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RESEARCH ARTICLE

Oxidative stress and early DNA damage in workers exposed to iron-rich metal fumes

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Abstract Occupational exposure to metal fumes occurs routinely in many occupational settings. The inflammatory response to fumes and metals after exposure could lead to an increase in reactive oxygen species and level of DNA damage. In this study, the level of early DNA damage and oxidative stress was evaluated in a group of steel company ($n = 30$) and compared to the non-exposed ($n = 28$) subjects. All DNA damage markers in workers were significantly higher in exposed group in comparison with controls ($p < 0.001$). Stratified analysis based on smoking showed no significant differences between smoking and comet assay parameters. There was no significant difference between workers and controls in terms of HCT, TIBC, iron, and ferreting. However, HB in controls was significantly lower than exposed group ($p < 0.001$). A significant increase in catalase activity and MDA serum levels were observed in workers in comparison with controls. These findings suggest for the potential genotoxic effect of iron reach dust. Due to recent findings on the predictive potential of comet assay for cancer development, further, researches should be conducted to investigate the possible biochemical mechanism of such finding.

Introduction

Fumes are a complex mixture of gaseous and fine particulate pollutants which are produced based on thermal process in different activities such as welding and metal smelting. Occupational exposure to metal fumes occurs routinely in many industrial settings (Cheng et al. 2008). The small diameter of fumes leads to their transportation to the deep alveoli region of the lung and thereby more internal dose of the toxic compounds. Iron is an essential trace element for the human body and also the most dominant fraction of metals in the metal fumes. Despite the very important biochemical role of iron, its overload in the human body can induce oxidative stress. Most of the available literature reported the role of low iron diet and iron deficiency in human health. However, recent findings show an adverse effect of iron overload on inflammation and probably cancer.

Epidemiological evidences on workers exposed to metal fumes have consistently concluded an increased relative risk of cancer (Humfrey et al. 1996). However, it is not clear that observed adverse effect belongs to which metal fraction of the welding fume. Iron is the most prominent fraction of welding fume followed by chromium, magnesium, and nickel (depending on the type of base metal and consumable electrode). Genotoxic effects of metal fumes in experimental animals are not conclusive. Metals' ability to interfere with DNA repair processes, DNA methylation, and metabolic processes during

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DNA replication and expressions involved oxidative reactions. Recent in vivo and in vitro studies found an increased level of DNA damage in exposure to metal fume. In an in vitro study, exposure to metal fume PM10 in electric steel plants, on A549 cells leads to increase in DNA damage (Cavallo et al. 2008). The inflammatory response to metal fumes could lead to an increase in reactive oxygen species. Thus, metals elevated the ROS levels and so can induce either oxidative damage to DNA or other biomolecules (Pesch et al. 2015). However, in a study on a group of welders exposed to high levels of metal fumes, slightly differences were observed in inflammation markers in comparison to controls (Palmer et al. 2006). Some host factors such as folate deficiency can exacerbate the effects of metals on genome instability and corresponding genotoxicity (Alimba et al. 2016). The International Agency for Research on Cancer (IARC) has focused on the carcinogenicity of welding fume. The comet assay (single cell gel electrophoresis) is a simple and easy to use DNA damage test which was developed by Singh et al. Applicability of the comet assay in occupational biomonitoring has recently been reviewed by Valverde and Rojas (2009).

The aim of this study was to evaluate the level of oxidative stress and primary DNA damage in copper smelter how were exposed to metal fumes in their work environments. The results compared with non-exposed group. The effect of exposure modifying factors such as smoking on observed oxidative stress level and DNA damage was studied.

Materials and methods

Subjects

Exposed ($n = 30$) and control ($n = 28$) subjects were all from Yazd city. All workers had a working history of at least 1 year in the furnace unit of a steel company. Controls were selected from administrative workers form the same factory with no history of exposure to known carcinogens in the same time span. To overcome possible effect of dietary pattern or the therapeutic effect of antioxidant supplements, two questions were asked in a questionnaire about "being under specific dietary plan" or "use of dietary supplementation." Those with positive answer to each of these questions were excluded from the study. Subjects with therapeutic or diagnostic radiation exposure were also excluded from the study. The study was approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences (Ethics committee approval number: IR.ssu.medicine.rec.1394.250). Informed verbal and written consent were obtained from all participants. All workers were selected from melting unit, apparently the most polluted unit in the factory. Previous results of inhalation exposure assessment in this unit showed inhalation exposure intensity to metal dust and fumes equal to 4.3 mg/m^3.

