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Letter

Health survey of workers in a 2,4,6-trinitrotoluene explosives factory in Fuxin, China

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ABSTRACT — 2,4,6-Trinitrotoluene (TNT) is a serious occupational and environmental pollutant. We conducted a cross-sectional health survey of workers in a TNT explosives factory in Fuxin, China. For each subject, we determined their blood pressure, hematotoxicity parameters, glutathione concentration, lipid hydroperoxide concentration, superoxide dismutase activity, and nitrite/nitrate (NOx) concentration in serum. Significantly fewer white blood cells were found in samples from male workers exposed to TNT than in samples from control male workers, but hematological parameters (such as the amount of hemoglobin present, the hematocrit value, and the formation of methemoglobin) varied little between the exposed and control workers. Exposure of male workers to TNT was found to cause their blood pressure to decrease significantly, concomitant with a tendency towards increased NOx concentrations in serum. On the other hand, lipid hydroperoxide (an oxidative stress marker) concentrations were significantly higher in female workers exposed to TNT than in control female workers. Our results suggest that TNT has different, deleterious effects in males and females, causing hematotoxic stress in males and oxidative stress in females.

Key words: 2,4,6-Trinitrotoluene, Clinical symptoms, Hematotoxicity, Oxidative stress

INTRODUCTION

2,4,6-Trinitrotoluene (TNT) is a common explosive that is used for both military and industrial purposes. Exposure (both acute and chronic) of factory workers to TNT has been shown to lead to toxic effects in the liver, hematopoietic system, and eyes (Harkonen *et al.*, 1983; Hathaway, 1977; Sabbioni *et al.*, 2005). The hematotoxic effects of TNT have been found to be manifested as hemolysis, the destruction of hemoglobin (Hb) in erythrocytes, and the formation of methemoglobin (metHb) (Crawford, 1954; Djerassi and Vitany, 1975; Levine *et al.*, 1984). The authors have previously found that zeta-crystallin catalyzes the reductive activation of TNT, which generates reactive oxygen species (Kumagai *et al.*, 2000), and that 4-hydroxylamino-2,6-dinitrotoluene (HADNT), which is an active metabolite of TNT, mediates TNT-induced hematotoxicity through the formation of pro-oxidants such as hydrogen peroxide (Shinkai *et al.*, 2015a). We also found that TNT inhibits endothelial nitric oxide synthase and increases blood pressure in rats (Sun *et al.*, 2005). These findings suggest that TNT is associated with oxidative stress and hypertension. However, little information is available on TNT having such deleterious effects on humans. In this study, we assessed the health risks posed by TNT by determining the blood pressures of subjects who had and had not been exposed to TNT, and by determining their glutathione (GSH) concentrations, lipid hydroperoxide (LPO) concentrations, superoxide dismutase (SOD) activities, and nitrite/nitrate (NOx) concentrations in serum samples. We also attempted to identify interactions between TNT and hematotoxic parameters, including white blood cell count (WBC), Hb and metHb concentrations, and hematocrit value.

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MATERIALS AND METHODS

Subjects

A cross-sectional survey was carried out at a TNT explosives factory in Fuxin, China, in summer 1998. The study was approved by the University of Tsukuba medical ethics committee. A total of 124 subjects were selected. The average age of the males exposed to TNT was 32 y; the male control group, the females exposed to TNT, and the female control group each had an average age of 35 y. The male and female workers had been exposed to TNT for an average of 10.6 y and 12.0 y, respectively. The TNT-exposed and control workers did not have significantly different smoking habits or alcohol intakes.

Determination of TNT

Ambient air in the factory was sampled using a high-volume air sampler, and the TNT concentrations in the samples were determined by high-performance liquid chromatography using a method described previously (Shinkai *et al.*, 2015a).

Interviews

The TNT-exposed and control workers were asked about the extent to which they had been exposed to TNT and their health status. The interviews included questions on the subjects' clinical symptoms, whether the subjects were currently or had previously suffered from any diseases, and the subjects' smoking habits and alcohol intakes.

Blood samples

Blood samples were collected from the subjects to measure hematological and biological parameters: Hb, hematocrit, GSH, metHb, LPO, SOD, and WBC. Total WBC was measured using an automated cell counter. Hb and metHb concentrations were determined using a previously published method (Evelyn and Malloy, 1938). The hematocrit value was measured using the microcapillary tube centrifugation technique. The resting blood pressure of each subject was determined using a sphygmomanometer.

