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OXIDATIVE STRESS AND GENOTOXICITY AMONG WORKERS EXPOSED TO COPPER IN A FACTORY FOR NON-FERROUS INDUSTRY IN EGYPT

By

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Abstract

Introduction: Exposure to high levels of copper at the workplace results in many adverse health effects with possible genotoxicity and carcinogenicity. **Aim of work:** To evaluate oxidative stress and detect the extent of DNA damage among workers at a copper processing factory. **Materials and methods:** The studied group is composed of 36 male workers from a copper processing factory and 34 male as a control group non-exposed to copper matched for age and socioeconomic status. Total antioxidant capacity was measured for all participants as an oxidative stress parameter, and urinary 8-OHdG was assayed by ELISA. Extent of DNA damage in leucocytes was also evaluated by comet assay as a biomarker of genotoxicity. **Results:** In the exposed workers, mean serum copper and urinary 8-OHdG were higher when compared to controls (155.1 ± 23 versus 77.9 ± 8.5 ug/dL and 9.7 ± 5 versus 4.1 ± 1.2 ng/mg creatinine, respectively; $p<0.001$ in each). T-AOC measured in exposed workers was significantly lower than that of the comparison group. There was significant DNA damage in leucocytes of exposed workers compared to the control group with mean comet tail length (9.5 ± 3.7 versus 5.7 ±1.4 mm; $p < 0.001$). T-AOC was negatively correlated with comet tail length; r=-0.64 and 8-OHdG showed positive correlation; r=0.71 ($p < 0.001$ for each). Linear regression models revealed that 8-OHdG is the significant predictor of DNA damage assayed by comet test whereas smoking, work duration and age had no significant effect on DNA damage. **Conclusion:** copper-exposed workers are at risk of oxidative stress with consequent DNA damage and potential genotoxic effect.

Keywords: Copper; Oxidative stress; Comet assay; 8-OHdG; Occupational exposure and DNA damage.

Introduction

Copper is important for good health as it is an essential trace element, a constituent of many proteins and of more than 20 enzymes. However, it is a persistent environmental contaminant and exposure to high doses can be harmful. The highest exposure occurs among occupationally exposed groups who spend most of their lives at the workplace (Ivo et al., 2013).

The world's production of copper has steadily increased since the 1900s and is mostly used for electrical/electronic consumption, construction, transport and other industrial applications. Workers in copper mines or in copper processing plants are at high risk for exposure to dangerous levels of copper (Fage et al., 2014).

Acute exposure to copper can irritate the nose, mouth and eyes, and cause headache, dizziness, nausea, vomiting and diarrhea. Chronic exposure to excessive levels results in a number of adverse health effects including liver and kidney damage, immunological and developmental toxicity (ATSDR, 2004).

Excess copper could enhance endogenous oxidative reactions by generating oxygen radicals via a Fenton-type reaction that might cause cellular injury via an oxidative pathway giving rise to enhanced thiol oxidation and DNA damage (Ian, 1998). Total Antioxidant Capacity (TAC) is a biomarker often used in order to investigate oxidative stress in many pathological conditions by measuring the antioxidant potential of body fluids (Niki, 2010).

In-vitro studies (in experimental animals) indicate a clastogenic activity of copper compounds, in the form of chromosome aberrations and micronuclei, however, the carcinogenic potential of copper and its inorganic compounds in animals cannot depend on the available studies (Yada and Trivedi, 2009).

Results of mutagenic studies in humans are conflicting. Although some studies have shown genotoxicity and increased cancer risk in workers exposed to copper, they were also exposed in the workplace to other chemicals with carcinogenic potential. Other studies found an association between high serum copper and various cancers but they are complicated by the fact that serum copper concentrations were measured after diagnosis and

high levels might be due to increased ceruloplasmin (Cetinkaya et al., 1988). No sufficient studies have adequately addressed the question of copper genotoxicity and hence carcinogenicity. Copper is currently classified by the Environmental Protection Agency (EPA) as a Group D carcinogen (inadequate evidence) and has not yet been reviewed for placement into one of the new cancer classification categories (EPA, 2011).