Comet assay

Peripheral blood sampling for exposed and control subjects was performed via venipuncture into a heparinized tube at morning. Blood sampling of exposed subjects were performed during routine annual occupational medicine screening program. All blood samples were transported to the laboratory in a cold box within 2 h. Whole blood was diluted 1:1 with phosphate buffer saline (PBS); then Ficoll density gradient solution (Baharafshan, Iran) was added and centrifuged for 20 min at 1425 rpm. Lymphocytes were isolated at the end of another 15 min spin down at 1425 rpm and diluted in 900 μL of PBS.

The comet assay was performed according to the protocol developed by Singh et al. (1988) with slight modifications (Dhawan 2013). Briefly, a frosted microscopic slide was cleaned with ethanol and then dipped into the 1% normal melting point agarose (NMA). Twenty μL of blood cell suspensions was mixed with 80 μL of 0.7% low melt agarose (LMA), vortex mixed and 30 μL of mixed agarose/cell suspension was placed on the microscopic slide. The slides finally were covered with the cover slip, left for 10 min on an ice-cooled metal surface and then 100 μL of LMA was dropped on prior layer of LMA. After 5 min, slides were dipped in an alkaline lysis solution for an hour, then removed and washed gently with deionized water. Slides were placed horizontally in an electrophoresis tank filled with electrolysis buffer (previously placed in 4 °C) and kept for 30 min at 4 °C in the refrigerator. Electrophoresis parameters were set for constant conditions of 300 mA and 0.8 V/CM based on the electrophoresis tank dimensions (20 V). After completion of 30 min electrophoresis, slides cured for 5 min in neutralization buffer and then washed with deionized water. Slides were stained with Ethidium bromide solution for 5 min. All slides prepared in triplicate. Microscopic analysis was performed under 400 magnifications with a fluorescent microscope (NikonE200, Nikon, Japan). At least 50 cells were counted for each comet slide. Open comet software was used for image analysis (Gyori et al. 2014).

Plasma malondialdehyde (MDA)

Plasma MDA levels were quantified by the procedure based on thiobarbituric acid (TBA) reactivity (Draper and Hadley 1990; Hammouda et al. 1995; Jentzsch et al. 1996). At first, 200 μL of plasma was added to 25 μL butylated hydroxytoluene (BHT) in ethanol (0.2%) and 200 μL orthophosphoric acid (0.2 mol/L) then vortexed for 10s.Then, 25 μL of TBA (0.11 mol/L, in 0.1 mol/L NaOH) was added and vortexed. After 45 min incubation at 90 °C in

Table 1 Summary of demographic and background exposures of participants

Parameter		Workers ($n = 30$)	Controls ($n = 28$)	p value
Age (years)		32.93 ± 6.23	29.64 ± 3.86	0.02
Work history (years)		7.73 ± 4.37	–	–
Smoking (N/day)		9.63 ± 13.13	5.96 ± 6.88	0.18
Smoking	No	13 (43.3%)	12 (42.8%)	0.97
	Yes	17 (56.7%)	16 (57.2%)	
Agriculture	No	26 (13.3%)	28 (100%)	0.047
	Yes	4 (86.7%)	0 (0%)	

the water bath and cooling, TBA-reactive substances (TBARS) were extracted with 500 μL n-butanol and 50 μL saturated NaCl solution. The mixture was centrifuged at 12000 rpm for 1 min and the absorbance of the upper butanol phase was measured at 535 and 572 nm by a microplate reader (BioTek Epoch; USA). The concentration of the TBARS was calculated by using the difference between two optical density (Jentzsch et al. 1996).

Total antioxidant capacity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) reduction assay was performed by adding 20 μL of plasma plus 380 μL phosphate buffered solution (pH: 7.4) to 400 μl of 0.1 mM methanol solution of DPPH and incubation at ambient temperature (21 °C) for 30 min. The absorbance of the samples was read at 517 nm (Janaszewska and Bartosz 2002).

Serum praoxonase-1 (PON1) activity

Serum paraoxonase activity was determined spectrophotometrically using paraoxon (O,O-diethyl-o-p-nitro-phenyl phosphate; Sigma Chemical Co) as the substrate and measurement of the rate of formation of 4-nitrophenol in the absorbance at 412 nm (Eckerson et al. 1983). PON1 activity calculated (PON activity = AΔOD/K) and expressed in U/l serum. One unit of PON1 activity was defined as 1 nmol of 4-nitrophenol formation per minute (Eckerson et al. 1983).

Serum catalase activity

Serum catalase activity was determined spectrophotometrically according to Goth (1991). This method uses three Blanks and the serum catalase calculated according to the

formula: Ku/L = A (sample) − A (blank 1) /A (blank 2) − A (blank 3) × 271. The catalase activity was expressed in Ku/L serum.

