

INDUCTION OF MICRONUCLEI IN MICE BONE MARROW CELLS
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Keywords: Cobalt chloride, copper chloride, heavy metal, mice, micronucleus assay.

Abstract: The aim of our research was to investigate the genotoxic effects of cobalt chloride and copper chloride in mouse bone marrow cells using the micronucleus (MN) assay. The three different concentrations of cobalt chloride (11.2, 22.5 and 45 mg kg⁻¹) and copper chloride (1.17, 2.35 and 4.70 mg kg⁻¹) were injected intraperitoneally to mice for 24 and 48 hours. It was observed that both of these heavy metals induced a significant increase in frequency of micronucleated polychromatic erythrocytes (MNPCE) at different concentrations in mice for 24 and 48 hours when compared with the control. Furthermore, the significant reduction for the polychromatic erythrocyte/normochromatic erythrocyte (PCE/NCE) ratio which is indicative of bone marrow cytotoxicity was observed in bone marrow cells which were treated with copper chloride at all concentrations for 24 and 48 hours. No reduction of the PCE/NCE ratio was observed both 24 and 48 hours after all the doses of cobalt chloride tested as compared to the negative control. These results lead us to the conclusion that copper chloride may have genotoxic and cytotoxic properties due to induction in the frequency of MN and a reduction in PCE/NCE ratio in bone marrow cells of mice, whereas cobalt chloride induced only genotoxic effect in mice bone marrow.

INTRODUCTION

Nowadays, air pollution resulting from the development of industry is the most important problem of all living creatures. Many pollutants and heavy metals are spreading rapidly to the environment through natural, industrial and agricultural sources, municipal wastes and atmospheric pollutants. Heavy metals are the main factors for the environment pollution due to their toxic effects and accumulation features.

Some heavy metals such as chromium, cobalt, copper, manganese, and zinc are the essential micronutrients for plants and animals. However, they are easily assimilated by plants and animals and accumulated in their structures. Diagonanolin et al., [13] and Beijer and Jernelov [3] reported that heavy metals are critical because of their easy uptake into the food chain and bioaccumulation processes [39]. According to the studies of Knasmuller et al., [27] and Hartwig [22], toxic heavy metals cause DNA damage and

their carcinogenic effects in animals and humans are most probably caused by their mutagenic ability [28]. Reporting on the mutagenic activity of heavy metals by using different assays on the genetic system of living organism is important in environmental studies.

Different testing methods have been used to assess the genotoxicity of heavy metals [16, 19, 21, 26, 55]. However, the mouse bone marrow micronucleus test is one of the most widely used genetic toxicology assays. Micronuclei consist of chromosome fragments or whole chromosomes which lag behind at anaphase of mitosis and are not incorporated into daughter nuclei. They form single or multiple micronuclei in the cytoplasm. The assay is based on the increase in the frequency of micronucleated PCEs in bone marrow of the treated animals [14].

Cobalt is used for the production of alloys and hard metal (cemented carbide), in diamond polishing, as drying agents, pigment and catalysts [9]. The available genotoxicity data suggest that cobalt is a toxic agent [9, 33, 44]. But in recent studies, it is stated that negative results were obtained in bacterial assays [4].

Copper is a naturally occurring metal that possesses high electrical and thermal conductivity and resists corrosion. It is essential for human health [34]. Copper is also an essential micronutrient for higher plant growth and metabolism [31]. However, Hall [20], Schutzendubel and Polle [42], Yruela [56] declared that its high bioavailability in soils makes it a potentially toxic substance causing the inhibition of growth and oxidative injuries [49]. Several testing methods which have been used to assess the genotoxicity of copper compounds in different test systems produced positive results [11, 18, 19, 23, 43]. However, negative results have also been reported in different test systems [32, 35, 45, 52, 54,].

Although cobalt and copper toxicities have been extensively investigated at different test systems, there are few reports of genotoxicity in mice *in vivo* assays [33, 38, 40].

Because of the controversial results stated in previous studies and the widespread use of pesticides, fertilizers, continuous air emissions from industrial sources and vehicular traffic throughout the world, additional studies are needed to evaluate the potential toxic risks of these heavy metals. The aim of this study was to investigate the genotoxic effects of the heavy metals cobalt and copper chloride by the use of mice bone marrow micronucleus assay *in vivo*.

MATERIAL AND METHODS

Animals

In the present investigation, *in vivo* studies were carried out in 8–10 weeks old (25–30 gr) Swiss albino mice (*Mus musculus*), obtained from the Laboratory Animal Center of Trakya University (Edirne, Turkey). They were housed in plastic cages with a bedding of wood shavings. They were fed fresh standart pellet and given water *ad libitum*. All mice were kept under constant environmental conditions within a 12/12h ligh/dark cycle.

Chemicals, dose and treatment

Cobalt chloride (CAS No:7791-13-1) and copper chloride (CAS No: 10125-13-0) were purchased from Merck (Whitehouse Station, NJ, USA). Each chemical was dissolved in distilled water.

The intraperitoneal (i.p.) route of application was used in all experiments. For mice, the LD₅₀ concentrations (i.p.) of cobalt chloride and copper chloride were 90 mg kg⁻¹bw [44] and 9.4 mg kg⁻¹bw [7], respectively. Cobalt chloride (45, 22.5, 11.25 mg kg⁻¹bw) and copper chloride (4.70, 2.35, 1.17 mg kg⁻¹bw) were injected i.p. to mice for 24 and 48 hours. The lowest concentrations were 1/8 of the LD₅₀ concentration of cobalt and copper chloride.

Distilled water was used as a negative control. Mitomycin C (CAS No: 50-07-7) was used as a positive control and given i.p. in a single dose of 2 mg kg⁻¹bw per mouse.