For free radical attack, guanine base in DNA is the most sensitive; it is converted to various oxides such as 8-hydroxyguanine, 8-hydroxyguanosine, and 8-hydroxy-2-deoxyguanosine (8-OHdG). However, 8-OHdG adduct is one of the most abundant base modifications excreted in urine, and because of its easy collection, it is regarded as a suitable biomarker for oxidative DNA damage and genotoxicity (Valavanidis et al., 2009). In the early studies on the genotoxicity and carcinogenicity of copper compounds, they were observed to be genotoxic in experimental studies, including increase in the frequency of chromosomal aberrations in White Leghorn chick bone marrow cells (Bhunya and Jena, 1996) and of chromosomal aberrations in Swiss mice (Agarwal et al., 1990).

For biomonitoring of human populations exposed to potential mutagens or carcinogens, the comet assay has become the method of choice in assessing oxidative DNA damage over the past 20 years. It is one of the most sensitive and accurate methods, being relatively free of artifacts and can provide an early detection system for the initiation of cell disregulation in the development of cancer (Valverde and Rojas, 2009).

While (EPA) has not yet classified copper as a human carcinogen, questions regarding its safety, long-term effects and how it affects humans are still up for debate.

Aim of work

To evaluate the oxidative stress and detect extent of DNA damage among workers at a copper processing factory.

Materials and methods

Study design: It is a comparative cross-sectional study.

Place and duration of the study: This study was conducted at a factory for non-ferrous industries located in Ain-Helwan region in Egypt, from

October 2017 to January 2018. The factory produces copper sheets, strips, wires, rods and pipes, as well as air conductors and insulated cables through smelting and refining of primary copper ingots and copper slab. During different stages of the process, a lot of smoke and copper dusts were disseminated into the workplace environment.

Study sample: All exposed workers were invited to participate in this study. The study sample included 50 male exposed workers who worked 8 hours/day , 5 days/week and whose mean age was 39.7+11.6 years (range 25-59) and mean duration of employment was 17.3+11.2 years (range 3- 34). Forty five accepted to participate in the study 9 of whom were excluded according to the exclusion criteria of the study. The study sample thus consisted of 36 exposed workers. The control group consisted of 34 subjects, selected from the administrative workers at the same factory in a place away from the copper processing workplace, with no history of occupational exposure to copper or any known physical or chemical agent in the workplace.

The inclusion criteria for the study: participants were males who had worked in the copper processing factory for a minimum of 2 years. The exclusion criteria consisted of: the receiving of radiation treatment or chemotherapy or having history of chronic diseases like diabetes, renal disease or malignancy in the last 2 years.

Study methods:

I- Questionnaire: The questionnaire determined the personal data, occupational and medical history of the participants under this study including age, smoking habit, type and place of work, duration of employment, personal protective equipment usage such as masks, gloves and lab-coat, history of chronic diseases and exposure to chemotherapy or radiotherapy and medicine usage.

II- Laboratory investigations

-Samples collection:

a- Peripheral blood sampling for exposed and control participants was performed in the morning via venipuncture; the sample was divided into two parts: one kept in a heparinized tube and the other into a plain tube for serum separation.

b- Urine samples were collected in special containers. Sampling of exposed workers was performed during routine annual periodic

medical examination given by the Health Insurance Organization.

All samples were transported to the laboratory in a cold box within 2 hours. Samples were kept frozen at -18°C until the time of analysis.

