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Increased nitrite levels in exhaled breath condensate in asthma-like syndrome induced by occupational sulfur dioxide exposure*

Zeki YILDIRIM[1], Sadık SÖĞÜT[2], Nurhan KÖKSAL[3], Talat KILIÇ[4]

Aim: To investigate whether nitrite levels were abnormally high in expired breath condensate (EBC) from workers with asthma-like syndrome.

Materials and methods: We measured the nitrite levels in EBC from 48 volunteer workers, including 25 smokers, with asthma-like syndrome before and after SO_2 exposure and compared these levels with those from 44 nonexposed control subjects, including 23 smokers.

Results: Baseline concentrations of nitrite in the workers before SO_2 exposure were higher (19.31 ± 1.06 nmol/mL) than in the controls (13.08 ± 1.36 nmol/mL; $P < 0.001$). Acute SO_2 exposure produced a significant increase in concentrations of nitrite (23.62 ± 1.36) compared to the baseline value ($P < 0.05$). Preexposure nitrite concentrations were similar in smoking and nonsmoking workers (17.85 ± 1.72 and 20.20 ± 1.59; $P > 0.05$).

Conclusion: We concluded that nitrite levels in EBC are increased in the asthma-like syndrome induced by occupational SO_2 exposure in an agricultural environment, suggesting the role of inflammation in the syndrome; this first application of the EBC method may be suitable in other field studies in occupational medicine.

Key words: Nitrite, asthma-like syndrome, sulfur dioxide, agriculture, apricot sulfurization

Mesleki kükürt dioksit maruziyetine bağlı astım benzeri sendromunda yoğunlaşmış ekspirium havasında nitrik oksit düzeyinin artması

Amaç: Bu çalışmanın amacı bu işçilerde yoğunlaşmış ekspirum havasında (YEH) nitrit seviyesinin artıp artmadığını araştırmaktır.

Yöntem ve gereç: Çalışmaya 25'i sigara içenlerden olmak üzere 48 gönüllü ABS'u olan işçi ve benzer yaş ve cinsiyetten 23'ü sigara içenlerden olmak üzere 45 sağlıklı gönüllü alındı. İşçilerin SO_2 maruziyeti öncesi ve sonrasında elde edilen YEH'de nitrit düzeyleri ölçüldü ve sağlıklı kişilerinki ile karşılaştırıldı.

Bulgular: ABS'li işçilerin YEH'de ölçülen bazal nitrit düzeyleri (19,31 ± 1,06 nmol/mL) sağlıklı gönüllülerin nitrit düzeylerinden (13,08 ± 1,36 nmol/mL, $P < 0,001$) anlamlı yüksek bulundu. Akut SO_2 maruziyeti nitrit düzeylerini bazal düzeye göre anlamlı olarak yükseltti (23,62 ± 1,36, $P < 0,05$). Sigara içen ve içmeyenlerin SO_2 maruziyeti öncesi nitrit düzeyleri benzer olarak bulundu (17,85 ± 1,72 ve 20,20 ± 1,59, $P > 0,05$).

Sonuç: Bu çalışmada tarım iş kolunda kayısı kükürtleme işlemi sırasında SO_2 maruziyeti sonucu oluşan ABS'li işçilerin YEH nitrit düzeylerinin arttığı gösterildi. Bu artış bu sendromun patogenezinde inflamasyonun olabileceğini düşündürmektedir. Bu çalışma tarım iş kolunda yapılan ilk çalışmadır ve bu yöntemin alan çalışmalarında uygun olabileceğini göstermektedir.

Anahtar sözcükler: Nitrit, astım benzeri sendrom, tarım, SO_2, kayısı kükürtleme

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[1] Department of Pulmonary Medicine, Faculty of Medicine, Fatih University, Ankara - TURKEY
[2] Department of Biochemistry, Faculty of Medicine, Bezmiâlem Vakıf University, İstanbul - TURKEY
[3] Department of Pulmonary Medicine, Faculty of Medicine, Sütçü İmam University, Kahramanmaraş - TURKEY
[4] Department of Pulmonary Medicine, Malatya State Hospital, Malatya - TURKEY
Correspondence: *Zeki YILDIRIM, Department of Pulmonary Medicine, Faculty of Medicine, Fatih University, Ankara - TURKEY*
E-mail: zyildirim@inonu.edu.tr

* This study was performed at the İnönü University School of Medicine between 2003 and 2004 in Malatya, Turkey.

