groups [13].

However, as shown in Table 1, the difference between the serum levels of NO in the SM-exposed groups with skin disorders was significantly higher than NO level in the exposed group without skin disorders (1483.00±488.754µg/mL vs. 1364.50±487.887µg/mL) (P<0.05). The data presented in Table 1 shows NO level in the exposed patients with mild, moderate, and severe skin disorders in comparison with the controls. As it is shown, a significantly higher level of NO was seen in exposed groups with skin disorders compared to the exposed group without skin disorders (P<0.05). The highest value was observed in the exposed persons with moderate to severe skin disorders, which was significantly different from normal controls (P=0.008).

The serum NO levels were evaluated based on skin complications and compared between the exposed and control groups. As indicated in Table 2, the serum NO level in the exposed group with the mustard scar was significantly higher than that in the exposed group without mustard scar (P=0.022). A significant elevation in the serum level of NO was revealed in the exposed group with hyperpigmentation compared to those without hyperpigmentation (P<0.05) (Table 3). Table 3 also shows the levels of NO in the exposed participants with hypopigmentation, which are significantly higher than those without hypopigmentation disorder (P<0.05).

As shown in Table 4, there is a significant difference in the serum level of NO between the exposed and control groups without cherry angioma (P<0.05). In addition, Table 5 shows no statistically significant difference in the NO levels between the exposed subjects with and without cherry angioma. According to our data, there is no statistically significant association between the serum NO levels and the skin findings, such as eczema, seborrheic dermatitis, xerosis, and urticaria.

Table 1. Association of serum levels of NO in the SM-exposed and control groups with dermatological assessment

Study Groups	Dermatological Assessment	Serum Nitric Oxide (µg/mL)					P1	P2
		n	Median	Q1	Q3	Mean±SD		
Control	No skin finding	108	1275.00	1039.00	1580.00	1416.48±572.190	0.188	0.076
	Skin finding	15	1340.00	1148.00	1961.00	1559.37±512.276		
Exposed	No skin finding	240	1364.50	1152.50	1619.00	1451.99±487.887	0.024	0.931
	Skin finding	101	1483.00	1252.00	1744.00	1551.17±488.754		
Control	Normal	108	1275.0	1039.0	1580.0	1416.5±572.2	0.335	0.076
	Mild	14	1329.0	1148.0	1961.0	1556.0±531.4		0.954
	Moderate-severe	1	1607.0	1607.0	1607.0	1607.0		
Exposed	Normal	240	1364.5	1152.5	1619.0	1452.0±487.9	0.013	0.008
	Mild	90	1453.0	1181.0	1734.0	1532.4±502.6		
	Moderate-severe	11	1572.0	1480.0	1945.0	1704.5±333.5		

IMMUNOREGULATION

The serum levels of NO in volunteers with and without skin findings, were assessed, and a comparison was made between the control and exposed groups, as well as, within each group.

P1: Comparison of the serum NO level between participants with or without skin findings within each group (Mann–Whitney test) ; P2: Comparison of the exposed participants with corresponding controls (Mann-Whitney test). Bold data shows significant differences with P<0.05; P3: Comparison between the exposed group with moderate to severe problems and normal group (Mann-Whitney test)

Participants subgroups based on the severity of the skin findings: 1. The participants with moderate to severe problems; 2. Participants with mild problems; 3. Normal group

Table 2. Association of serum NO levels in the SM-exposed and control groups with and without Mustard scar (Diagnosis)

Study Groups	(Diagnosis) Mustard Scare	Nitric Oxide (Serum) (µg/mL)					P1	P2
		N	Median	Q1	Q3	Mean±SD		
Control	No	123	1293.0	1051.0	1588.0	1433.9±565.2	-	0.088
	Yes	0	-	-	-	-		
Exposed	No	291	1375.0	1155.0	1644.0	1466.0±491.6	0.022	-
	Yes	51	1520.0	1307.0	1874.0	1608.3±539.2		

IMMUNOREGULATION

The serum NO levels in volunteers with (Yes) and without (No) mustard scar were assessed, and a comparison was made between the control and exposed groups, as well as within each group.