1-Analysis for blood copper: The concentrations of serum copper were analyzed by the atomic absorption spectrophotometer (AAS) at the department of Occupational and Environmental Medicine, Faculty of Medicine, Cairo University. Blood copper level was measured with Zemman background (Thermo elemental M-6 Type). The samples were prepared by dilution of 0.5 ml of blood with 2 ml deionized water and then centrifuged to obtain hemolysate. External calibrators for copper were prepared by serial dilution of parent stock which contains 1000 µl /ml using the diluents (deionized water). By plotting standard curve, the reading of the absorbance of the sample and calibrator was plotted on semi-log curve; the concentration of copper in the samples was interpreted from the standard curve (Fernandez and Kahn, 1971).

2-Total antioxidant capacity (T-AOC): 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 0.1mM 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).

3-Analysis of urinary 8-OHdG: Urinary 8-OHdG was measured with an ELISA kit (MyBioSource Inc, San Diego, USA), using competitive binding enzyme immunoassay technique. The amount of 8-OHdG was calculated by comparison with a standard curve. Urinary 8-OHdG concentrations were adjusted by urinary creatinine and expressed as ng/mg creatinine (Saito et al., 2000).

4-Comet assay: Frosted microscopic slides were cleaned with ethanol and dipped into the 1% normal melting point agarose (NMA). Twenty µL of blood cell suspensions were mixed with 80 µL of 0.7% low melt agarose (LMA), vortex mixed and 30 µL of mixed agarose/cell suspension and placed on the microscopic slides. The slides finally were covered with the cover slip, left for 10 minutes on an ice-cooled metal surface and

then 100 µL of LMA was dropped on prior layer of LMA. After 5 minutes, 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 at 4 °C) and kept for 30 minutes at 4 °C in the refrigerator. Electrophoresis parameters were set for constant conditions of 300 mA and 0.8V/CM. After completion of 30 minutes electrophoresis, slides were cured for 5 minutes in neutralization buffer and then washed with deionized water. Slides were stained with Ethidium bromide solution for 5 minutes. Microscopic analysis was performed under 400 magnifications with a fluorescent microscope (NikonE200, Nikon, Japan). At least fifty cells were counted for each comet slide. Open comet software was used for image analysis (Singh et al., 1988 and Collins et al., 2001).

Consent

Approval from the factory manager was obtained and all participants were informed of the objective of the study and gave their consent.

Ethical approval

The Institutional Ethical Committee of the department of Occupational and Environmental Medicine, Faculty of Medicine, Cairo University approved the procedures used in this study. All participants were treated according to the Helsinki Declaration of biomedical ethics.

Data management

Data were entered and statistically analyzed on the Statistical Package of Social Science Software program, version 23 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). Data were presented using mean and standard deviation (SD) for quantitative variables; frequency and percentage for qualitative ones. Comparison between groups for qualitative variables was performed using Chi square or Fisher's exact tests while for quantitative variables the comparison was conducted using independent sample t-test. Pearson correlation coefficients were calculated to explore the association between different quantitative variables. Multivariate linear regression model was conducted to explore predictors of comet tail length. p values less than 0.05 were considered statistically significant

Results

Table 1: Personal characteristics of the studied groups (exposed workers and the controls).

Variables	Exposed workers (No=36)	Controls (No =34)	t/X^2	p
Age (yrs.)				
Mean ± SD	39.7 ± 11.6	40.5 ± 11.6	**t=** -0.290	0.772
< 35 No %	15 (41.7)	13 (38.2)	**X^2=** 0.086	0.770
≥ 35 No %	21 (58.3)	21 (61.8)		
Smoking: No %				
Yes	25 (69.4)	25 (73.5)	**X^2=** 0.143	0.705
NO	11 (30.6)	9 (26.5)		
Smoking Index				
Mean ± SD	206.0 ± 102.8	219.2 ± 89.3	**t=** -0.485	0.630
Use of PPE: No %				
Yes	7 (19.4)			
NO	29 (80.6)	----------	**----------**	----------
Years of exposure				
Mean ± SD	17.3 ± 11.2			
<10 No %	17 (47.2)	----------	----------	---------
≥10 No %	19 (52.8)			

- Values were compared by Student t-test and the X2 test.
- Smoking index: number of cigarettes/day × duration of smoking in years.
- PPE: Personnel protective equipments.