Introduction

The term "asthma-like syndrome" is used to describe an acute nonallergic airway response arising from the inhalation of various agents (1). The symptoms consist of chest tightness, wheeze, and/or dyspnea and can be associated with a cross-shift decline in forced expiratory volume in the first second (FEV_1) (usually less than 10%). Recently, it was reported that the syndrome is associated with airway inflammation, neutrophils, and proinflammatory cytokines (1). A number of agents, such as cotton and grain dust, have been shown to give rise to the syndrome (1,2). Poultry and swine confinement workers also develop asthma-like syndrome due to exposure to organic dust (3-7). Swine confinement workers are additionally exposed to gases such as ammonia, hydrogen sulfide, and carbon dioxide (6,7). In a previous study (8), it was reported that apricot sulfurization workers (ASWs) were exposed to high concentrations of sulfur dioxide (SO_2) during the process of apricot sulfurization. This study also showed that the SO_2 inhaled by these workers caused an "asthma-like syndrome" manifested by a decrease in pulmonary functions and an increase of symptom scores of dyspnea and cough.

Although the mechanism by which inhaled SO_2 induces bronchoconstriction is unknown, the possibilities include a neuronal reflex mechanism (9), a direct effect of SO_2 on mast cells or other cells of the airways (10-12), and an altered pH in the airways due to SO_2 (13). Previous studies revealed that increased serum malondialdehyde (14) and nitrite levels (15) and decreased serum antioxidant enzyme activities occur in ASWs who develop an asthma-like syndrome during apricot sulfurization, suggesting the possible role of oxidative and nitrative stress in the pathogenesis of this SO_2-induced asthma-like syndrome.

Exhaled breath condensate (EBC), the liquid phase of exhaled breath, contains aerosol particles in which both nonvolatile and volatile substances have been identified. Markers of free radical production such as hydrogen peroxide (16), leukotrienes, 3-nitrotyrosine (17), and isoprostanes (18) have been detected in asthma and chronic obstructive pulmonary diseases, confirming that these groups of patients have increased oxidative stress in their airways. The analysis of EBC is a novel, noninvasive method with which to monitor airway inflammation, which is likely to reflect the local rather than systemic production of free radicals (19). Norris et al. studied the effect of 200 ppm SO_2 exposure for a period of 2 h on the cellular permeability and inflammatory changes in the bronchoalveolar lavage (BAL) fluids in dogs and revealed an increase in the number of neutrophils, macrophages, and epithelial cells. They also found an increase in airway permeability to plasma proteins and an increase in BAL proinflammatory prostaglandin E_2 (PGE_2) content (20), as occurred in patients with chronic obstructive pulmonary disease (COPD) or asthma. Given these data, our previous findings (15), and the increased oxidative stress marker in EBC in asthma and COPD (16-18), we speculated that the measurement of nitrite and the oxidized metabolite of nitric oxide (NO) in EBC would provide further information about nitrogen-reactive species in the airways of workers with SO_2-induced asthma-like syndrome. We hypothesized that, on the basis of the increased levels of serum nitrite, the exhaled nitrite level would be high. The aim of this study was thus to assess the level of nitrite in the EBC of workers with SO_2-induced asthma-like syndrome in comparison with healthy subjects without SO_2 exposure.

Materials and methods

Subjects

We visited 8 different apricot farms where a total of 60 workers were working in the apricot sulfurization process. Of these ASWs, 51 volunteered to participate in the study, 25 of whom were smokers. All of the workers had been working in apricot sulfurization seasonally, for a period of 20-25 days per year each summer. Each worker provided a complete medical history including previous and current pulmonary diseases and drug use. Three subjects with asthma, other known pulmonary or systemic diseases, or respiratory infection within the 3 weeks preceding the study were excluded. The control subjects consisted of 44 healthy, age-matched, male volunteers who had no exposure to SO_2 or any other toxic gas, 23 of whom were smokers. The study was approved by the Research Ethics Committees of the İnönü University Medical Faculty and all of the participants gave written informed consent before enrollment.

Pulmonary function tests

Pulmonary function measurements were obtained at the apricot farms, with the subjects in the seated position, using a portable spirometer (Cosmed, P/N: CO9002-02-99, S/N: 7012154, Rome, Italy) according to the guidelines of the American Thoracic Society (21). A set of pulmonary function parameters was measured before (baseline) and immediately after hours of SO_2 exposure during the apricot sulfurization process, as described below. Forced vital capacity (FVC), FEV_1, FEV_1/FVC %, and forced midexpiratory flow rate ($FEF_{25\%-75\%}$) were measured.