Determination of LPO in serum

The LPO concentration in each serum sample was determined by measuring the concentration of thiobarbituric-reactive substances, using a previously described method (Pi *et al.*, 2002). Briefly, serum (300 μL) was mixed with 20% trichloroacetic acid (2,700 μL) and 0.67% thiobarbituric acid (1,000 μL), then the mixture was incubated at 100°C for 30 min. The mixture was then cooled with tap water, and the resulting chromogens were extracted by vigorously shaking the mixture with n-butyl alcohol (4,000 μL). The mixture was then centrifuged at 3,000 rpm for 10 min, and the absorbance of the supernatant at 535 nm was measured using a spectrophotometer (UV-1600; Shimadzu, Kyoto, Japan). 1,1,3,3-Tetraethoxypropane was used as an external standard, and the LPO concentration in each sample was expressed as the equivalent of malondialdehyde, in nanomoles.

Determination of GSH in whole blood

The GSH concentration in each whole blood sample was determined by treating the sample with 5,5′-dithiobis-2-nitrobenzoic acid and then measuring the absorbance at 412 nm, following a previously published method (Ellman, 1959). Briefly, whole blood (100 μL) was diluted with cold, deionized distilled water (0.4 mL), then 10% trichloroacetic acid (0.5 mL) was added to cause the proteins to precipitate. The sample was then mixed well and centrifuged at 15,000 rpm for 5 min, then the absorbance of the supernatant at 412 nm was determined.

Determination of SOD activity in whole blood

Whole blood (50 μL) was washed with saline (5 mL), then centrifuged at 2,000 rpm for 3 min. The rinsed cells were diluted with cold deionized distilled water to give a final volume of 0.2 mL. Ethanol (0.1 mL) and chloroform (0.1 mL) were mixed with the erythrocyte lysate to precipitate the hemoglobin. The tube was shaken vigorously for 15 min and then centrifuged at 4,000 rpm for 3 min. The water and ethanol layer was analyzed to determine the SOD activity using a previously published method (Elstner and Heupel, 1976).

Measurement of NO metabolites

The NOx concentration in each serum sample was determined using a previously published method (Green *et al.*, 1982) with slight modifications. Briefly, serum was deproteinized, and the supernatant (1.5 mL) was mixed with an equal volume of a glycine-NaOH buffer (pH 9.7) and cadmium filings (2.5 g) that had been treated with 5 mM $CuSO_4$ for 5 min. The mixture was heated to 65°C for 30 min to convert nitrate to nitrite, then an aliquot (2 mL) was reacted with the Griess reagent (1 mL), which was 1% sulfanilamide in 3 M HCl and 0.2% *N*-(1-naphthyl)-ethylenediamine HCl. The absorbance of the extract at 540 nm was then measured, and $NaNO_2$ was used as a standard.

Statistical analysis

The data are all expressed as the mean ± the standard deviation. Statistical significance of the differences between the results for the TNT-exposed and control workers was determined using the two-tailed paired Student's t-test, and $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The mean TNT concentration in the factory air was 1.42 mg/m^3, which was higher than the maximum allowable TNT concentration (1 mg/m^3) in China. The prevalence of clinical symptoms reported by the TNT-exposed and control workers in the interviews are shown in Table 1. The prevalence of most of the symptoms was the same in the TNT-exposed and control workers, but poor memory and abdominal pain were reported more often by the TNT-exposed workers than by the control workers. Changes in the hematological parameters caused by exposure to TNT are shown in Table 2. WBC was significantly lower in the males exposed to TNT than in the male control workers, whereas WBC was the same for the female TNT-exposed and control workers. This suggests that TNT has different hematotoxic effects on males and females. No significant differences were found in the other parameters (Hb concentration, Ht value, and formation of metHb) between the TNT-exposed and control workers.