Table 1 showed that there was no statistically significant difference between the two studied groups as regards age, smoking habit, smoking index and years of exposure. Approximately, one fifth of exposed workers regularly used personal protective equipments.

Table 2: The concentration of blood copper, T-AOC, 8-OHdG and mean DNA damage (comet tail length in mm) in the studied groups.

	Exposed (No =36) (Mean ± SD)	Controls (No =34) (Mean ± SD)	Mean difference (95% CI)	t	p
Copper level *(µg/dl)*					
Total	155.1 ± 23	77.9 ± 8.5	77.2 (69.0 - 85.5)	18.859	**<0.001****
Smokers	156.6 ± 23.8	80.5 ± 8.1	76.2 (65.9 - 86.4)	15.167	**<0.001****
Non-smokers	151.6 ± 21.7	70.7 ± 4.7	81.0 (66.2 - 95.8)	12.041	**<0.001****
T-AOC *(mIU/L)*					
Total	18.2 ± 7.2	25.7 ± 5.5	-7.5 (-10.6 - -4.5)	-4.941	**<0.001****
Smokers	18.6 ± 7.3	27 ± 5.5	-8.4 (-12.1 - -4.7)	-4.613	**<0.001****
Non-smokers	17.3 ± 7.3	22.3 ± 4	-5.0 (-10.7 - 0.8)	-1.813	0.086
Comet tail length *(mm)*					
Total	9.5 ± 3.7	5.7 ± 1.4	3.8 (2.5 - 5.1)	5.723	**<0.001****
Smokers	8.9 ± 3.7	6.1 ± 1.2	2.9 (1.3 - 4.5)	3.669	**<0.001****
Non-smokers	10.8 ± 3.4	4.8 ± 1.5	6.0 (3.5 - 8.4)	5.220	**<0.001****
8-OHdG *(ng/mg creatinine)*					
Total	9.7 ± 5	4.1 ± 1.2	5.6 (3.8 - 7.3)	6.467	**<0.001****
Smokers	9.5 ± 5.3	3.7 ± 0.6	5.7 (3.5 - 7.9)	5.376	**<0.001****
Non-smokers	10.3 ± 4.5	5.2 ± 1.8	5.0 (1.8 - 8.2)	3.380	**0.005****

**: Highly statistically significant.

Table 2 showed statistically significantly lower levels of T-AOC and higher levels of serum copper, 8-OHdG and comet tail length among exposed group compared to non-exposed one; with similar results when comparing the same variables between the smokers of exposed workers and those of non-exposed; and also between the non-smokers of exposed and those of non-exposed except for the case of T-AOC in the non-smokers where the difference is not statistically significant.

Table 3: Comet tail length (mm) with respect to age, smoking habit, smoking index, work duration, years of exposure, oxidative stress parameters and copper level among exposed workers.

	Comet tail length (mm)			
	Exposed (No =36)			
	No	**Mean± SD**	**Range**	**p**
Age/years	36	r= -0.059		0.731
Age groups				
< 35 years	15	9.7 ± 3.8	3.4 - 14.9	0.819
≥ 35 years	21	9.4 ± 3.7	3.8 - 16.1	
Smoking				
Smokers	25	8.9 ± 3.7	3.4 - 16.1	0.166
Non-smokers	11	10.8 ± 3.4	5.1 - 14.9	
Smoking index	25	r= -0.196		0.347
Duration of exposure (years)	36	r= -0.079		0.645
Years of exposure				
< 10 years	17	9.6 ± 3.8	3.4 - 14.9	0.863
≥ 10 years	19	9.4 ± 3.7	3.8 - 16.1	
Copper level *(µg/dl)*	36	r= -0.106		0.539
T-AOC *(mIU/L)*	36	r= **-0.637**		**<0.001****
8-OHdG *(ng/mg creatinine)*	36	r= **0.706**		**<0.001****

r = simple correlation coefficient. **: Highly statistically significant

Table 3 showed that Comet tail length had statistically significant positive associations with 8-OHdG and significant negative associations with T-AOC ; while no significant correlations were found between comet tail length and each of serum copper levels, smoking index, age or duration of employment among the exposed group.