SO_2 exposure

As previously described (8), apricot sulfurization has traditionally been performed in well-isolated rooms called sulfurization chambers (SC). Sulfur is melted and converted to gaseous SO_2 by heating it on a plate over the stove in the SC. Apricots are exposed to SO_2 for a period of 8-10 h. When the process of sulfurization is completed, the door of the SC is opened and the SO_2 escapes into the open air. The workers use no protective equipment, and although they try to hold their breath, ASWs in these areas are exposed to SO_2 while replacing the boxes of processed apricots with the unprocessed ones. This replacement work is completed within 1 h, during which the exposure of the workers to SO_2 is maximal. This study was performed at the same farms as our previous study, in which the SO_2 concentration ranged between 106.6 and 721.0 ppm (8). Since the pulmonary function changes and the serum nitrite and malondialdehyde concentrations did not correlate with the concentration of SO_2 in our previous studies (14,15), and because apricot sulfurization has been performed in the same manner for a long time, we did not measure the SO_2 concentration in this study.

EBC collection

We visited apricot farms to collect EBC. The EBC was collected using a portable condenser specially designed as previously described by Kharitonov and Barnes (22), with some modifications. EBC was obtained twice from each worker, before and at least 1 h after SO_2 exposure. After rinsing their mouths, the subjects breathed through a mouthpiece and a 2-way nonrebreathing valve block in which inspiratory and expiratory air was separated. The valve also served as a saliva trap. During expiration, the breathing air flowed through the condenser, which was cooled at −20 °C. Subjects were asked to breathe at a normal frequency and tidal volume, wearing a nose clip, for a period of 15 min. At least 1 mL of condensate was immediately stored at −20 °C, transferred on dry ice within a few hours, and stored in the laboratory at −70 °C.

Nitrite measurements

Since NO measurement is very difficult in biological specimens, EBC nitrite (NO_2^-) and nitrate (NO_3^-) were used as an index of NO production. The method for measuring EBC nitrite and nitrate levels was based on the Griess reaction (23). Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite + nitrate) was measured after the conversion of nitrate to nitrite by copperized cadmium granules with a spectrophotometer at 545 nm (Ultraspec Plus, Pharmacia LKB Biochrom Ltd., England). A standard curve was established with a set of serial dilutions (from 10^{-8} to 10^{-3} mol/L) of sodium nitrite. Linear regression was done using the peak area from the nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations.

Amylase measurement

The amylase concentration of the EBC was measured with an autoanalyzer (Olympus AU600) using commercial Olympus kits.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). A paired t-test was used for comparison of the pulmonary function parameters and nitrite level before and after SO_2 exposure. An unpaired t-test was used to compare nitrite in the EBC between the ASWs and the healthy subjects. $P < 0.05$ was considered statistically significant. Statistical analysis of all of the data was performed using SPSS (SPSS Inc., Chicago, IL, USA).

Results

The characteristics of the ASWs and control subjects are shown in Table 1. Baseline spirometric values of the ASWs and control subjects were similar. SO_2 exposure for a 1-h period caused significant decreases

Table 1. Characteristics of the study populations.

	Workers n = 48	Controls n = 44
Sex, m/f	48/0	44/0
Smoker/nonsmoker	25/23	23/21
Age (years)	31 ± 11	30 ± 10
FEV_1 (L)	4.08 ± 0.12	4.15 ± 0.14
FEV_1 (% predicted)	96.8 ± 2.11	97 ± 2.14
FVC (L)	4.33 ± 2.19	4.40 ± 1.19
FVC (% predicted)	90.48 ± 1.91	92 ± 2.01
$FEF_{25\%-75\%}$ (L/s)	5.22 ± 0.2	5.32 ± 0.24
$FEF_{25\%-75\%}$ (% predicted)	112 ± 4.03	111 ± 4.12

in pulmonary functions (Table 2). The mean rates of decrement in the FVC, FEV_1, FEV_1/FVC %, and $FEF_{25\%-75\%}$ values were 2% ($P < 0.05$), 4% ($P > 0.001$), 0.32% ($P > 0.05$), and 7.96% ($P < 0.001$), respectively. The declines in the FEV_1 and $FEF_{25\%-75\%}$ ($P < 0.001$) were higher than that of the FVC, revealing an obstructive pattern.