P1: Comparison of the serum level of NO between participants with (Yes) or without (No) mustard scare within each group (Mann–Whitney test); P2: Comparison between the exposed subjects and corresponding control group (Mann-Whitney test). Bold data shows significant differences with (P<0.05)

Discussion

During the Iran-Iraq conflict (1980-1988), chemical warfare agents were frequently used by the Iraqi army forces against Iranian soldiers and civilians. Sardasht, one of the border towns in northwestern Iran, with 12000 population was struck with SM bombs in 1987 which resulted in 128 dead and 1500 seriously-injured people with chronic and delayed respiratory, visual, and skin disorders [14].

Nitric oxide seems to have various biological functions on the skin, from the orchestration of the normal regulatory processes to some of the pathophysiological changes. In the skin, keratinocytes, Langerhans, melanocytes, endothelial cells, and fibroblasts express NOS and appear to be capable of releasing NO [8-10]. Under certain conditions, almost all human skin cells can express the inducible NOS isoforms (NOS2). The iNOS synthase expression implies that NO may play an important role in psoriasis and other inflammatory diseases [10].

Table 3. Association of serum levels of NO in the SM-exposed and control groups with pigmentation disorders

Study groups	(Diagnosis) Hyperpigmentation	NO	n	Median	Q1	Q3	Mean±SD	P1	P2
Control	No	Nitric Oxide (Serum) (µg/mL)	117	1293.0	1051.0	1587.0	1438.9±574.644	0.907	0.091
	Yes		6	1297.0	1189.0	1627.0	1336.6±349.289		
Exposed	No		286	1373.5	1162.0	1606.0	1464.5±486.013	0.036	0.287
	Yes		56	1499.5	1245.5	1873.0	1603.2±560.465		
Control	No	Nitric Oxide (Serum)	121	1297.00	1107.00	1588.00	1442.66±565.59	0.035	0.105
	Yes		2	904.10	812.50	995.70	904.10±129.54		
Exposed	No		322	1380.00	1164.00	1641.00	1468.27±481.85	0.012	0.035
	Yes		20	1515.00	1356.00	2020.50	1792.59±687.74		

IMMUNOREGULATION

The serum levels of NO in volunteers with (Yes) and without (No) pigmentation disorders were assessed, and a comparison was made between the control and exposed groups, as well as within each group.

P1: Comparison of the serum level of NO between participants with (Yes) or without (No) pigmentation disorders within each group (Mann-Whitney test); P2: Comparison of the exposed participants with corresponding controls (Mann-Whitney test). Bold data shows significant differences with (P<0.05)

Table 4. Association of serum levels of NO in the SM exposed and control groups with cherry angioma

Study Groups	(Diagnosis) Cherry Angioma	Nitric Oxide (Serum) (µg/mL)					P1	P2	P3
		n	Median	Q1	Q3	Mean±SD			
Control	No	111	1293.0	1079.0	1597.0	1439.4±569.4	0.724	0.039	-
	Yes	12	1294.0	1018.4	1443.5	1383.1±545.7		0.417	
Exposed	No	270	1403.5	1177.0	1657.0	1500.8±518.8	0.274	-	0.41
	Yes	72	1353.5	1159.5	1539.5	1436.5±425.6			

IMMUNOREGULATION

The serum levels of NO in volunteers with (Yes) and without (No) cherry angioma were assessed, and a comparison was made between the control and exposed groups, as well as, within each group.

P1: Comparison of the serum level of NO between participants with (Yes) or without (No) cherry angioma within each group (Mann-Whitney test); P2: Comparison of the exposed subjects and corresponding controls (Mann-Whitney test); P3: Comparison between the exposed group with skin findings and controls without cherry angioma (Mann-Whitney test). Bold data shows significant differences with (P<0.05)

Table 5. Association of serum levels of NO in the SM-exposed and control groups with eczema, seborrheic dermatitis, and xerosis

Study Groups			Nitric Oxide (Serum)					P1	P2
			n	Median	Q1	Q3	Mean±SD		
Control	No	(Diagnosis) Eczema	87	1316.00	1107.00	1597.00	1456.03±589.72	0.453	0.137
	Yes		36	1191.50	1030.50	1587.50	1380.45±504.86		
Exposed	No		248	1378.50	1174.00	1645.00	1484.99±478.18	0.892	0.106
	Yes		94	1408.00	1184.00	1659.00	1493.17±558.55		
Control	No	(Diagnosis) Xerosis	109	1280.00	1037.00	1587.00	1422.62±575.65	0.173	0.016
	Yes		14	1548.00	1189.00	1599.00	1521.79±485.48		
Exposed	No		257	1399.00	1170.00	1653.00	1482.81±494.94	0.938	0.564
	Yes		85	1378.00	1180.00	1602.00	1500.62±520.60		
Control	No	(Diagnosis) Seborrheic Dermatitis	108	1272.50	1065.00	1578.50	1425.26±579.32	0.308	0.015
	Yes		15	1483.00	995.70	1758.00	1496.17±462.75		
Exposed	No		305	1388.00	1175.00	1644.00	1485.09±498.04	0.955	0.848
	Yes		37	1366.00	1119.00	1770.00	1504.88±529.10		