Table 4: Predictors of DNA damage expressed as comet tail length (mm) through multiple linear regression model among the exposed workers (R2= 0.586).

	β	95% CI for β			p value	r	Partial r
Age	0.038	-0.089	-	0.165	0.555	-0.068	0.075
Duration of work (years)	-0.009	-0.141	-	0.122	0.889	-0.076	-0.018
Smoking	0.337	-0.861	-	1.534	0.576	-0.082	0.071
Copper level (µg/dl)	-0.023	-0.056	-	0.010	0.165	0.48	-0.176
T-AOC (mIU/L)	-0.026	-0.144	-	0.093	0.669	-0.638	-0.055
8-OHdG (ng/mg creatinine)	0.492	0.288	-	0.697	**<0.001****	0.773	0.522

β= beta coefficient, CI= confidence interval.
r = simple correlation coefficient. **: Highly statistically significant

Table 4 showed that 8-OHdG is the best predictor of DNA damage expressed as comet tail length while age, duration of work, smoking, serum copper and T-AOC had no significant effect.

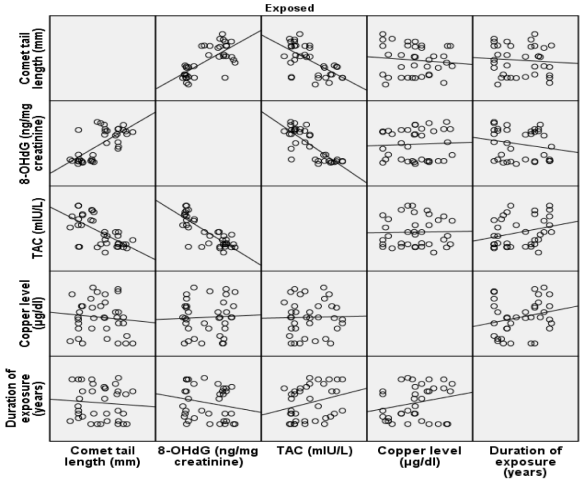

Figure 1: Correlation between early DNA damage (comet tail length) and each of 8-OHdG, T-AOC, copper level and duration of exposure among the exposed workers.

Figure 1 showed significant positive associations between comet tail length and 8-OHdG and significant negative association with T-AOC among the exposed group. No significant correlations were found between comet tail length and each of serum copper levels or duration of employment among the exposed group.

Discussion

Production of copper has grown significantly in the recent years. It is used to make many very valuable alloys and is used in many forms for electrical engineering and electronics, and in pipes for water installation. So, a wide range of workers is exposed to copper in various industrial settings with variable health effects; and possible genotoxicity and carcinogenicity (EPA, 2011).

The current study was conducted to determine oxidative stress and extent of DNA damage using the comet assay in a group of copper processing workers in one of the factories for non-ferrous industries in Egypt and to estimate the prevalence of genotoxicity from copper among exposed workers and the correlation of the study variables with the genotoxicity level.

The study results showed a significant difference in the mean values of serum copper levels between exposed workers and the control group (155.1±23 versus 77.9±8.5 ug/dL; p value <0.001) (Table 2). Normal levels of total copper in serum range from 63.7 to140.2 ug/dL (ATSDR, 2004). Results of the present study are in accordance with a recent study on copper smelters which found high serum copper levels among exposed workers compared to the non-exposed (148.4±15.6 versus 90.7±9.7ug/dL) (El-Safty et al., 2014). The increase in body burden of metals in case of exposure at the workplace was observed in other studies (Gurgueira et al., 2002).