Changes in the biochemical parameters caused by exposure to TNT are shown in Table 3. As expected, LPO (an oxidative stress marker) concentration was significantly higher in the female TNT-exposed workers than in the female control workers. This suggests that females are particularly susceptible to oxidative stress caused by TNT. No differences were found between the LPO concentrations in the male TNT-exposed and control workers. SOD (an antioxidant enzyme) activity was significantly higher in the male TNT-exposed workers than in the male control workers, whereas the SOD activity was lower in the female TNT-exposed workers than in the female control workers. There are adaptive systems that respond to oxidative stress by inducing the production of antioxidant enzymes in humans (Landriscina *et al.*, 2009). The exact mechanism responsible for the different oxidative stress

Table 1. Prevalence of clinical symptoms in workers exposed to TNT and control workers.

Symptom	Unexposed (n = 29)		TNT-exposed (n = 95)	
	n	%	n	%
Headache	4	(13.8)	14	(14.7)
Vertigo	4	(13.8)	14	(14.7)
Insomnia	4	(13.8)	8	(8.5)
Poor memory	0	(0)	7	(7.4)
Loss of appetite	1	(3.5)	4	(4.2)
Nausea	1	(3.5)	5	(5.2)
Vomiting	5	(17.2)	17	(17.9)
Abdominal pain	0	(0)	4	(4.2)

Table 2. Change in hematological parameters caused by TNT exposure.

Group	WBC (10^3/mm^3)	Hb (g/L)	Ht (%)	MetHb (n, > 1%)
Male				
Unexposed (n = 17)	7.3 ± 1.7	139.7 ± 9.5	48.5 ± 3.6	1
TNT-exposed (n = 59)	6.5 ± 1.3*	138.6 ± 10.5	48.7 ± 3.9	1
Female				
Unexposed (n = 12)	6.7 ± 1.1	125.0 ± 10.5	42.7 ± 3.4	0
TNT-exposed (n = 36)	6.7 ± 1.5	126.4 ± 8.8	44.2 ± 2.5	2

Each value is the mean ± the standard deviation, * indicates $P < 0.05$ (versus the unexposed group), WBC = white blood cell count, Hb = hemoglobin concentration, Ht = hematocrit value, MetHb = number of subjects with methemoglobin concentrations greater than 1%

Table 3. Change in biochemical parameters caused by TNT exposure.

Group	GSH (nmol/mg)	LPO (nmol/mL)	SOD (NU/mg Hb)
Male			
Unexposed (n=15)	7.5 ± 3.5	6.8 ± 0.5	16.2 ± 1.0
TNT-exposed (n=37)	8.4 ± 4.9	6.9 ± 1.3	17.2 ± 1.7*
Female			
Unexposed (n=11)	9.4 ± 4.4	6.1 ± 0.7	18.5 ± 1.7
TNT-exposed (n=22)	9.2 ± 7.1	6.7 ± 0.7*	17.7 ± 1.4

Each value is the mean ± the standard deviation, * indicates $P < 0.05$ (versus the unexposed group), GSH = glutathione concentration, LPO = lipid hydroperoxide concentration, SOD = superoxide dismutase activity.

Table 4. Change in the blood pressure and nitrite/nitrate (NOx) concentration in male subjects caused by TNT exposure.

	Unexposed	TNT-exposed
Systolic blood pressure (mm Hg)	101.7 ± 3.9 (n = 19)	95.2 ± 1.3* (n = 52)
NOx concentration in serum (μmol/L)	56.2 ± 8.0 (n = 16)	62.9 ± 5.8 (n = 50)

Each value is the mean ± the standard deviation, * indicates $P < 0.05$ (versus the unexposed group)

responses in males and females is not known. However, our results suggest that the higher LPO concentrations in the female TNT-exposed workers than in the male TNT-exposed workers may have been caused by the females having lower SOD activities than the male workers. No differences were found between the GSH (the most abundant antioxidant in the body) concentrations in the TNT-exposed and control workers.

As is shown in Table 4, the exposure of male workers to TNT significantly decreased their blood pressures, concomitant with a tendency towseards increased NOx concentrations in their sera. This contrasts with previous findings that TNT inhibits endothelial nitric oxide synthase and increases the blood pressure in rats (Sun *et al.*, 2005). An explanation for this discrepancy is that TNT may increase the production of NOx found in the serum through an alternative pathway. We suggest this because we recently found that NADPH-cytochrome P450 reductase catalyzes the reduction of TNT to form HADNT, resulting in denitration reaction through the interaction between TNT and HADNT to release nitrite (Shinkai *et al*., 2015b). It has previously been reported that nitrite can be reduced to NO through several pathways (Lundberg and Weitzberg, 2005; Shiva, 2013). Therefore, exposure to TNT could decrease blood pressure by increasing the production of NO.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Crawford, M.A. (1954): Aplastic anaemia due to trinitrotoluene intoxication. Br. Med. J., **2**, 430-437.