IMMUNOREGULATION

The serum levels of NO in volunteers with (Yes) and without (No) cherry angioma were assessed, and a comparison was made between the control and exposed groups, as well as, within each group.

P1: Comparison of the serum level of NO between participants with (Yes) or without (No) cherry angioma within each group (Mann-Whitney test); P2: Comparison of the exposed subjects and corresponding controls (Mann-Whitney test)

Bold data shows significant differences with (P<0.05)

Oxidative stress damages both the skin epidermal and dermal cells, because of the high occurrence of suitable and potential biological targets for such reactions. These reactions may originate in the outer environment, in the skin itself, in various endogenous and exogenous sources, in exogenous chemicals, and toxins [15-17].

The physiological functions of NO in the skin are being elaborated. It has been shown that NO has the potential to switch from being an essential regulator to a harmful destroyer. The overstimulation of NOS can cause disease generation, inflammatory pathologies, neurodegenerative diseases, and cancer progression [18].

In Sardasht-Iran cohort study, it has been shown that (79.9%) of the Iranian SM-exposed individuals suffer from chronic skin disorders [2]. The most common non-significant chronic skin complications were xerosis, eczema, seborrheic dermatitis, cherry angioma, hyperpigmentation, urticaria, and vitiligo while mustard scar was the most significant finding among chemical victims in Sardasht City [2, 3, 14].

Previously, it has been found that the serum levels of pro-inflammatory cytokines (IL-1α and TNF-α) in SM-exposed individuals with chronic and delayed skin disorders were lower than those in the healthy controls [15]. This study is focused on the serum levels of NO in SM-exposed individuals compared to their matched controls with or without skin disorders.

In the mentioned study, it has also been shown that the serum NO concentration is not different between SM-exposed individuals and controls, regardless of skin disorders [12]. In the present study, a significant association was seen between the elevation of serum levels of NO and the occurrence of skin disorders in the SM-exposed group. In other words, elevated NO levels are seen in SM-exposed group with skin findings. Based on the results of this study, it is suggested that the increased level of NO in serum is proportional to the severity of skin disorders in SM-exposed patients with various degrees of late skin complications.

In this study, the relationship between serum NO and a variety of skin disorders has also been studied. A significant association was seen between the elevation of the serum NO levels and the presence of pigmentation disorders (salt and pepper) in the SM-exposed group. As the Emadi et al. explained in mustard exposed case, pigmentary changes, known as salt and pepper pigmentation, hence low dose mustard exposure causes incomplete melanocyte destruction and subsequent hyperpigmentation. In contrast, high dose exposure will lead to complete local melanocyte destruction and depigmentation, which is permanent and different from transient post-inflammatory hyper or hypo-pigmentation (PIH) [19]. The incidence of hyper and hypo-pigmentation was reported to be (15.5%) and (5.5%), respectively in SM-exposed group 20 years after SM exposure [2].

Although, no relationship has been so far reported between SM-induced pigmentation disorders and NO, some results suggest that NO may contribute to UV-induced hyperpigmentation as a main physiological stimulus for human skin pigmentation [20]. In human pigmentation, melanin is produced and dispersed by epidermal melanocytes to neighboring keratinocytes [21, 22]. The rise in melanogenesis induced by NO-generating compounds is directly related to the high amount of both tyrosinase and tyrosinase-related protein. These investigations point out that NO has a vital role in the paracrine mediation of UV-induced melanogenesis [20]. The outcome of this study suggests that NO may also be involved in SM melanogenesis.

Hypopigmentation and hyperpigmentation may be the result of the agents that increase reactive oxygen species, since melanocytes containing relatively less catalase, peroxidase, and superoxide dismutase in comparison with other cells, including keratinocytes, they are more susceptible to reactive oxygen species [23]. In vitiligo, a chronic depigmented skin disorder, the derived keratinocytes from vitiligo lesions generate a rising number of superoxide anions (hyperactive oxygen and NO). Patients that suffer from generalized vitiligo display an imbalance between oxidant and antioxidant systems [24].