There was no detectable amylase concentration in the EBC in the study population, which showed no salivary contamination. Nitrite concentrations were detectable by the spectrophotometric method in the EBC of the ASWs and the healthy subjects. The results of measurements of nitrite in the EBC in the 2 groups are shown in Figure 1. Baseline concentrations of nitrite in the ASWs were higher than in the control subjects (19.31 ± 1.06 vs. 13.08 ± 1.36 nmol/mL; $P < 0.001$). SO_2 exposure produced a significant increase in concentrations of nitrite (23.62 ± 1.36 nmol/mL) compared to the baseline values in the ASWs. As shown in Figure 2, the nitrite concentration was higher in healthy smoking subjects than in the nonsmoking subjects; however, there was no significant difference between the smoking and nonsmoking ASWs. The decrements in pulmonary function tests and the increments in nitrite concentration did not correlate with each other.

Discussion

We have demonstrated that exhaled nitrite is increased in workers with an asthma-like syndrome induced by SO_2 exposure in this agricultural setting. The baseline level of nitrite before SO_2 exposure was higher in the ASWs than in the control subjects, and SO_2 exposure for 1 h caused a further elevation of the concentrations of nitrite. The increased concentration of nitrite before SO_2 exposure may be due to exposure of the workers to SO_2 for several days before the study day, and therefore reflects the subacute response to exposure. The observed further increments in the nitrite level after 1 h of SO_2 exposure appears to be an acute response to exposure.

Table 2. The results of pulmonary function tests (PFTs) measured before and after SO_2 exposure, the differences between these values, and the statistical significance of the measured differences.

PFT parameter	Prior to the exposure	After the exposure	Difference	P
FVC (L)	4.33 ± 1.34	4.29 ± 1.38	0.09 ± 0.06	NS
FVC (%)	89.41 ± 2.05	86.76 ± 1.97	2.23 ± 1.26	
FEV_1[c] (L)	4.04 ± 0.11	3.91 ± 0.12	0.12 ± 0.03	<0.001
FEV_1 (%)	97.91 ± 2.11	93.80 ± 2.12	4.11 ± 1.09	
FEV_1/FVC (%)	93.02 ± 1.01	92.48 ± 1.07	0.32 ± 0.88	NS
$FEF_{25\%-75\%}$[e] (L/s)	5.18 ± 0.19	4.94 ± 0.17	0.28 ± 0.07	<0.001
$FEF_{25\%-75\%}$ (%)	109.01 ± 3.88	101.04 ± 3.64	7.96 ± 2.13	

The results are expressed as mean ± SEM; FVC: forced vital capacity; FEV_1: forced expiratory volume in the first second; and $FEF_{25\%-75\%}$: forced midexpiratory flow rate.

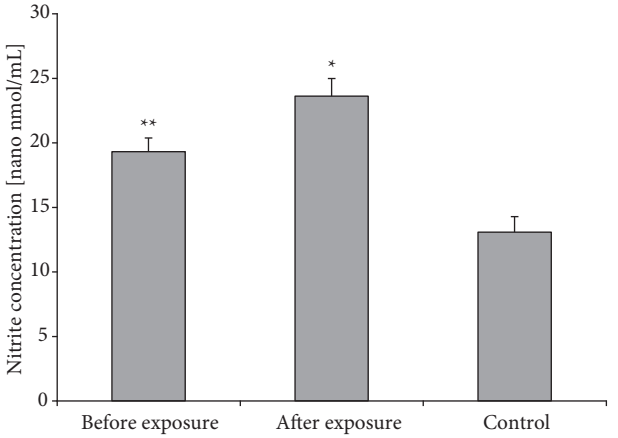

Figure 1. Nitrite concentration in the EBC of workers with asthma-like syndrome and in normal subjects. Each value represents mean ± SEM. Baseline exhaled nitrite level before SO_2 exposure was significantly greater in the workers than in the control subjects and was further increased after the exposure. *Significantly higher than in other groups ($P < 0.001$) and **significantly higher than in the control group ($P < 0.0001$).