Hemoglobin (Hb), hematocrit (HCT)

Hemoglobin concentrations (Hb), hematocrit (HCT) were determined by Sysmex KX-21N Hematology Analyzer.

Serum iron, total iron binding capacity (TIBC) and ferritin

Serum iron, total iron binding capacity (TIBC) and Ferritin were estimated on autoanalyzer (BT-3000) using colorimetric assay (Pars Azmon, Iran).

Statistics

To overcome between observer and between laboratory variability, all biological analysis on blood samples was performed in the same laboratory. Image analysis was performed by a blind analyzer. Comet assay values were tested for normal distribution with the Shapiro–Wilk's test. In the case of violation of normal distribution assumption, significant differences between exposed and reference subjects were tested with the non-parametric Mann–Whitney U test. Two-sided P values, at 0.05 were regarded as statistically significant. Correlation analysis was performed to examine the possible correlation between protective behavior and demographic characteristics of subjects with observed DNA damage. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., IL, and USA).

Table 2 Levels of early DNA damage detected by alkaline comet assay in participants

Group	Tail length	Tail DNA%	Tail moment	Tail intensity
Workers ($n = 30$)	15.88 (8.94–20.44)	8.98 (5.81–11.37)	3.42 (1.60–6.01)	24.59 (22.74–29.53)
Control ($n = 26$)	6.17 (5.57–8.07)	3.97 (3.07–4.84)	0.68 (0.53–0.93)	20.19 (17.50–22.26)
p value	<0.001	<0.001	<0.001	<0.001

Table 3 Iron status in participants

Group	HB	HCT	TIBC	Iron	Ferritin
Workers	14.40 (13.50–15.10)	42.70 (40.90–43.40)	295.50 (271.00–318.00)	86.50 (70.00–99.00)	97.50 (62.00–136.00)
Controls	13.20 (12.80–13.80)	42.90 (41.25–43.95)	301.00 (283.50–315.50)	80.50 (65.00–99.50)	65.50 (44.00–135.50)
p value	<0.001	0.76	0.39	0.52	0.16

Results

Table 1 shows the distribution of age, smoking, and contact with agricultural activities in workers and controls. Age and contact with agricultural activities in workers group were significantly higher than control group ($p = 0.02$ and $p = 0.047$, respectively).

Table 2 shows a median and interquartile range of comet assay parameters (tail length, tail DNA%, tail moment, tail intensity) in workers and controls group. All DNA damage markers in workers were significantly higher than controls ($p < 0.001$). Stratified analysis based on smoking status in workers and controls groups separately or in overall mode showed no significant differences between smoking and comet assay parameters in the significance level of 0.05 ($p > 0.05$). To identify possible effect of exposure to agriculture activities on observed DNA damage in workers group, analysis were conducted with the exclusion of four cases with exposure to agriculture activities. There was no significant difference in the results after exclusion of these cases.

Table 3 shows the levels of iron status in workers and control group. There was no significant difference between workers and controls in terms of HCT, TIBC, iron, and ferritin. However, HB in controls was significantly lower than the workers group ($p < 0.001$). To exclude the possible role of smoking on the observed significance of HB in workers and controls group, the univariate general linear model with smoking adjustment was used. HB was higher in workers even after adjustment for smoking status ($p = 0.001$).

Results of oxidative stress markers in workers and controls showed the only significant increase in catalase activity and MDA serum levels in workers. However, PON-1 activity was higher in controls than workers, but it was not significant at the level of 0.05 (Tables 4 and 5).

Discussion

In this study, we examined the level of oxidative stress and primary DNA damage in the group of steel production workers which were in exposure to iron oxide dust. The results were compared with non-exposed control group. Steelworkers showed the higher level of DNA damage and oxidative stress in comparison with control group. Several other studies also revealed the association between inhalational exposure to air pollutants with elevation in oxidative stress markers and primary DNA damage (Halliwell et al. 2000; Moller et al. 2008; Valko et al. 2004). However, there are few studies pertained to the association between exposures to non-fibrogenic dust like iron oxide and oxidative stress and primary DNA damage especially in occupational environments (Malekirad et al. 2015). Inhalation exposure is the most important route of exposure to hazardous chemicals in the workplaces; therefore, the importance of gestational route is negligible and has no serious effect on an internal dose of chemicals. The increase in body burden of metals such as iron, magnesium, and copper was observed in the case of exposure to polluted air in other studies, which prove these facts about the contribution of inhalation route on the internal dose of chemicals (Gurgueira et al. 2002). This is in accordance with our findings that showed the significant difference in ferritin and iron in the blood of exposed and control groups. We also found an increase in hemoglobin level of workers in comparison with controls. Generally, increase in hemoglobin as a marker of iron deficiency in the human body, but it seems that this finding as for a compensatory mechanism to compensate deficiency in the respiratory system of workers (decrease in lung capacities). Elevated carbon monoxide concentration in the smelting unit is also another explanation for this finding.