Another delayed skin complication was cherry angioma. In SICS, the incidence of cherry angioma was reported to be (19.9%) in SM-exposed group 20 years after SM exposure [2]. Emadi et al. described the pathogenesis of abnormal angiogenesis in cherry angioma. They stated that vascular changes could be presented as cherry angioma and telangiectasia. They could be induced either by cytokine release in the acute inflammatory phase of bulla healing or by direct effects of SM on DNA [4, 19].

In this study, a significant association was seen between the elevation of the serum levels of NO and the absence of cherry angioma in the SM-exposed group as compared to the control group. No significant difference was seen in the serum level of NO between SM-exposed participants with cherry angioma and the control group without cherry angioma, while there was a significant elevation in NO level in SM-exposed without cherry an-

gioma compared to normal unexposed group. In other words, an elevated level of NO was seen in the exposed group without cherry angioma, and SM-exposed participants with cherry angioma did not show this alterations.

NO has been shown to play a critical role in the regulation of angiogenesis during several pathophysiological processes such as ischemia. Endothelial NOS is extremely important for ischemic remodeling, mural cell recruitment, and blood flow reserve [25, 26]. On the contrary, in diabetic nephropathy, the elevated NO derived from the endothelial cells plays an inhibitory role that hinders excessive endothelial cell proliferation, vascular smooth muscle cell proliferation, and macrophage infiltration. When NO bioavailability is lowered in diabetes, increased levels of Vascular Endothelial Growth Factor (VEGF) leads to excess endothelial cell proliferation, stimulation of macrophage chemotaxis, and vascular smooth muscle cell activation [27].

Furthermore, the same relationship was found for prolactin in our previous study, and it was shown that prolactin increased the same as NO in the SM-exposed population without cherry angioma [28]. It should also be noted that angiogenesis was a multifactorial issue, which numerous elements might be involved in its establishment in a dependent or independent manner, and many of these factors were affected by SM toxicity. SM exposure may cause a certain disorganization, which could lead to cherry angioma [19, 28]. Based on these results, it is suggested that increases in serum levels of NO might be involved in the protection against SM-induced cherry angioma. Because of the abovementioned reasons, we would like to claim that different dose, time duration, and frequency of exposure to SM might cause different pathogenesis and side effects which could be associated with different secretion of NO.

As an example, in this study, pigmentary changes with a low dose of SM could be associated with high serum level of NO while vascular changes (by cytokine release in acute inflammatory phase or direct damage on DNA) due to a high dose of exposure to SM might block the normal process of NO activity and production. Despite the presence of report supporting the role of NO in patients with atopic eczema/dermatitis syndrome, no significant association was found between NO and SM-induced eczema/dermatitis [29, 30].

According to the Moin et al. study, one of the most common skin lesions in the exposed groups was mustard scar (14.6%) [2]. The serum level of NO in the exposed group with mustard scar disorder was significantly higher than that in the exposed group without a scar. To our knowledge, there is no study on the association between NO and mustard scar, but there are many reports about the effects of NO on wound healing [31, 32]. Scar is a part of fibrous tissue (fibrosis) that is responsible for replacing normal skin when the injury takes place. The scar is formed by the biological process of wound repair in the skin and other human body tissues [33-35].

Overall, the skin is the largest organ in the body, and exposure to mustard gas can cause extensive damage to it. The existence of scar may indicate the severity of exposure or exposure-induced injury. Given the positive relationship between NO and scar, it can be told that NO levels 20 years after exposure can have a relationship with the severity of the primary injury. This finding on the alterations of NO could serve as a basis for further research on the molecular mechanisms and pathways involved in the pathogenesis of SM-induced skin disorders. To investigate the role of NO, it is necessary to evaluate the concentration of NO in the lesions of SM-exposed patients.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee Board of the Research Ethics of Janbazan Medical and Engineering Research Center (JMERC), the Board of Research of Ministry of Health and Medical Education, and the Ethics committee of Shahed University. All potential volunteers were informed about the purpose and procedure of the study. The volunteers who signed informed consent were recruited.

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Authors' contributions

All authors contributed in preparing this article.

Conflicts of interest

The authors declared no conflict of interest.

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