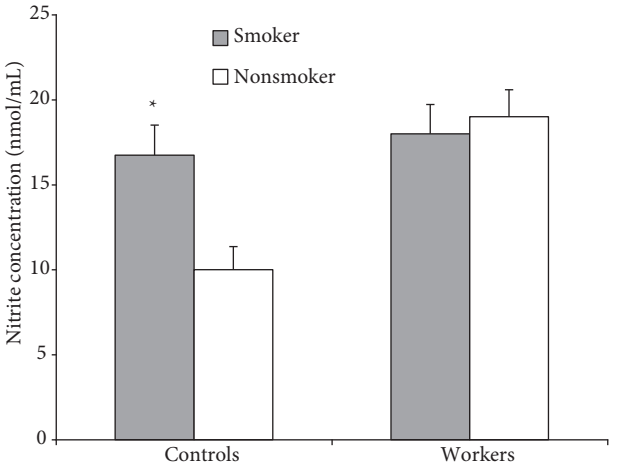

Figure 2. Nitrite concentration in the EBC of smoking and nonsmoking workers with asthma-like syndrome and of normal subjects. Each value represents mean ± SEM. There was no significant difference among the workers between smokers and nonsmokers. Nitrite concentration in exhaled breath was significantly higher in the smoking control subjects (*$P < 0.005$ when compared to the nonsmoking controls).

NO is an unstable molecule that is produced by various cells, including epithelial and vascular cells, macrophages, eosinophils, and neutrophils within the respiratory tract. It reacts rapidly to yield other reactive species such as peroxynitrite ($ONOO^-$), and also the more stable end product, nitrite. It is possible to measure the levels of nitrite in samples of EBC, and increased levels may reflect increased production of NO within the airways or lung parenchyma (24). Norris et al. demonstrated that a model of airway reactivity in dogs induced by SO_2 was associated with significant inflammatory changes within the lung after 1, 2, and 24 h of SO_2 exposure (20). They found that the number of neutrophils recovered increased at each of these times, and by 24 h, they accounted for approximately 80% of the total cells (20). Inflammatory cells such as neutrophils or macrophages produce several reactive oxygen and nitrogen-derived species, such as NO and superoxide radicals. Because the present study was performed on apricot farms, we were not able to obtain BAL from the ASWs. However, together with the data of Norris et al., it can be suggested that the increased nitrite concentration may be due to the excessive production of NO by increased numbers of inflammatory cells within the lungs induced by SO_2 exposure.

Our previous studies showed that nitrite and nitrate production were increased in the serum of ASWs, suggesting the role of nitrative stress in the pathogenesis of the disease (15). However, this evidence implicating airway inflammation in response to occupational SO_2 exposure comes from measurements in the venous blood of markers of oxidative stress, which may be produced by systemic inflammation. Since the analysis of EBC is likely to reflect local production of free radicals rather than systemic production, the present study suggests that increased nitrative stress plays an important role in the pathogenesis of the asthma-like syndrome induced by SO_2 exposure. In addition to studies showing high levels of nitrite in the EBC (25) and sputum of asthmatic patients (26), Von Essen et al. (27) recently showed that a small elevation in exhaled NO was seen in swine confinement workers with an asthma-like syndrome and that macrophages were significantly elevated in the induced sputum samples of these workers, indicating that they had lower respiratory tract inflammation.

There was no significant difference in the levels of nitrite between the smoking and nonsmoking ASWs. One possible reason for the lack of any difference may be that since ASWs had already produced excess nitrite due to high SO_2 exposure, cigarette smoke itself did not cause a further increment in nitrite levels. In the control subjects, the concentration of nitrite was elevated significantly in current smokers compared with nonsmokers. It has been shown that acute exposure to cigarette smoke causes a reduction in exhaled NO, possibly by the down-regulation of constitutive NO synthase or by the formation of the stable end products of NO metabolism, nitrite and nitrate. However, its effect on NO metabolites such as nitrite, nitrate, and nitrotyrosine in EBC is variable (28).

SO_2 exposure resulted in small but significant decrements in the FEV_1 of the ASWs, indicating the occurrence of an occupational SO_2-induced asthma-like syndrome, in agreement with our previous study (8). There was no correlation between the increments in the concentrations of nitrite in the EBC of the ASWs and the decrements in pulmonary function tests. Although a lack of measurements of SO_2 concentration may seem to be a limitation of this study, we performed this study at the same apricot farms where, in previous studies (8,14,15), the concentration of SO_2 during apricot sulfurization ranged between 106.6 and 721.0 ppm. There was no correlation between such SO_2 concentrations and changes in pulmonary function, in agreement with the data of Norris et al., and serum nitrite levels in previous studies (8,15). Therefore, we did not measure the concentration of SO_2 in the present study.

We found that SO_2 exposure in healthy workers caused an increase in nitrite concentrations in the EBC of ASWs with an asthma-like syndrome in an agricultural environment, suggesting the role of nitrative stress in the pathogenesis of this SO_2-induced bronchoconstriction. This is one of the first applications of the EBC method, which may be suitable for other field studies in occupational medicine.

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