Table 4 Levels of oxidative stress markers in participants

Group	Catalase activity	DPPH	MDA	PON-1 activity
Workers	20.32(17.88–22.47)	23(10–42)	3.11(1.78–3.78)	1.28(0.93–3.66)
Controls	16.97(14.24–27.81)	22(8.2–39)	1.17(0.89–2)	2.95(1.15–5.30)
p value	0.07	0.41	<0.001	0.16

Table 5 Correlation matrix between markers of oxidative stress and early DNA damage measured by comet assay (n = 56)

	Tail intensity	Tail length	Tail DNA%	Tail moment	Catalaz	DPPH	MDA	PON
Tail intensity	1.00	0.31*	0.55**	0.51**	0.22	0.12	0.58**	0.02
Tail length		1.00	.89**	0.90**	0.33*	−.03	0.22	−0.18
Tail DNA%			1.00	0.98**	0.34*	.08	0.39**	−0.14
Tail moment				1.00	0.32*	.06	0.38**	−0.20
Catalaz					1.00	−.03	0.31*	−0.01
DPPH						1.00	0.18	0.03
MDA							1.00	−0.08
PON								1.00

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

Chronic exposure to carbon monoxide is associated with an increase in the level of hemoglobin in long term (Malekirad et al. 2015) smoking is another explanation for this elevation; however, results of sensitivity analysis showed that there is no significant difference in association after excluding smokers from analysis. There was also no difference between hematocrit in two groups, which is in accordance with previous findings (Autrup et al. 1999).

Ferritin previously is used as a plasma marker for evaluation of chronic iron overload (Gurgueira et al. 2002). Despite no significant difference in serum iron in both groups, but our finding of an increased level of ferritin in exposed subjects suggests that long-term exposure to the iron-rich dust elevated the body storage of iron. This finding of the role of iron in inhaled dust should be interpreted with caution because of the heterogeneous composition of metal dust in the steel factories that is regularly is the composition of different heavy metals and even polyaromatic hydrocarbons. Maleki et al. in a study showed that the copper level in serum of exposed workers was higher than non-exposed despite no difference in serum iron levels (Malekirad et al. 2015). The most plausible explanation for oxidative stress increase in exposed subjects due to exposure to metal-rich dust is Fenton and Haber Weiss reaction which describe the process of production of hydroxyl radicals in the case of the reaction of copper and Iron with hydrogen peroxide in the human body (Zhong et al. 2016). Our findings of the level of iron and ferritin suggest that the long-term exposure to iron and copper and subsequent increase in ferroradicals cause the oxidative stress and DNA damage.

Significant elevation of MDA as a marker of lipid peroxidation was also found for exposed subjects, which is in accordance with other studies on metal exposures such as iron and copper (Bor-Sheng Ko et al. 2015; Porter et al. 2016; Sumida et al. 2001). An increase in catalase activity in this study seems to be related to increasing the production of hydrogen peroxide due to exposure to metal dust especially iron and copper (Arnal et al. 2010; Sagara et al. 1998). Previous studies showed a positive association between oxidative stress and hydrogen peroxide levels in the body can in catalase gene over-expression and activity (Gürel et al. 2004; Leonard et al. 2010; Valko et al. 2005).

We found a significant association between comet assay TL and %DNA in the tail with catalase activity. Overproduction of hydrogen peroxide leads to an increase in catalase activity. However, in long term, this protective effect cannot completely suppress the oxidative stress and subsequent DNA damage caused by hydrogen peroxide elevation, and finally leads to increase of DNA damage (Valko et al. 2005).

Comet assay is an inexpensive and sensitive method for detection of preliminary DNA damage in single cell level. Exposure to metals is related to an increase in comet assay results (Kobal et al. 2004; Nadif et al. 1998; Pease et al. 2016). In this study, we also found a significant elevation in all comet parameters in exposed subjects in comparison with controls. Several other studies also found a significant positive association between exposure to metals, oxidative stress markers such as MDA and primary DNA damage detected in comet assay (Rohrdanz and Kahl 1998).

This suggests the potential genotoxic effect of iron reach dust. Due to recent findings on a predictive potential of comet assay for cancer development, and the role of oxidative stress in this process (Tate et al. 1995), further researches should be conducted to investigate in more detail the exact effect size and possible biochemical mechanism of such finding. Results of these studies could be used as a milestone for a derivation of exposure limits and standards for work environments.

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