Djerassi, L.S. and Vitany, L. (1975): Haemolytic episode in G6 PD deficient workers exposed to TNT. Br. J. Ind. Med., **32**, 54-58.

Ellman, G.L. (1959): Tissue sulfhydryl groups. Arch. Biochem. Biophys., **82**, 70-77.

Elstner, E.F. and Heupel, A. (1976): Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. Anal. Biochem., **70**, 616-620.

Evelyn, K.A. and Malloy, H.T. (1938): Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. Biol. Chem., 655-662.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982): Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem., **126**, 131-138.

Harkonen, H., Karki, M., Lahti, A. and Savolainen, H. (1983): Early equatorial cataracts in workers exposed to trinitrotoluene. Am. J. Ophthalmol., **95**, 807-810.

Hathaway, J.A. (1977): Trinitrotoluene: a review of reported dose-

related effects providing documentation for a workplace standard. J. Occup. Med., **19**, 341-345.

Kumagai, Y., Wakayama, T., Lib, S., Shinohara, A., Iwamatsu, A., Sun, G. and Shimojo, N. (2000): Zeta-crystallin catalyzes the reductive activation of 2,4,6-trinitrotoluene to generate reactive oxygen species: a proposed mechanism for the induction of cataracts. FEBS Lett., **478**, 295-298.

Landriscina, M., Maddalena, F., Laudiero, G. and Esposito, F. (2009): Adaptation to oxidative stress, chemoresistance, and cell survival. Antioxid Redox Signal, **11**, 2701-2716.

Levine, B.S., Furedi, E.M., Gordon, D.E., Lish, P.M. and Barkley, J.J. (1984): Subchronic toxicity of trinitrotoluene in Fischer 344 rats. Toxicology, **32**, 253-265.

Lundberg, J.O. and Weitzberg, E. (2005): NO generation from nitrite and its role in vascular control. Arterioscler. Thromb. Vasc. Biol., **25**, 915-922.

Pi, J., Yamauchi, H., Kumagai, Y., Sun, G., Yoshida, T., Aikawa, H., Hopenhayn-Rich, C. and Shimojo, N. (2002): Evidence for induction of oxidative stress caused by chronic exposure of Chinese residents to arsenic contained in drinking water. Environ. Health Perspect., **110**, 331-336.

Sabbioni, G., Liu, Y.Y., Yan, H. and Sepai, O. (2005): Hemoglobin adducts, urinary metabolites and health effects in 2,4,6-trinitrotoluene exposed workers. Carcinogenesis, **26**, 1272-1279.

Shinkai, Y., Li, S., Kikuchi, T. and Kumagai, Y. (2015a): Participation of metabolic activation of 2,4,6-trinitrotoluene to 4-hydroxylamino-2,6-dinitrotoluene in hematotoxicity. J. Toxicol. Sci., **40**, 597-604.

Shinkai, Y., Nishihara, Y., Amamiya, M., Wakayama, T., Song, L., Kikuchi, T., Nakai, Y., Shimojo, N. and Kumagai, Y. (2015b): NADPH-cytochrome P450 reductase-mediated denitration reaction of 2,4,6-trinitrotoluene to yield nitrite in mammals. Free Radic. Biol. Med., in press.

Shiva, S. (2013): Nitrite: A Physiological Store of Nitric Oxide and Modulator of Mitochondrial Function. Redox Biol., **1**, 40-44.

Sun, Y., Iemitsu, M., Shimojo, N., Miyauchi, T., Amamiya, M., Sumi, D., Hayashi, T., Sun, G., Shimojo, N. and Kumagai, Y. (2005): 2,4,6-Trinitrotoluene inhibits endothelial nitric oxide synthase activity and elevates blood pressure in rats. Arch. Toxicol., **79**, 705-710.