In the present study, less than one fifth of exposed workers used personal protective equipments (PPE) regularly which may have contributed to the high levels of their serum copper. Total antioxidant capacity was 1.4 times lower and urinary 8-OHdG was 2.3 times higher among exposed workers compared to controls and the differences were statistically significant ($p< 0.001$ for each) (Table 2) which reflects the development of oxidative

stress and oxidative DNA lesions from long term excessive exposure to copper. Previous studies on copper smelters using malondialdehyde level and superoxide dismutase enzyme activity revealed that oxidative stress developed among exposed workers and might have resulted in the development of health effects with continued occupational exposure to copper fumes (De Oliveira et al., 2011 and El-Safty et al., 2014).

In the current study; participants were investigated for genotoxicity using comet assay which is increasingly being used to quantify basal DNA damage in occupationally exposed humans (Valverde and Rojas, 2009). The mean comet tail length was approximately twofold higher in exposed workers than the level of the control group; $p<0.001$ (Table 2). The results also showed significant association between comet tail length with each T-AOC and 8-OHdG ($r= -0.64$ and $r=0.71$ respectively; $p<0.001$ for each) among exposed workers (Table 3). Overproduction of free radicals could overcome the protective antioxidant effect and leads to oxidative stress in long term exposure to genotoxic agents, and finally leads to increase of DNA damage (Valko et al., 2005). Lewijska et al. (2007) reported increased frequency of micronucleus in peripheral blood lymphocytes and buccal epithelial cells among copper smelters which indicated chromosomal damage. Similar findings were obtained by De Olivera et al. (2011). A recent study on copper processing workers revealed DNA damage quantified by comet assay along with decreased antioxidant power of plasma and increased propensity for oxidative stress (Kumar et al., 2016). Several other studies also found significant positive associations between exposure to metals, oxidative stress parameters and primary DNA damage detected in comet assay (Kobal et al., 2004, Rohrdanz and Kahl, 2005 and Pease et al., 2016).

The comparison of the genotoxic effect between smokers of exposed workers and that of non-exposed, and also between the non-smokers of exposed and those of non- exposed revealed significant increases in comet tail length and 8-OHdG levels among exposed workers (smokers and non-smokers) compared to those of non-

exposed group ($p<0.001$ for each) (Table 2), which implies a more severe DNA damage in copper exposed workers. Also, the analysis did not show any statistically significant correlations between neither smoking nor exposure level and the frequency of DNA damage (comet tail length) among the exposed group (Table 3). In addition, the multiple regression analysis revealed a significant effect of 8-OHdG on comet tail length in peripheral blood lymphocytes as predictor of DNA damage $p<0.001$; and excludes age, duration of exposure, serum copper and smoking as predictors of DNA damage among workers exposed to copper (Table 4). So smoking has no effect on the genotoxicity among copper exposed workers in the present study.

In other studies done on smoked workers exposed to heavy metals, smoking did not significantly affect the comet assay values among lead exposed workers (Fracasso, et al., 2002); another study on the genotoxicity of chromium and nickel using comet assay found that smoking and age had no significant effect on DNA damage (Danadevi et al., 2004).

Conclusion and recommendations: Our results indicated that occupational exposure to copper would cause DNA damage which may be due to increased oxidative stress. This suggests the potential genotoxic effect of copper. Due to recent findings on potential of oxidative stress and comet assay for genotoxicity, further researches should be conducted to investigate in more details the exact biochemical mechanism of such findings which could be used as a milestone for the derivation of exposure limits and standards for work environments. Determination of biomarkers of oxidative stress and DNA damage is important for early detection of genotoxic effects in order to prevent health hazards in copper exposed workers.

Limitations: Certain limitations of the present study should be considered. The sample size was relatively small. Therefore, these results should be verified with large-scale, multicenter prospective cohort studies.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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