Micronucleus assay

In the MN test, each concentration group, negative and positive control group contained 5 male mice. Mice (8–10 weeks) were treated with the same concentrations intraperitoneally for 24 and 48 hours. The micronucleus test was performed according to Schmid [41] and Aaron et al. [1] with minor modifications. The bone marrow cells were flushed out with fetal calf serum, and the suspension was centrifuged for 10 min at 2000 rpm. The pellets were spread on a slide glass and fixed with methanol. The slides were stained with May-Grunwald (Cat. No. 101424) for 3 min, May Grunwald: distilled water (1:1) for 2 min, 10% Giemsa (CAS No. 51811-82-6) in Sörensen buffer for 10 min. A total of 1000 erythrocytes were scored for each animal at a magnification of ×1000. The numbers of MNPCE and MNNCE were also counted. PCE/NCE ratio was calculated to determine the cytotoxic effects of the chemicals.

Statistical evaluation

The normality of the distribution of the frequency of MNPCE scores was assessed using the non parametric Kruskal-Wallis and Mann-Whitney U test. All statistical analyses were carried out by the Statistica programme for Windows.

RESULTS AND DISCUSSION

The results obtained are presented Table 1. Cobalt chloride induced a significant increase in the frequency of micronucleated PCE at 22.5 and 45 mg kg⁻¹bw concentrations for 24 hours and at all the concentrations for 48 hours when compared with the control (Figures 1 and 2). No significant reduction for the PCE/NCE was observed at all the concentrations for 24 and 48 hours.

Copper chloride increased significantly the number of micronucleated PCE at 2.35 and 4.7 mg kg⁻¹bw concentrations for 24 hours and all the concentrations for 48 hours as compared with the control group (Figures 1 and 2). Also significant reduction for the PCE/NCE was observed at all the concentrations for 24 and 48 hours.

Heavy metals are a major class of environmental pollutants. Heavy metal intoxication is a new threat issue for public health at present. There are many reports about occupational and environmental intoxication [29, 46, 51, 53]. The detection of genotoxicity of heavy metals is very important because people exposed to these chemicals may be adversely affected [9, 24, 25]. So cobalt and copper chloride have been extensively investigated to determine the damaging effects of these heavy metals.

According to the present investigation, cobalt chloride induced significant frequencies of MN in mice at different concentrations and time intervals studied. Also

Table 1. Micronucleus induction in mice bone marrow cells injected with cobalt chloride and copper chloride.

Treatments	Concentrations mg kg ⁻¹	Total cell number mice number	Sampling time (h)	Total MNPCE %	PCE/NCE	Sampling time (h)	Total MNPCE %	PCE/NCE
Negative control	-	5000/5	24	0.70 ± 0.09	1.57 ± 0.04	48	0.67 ± 0.08	1.78 ± 0.08
Positive control	2	5000/5		4.60 ± 0.57***	0.94 ± 0.05*		4.25 ± 0.30***	0.85 ± 0.02*
Cobalt chloride	11.2	5000/5		1.10 ± 0.19	1.43 ± 0.10		2.35 ± 0.17***	1.70 ± 0.33
	22.5	5000/5		1.45 ± 0.25*	1.45 ± 0.08		1.90 ± 0.40**	1.80 ± 0.39
Copper chloride	45	5000/5		1.70 ± 0.02**	1.51 ± 0.01		2.55 ± 0.53***	2.81 ± 0.38
	1.17	5000/5		1.05 ± 0.09	0.33 ± 0.05***		1.30 ± 0.12*	0.29 ± 0.03***
	2.35	5000/5		1.35 ± 0.20*	0.25 ± 0.06***		1.35 ± 0.17*	0.20 ± 0.01***
	4.7	5000/5		1.55 ± 0.30**	0.26 ± 0.03***		2.00 ± 0.50***	0.41 ± 0.09***

MNPCE, micronucleated polychromatic erythrocyte; PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte. All data are presented as mean ± standard error.

* P<0.05

** P<0.01

*** P<0.001

in the studies of Sigma Aldrich [44] it is reported that cobalt chloride is mutagenic in human lymphocytes, mammalian somatic cells. In addition, it affects the reproductive system of mice at different concentrations. It has been reported by De Boeck et al. [9] that cobalt (II) ions are genotoxic *in vitro*, and *in vivo*, and carcinogenic in rodents; by De Boeck et al. [10] that cobalt caused DNA single strand breaks and micronuclei in mammalian cells *in vitro*. According to NTP studies [33] cobalt (II) chloride induced aneuploidies, micronuclei and chromosome aberrations in bone marrow in mice. Therefore, it is stated that cobalt chloride is genotoxic in mice bone marrow cells. These results are in agreement with our studies on the positive genotoxicity of cobalt chloride.

In this study, copper chloride is genotoxic in mice at different concentrations tested. Previous studies have also demonstrated that copper have mutagenic and genotoxic activities in different biological test systems [6, 12, 18, 30, 36, 43, 47, 49, 51, 55]. Furthermore, many earlier authors [32, 35, 37, 45, 48, 50, 52, 54] have adversely observed toxicity of copper against different test organisms. However, there are few studies addressing the genotoxicity of copper in mice. Bhunya and Pati [5] indicated that copper sulphate induced chromosomal aberrations, micronuclei and sperm abnormalities in mice bone marrow cells. It has been reported that copper sulphate induced chromosomal aberrations [2] and it was genotoxic in mice [15]. Pra et al. [38] and Saleha et al. [40] have observed the genotoxicity and mutagenicity of copper in mice, respectively. These statements are consistent with the results of the present study.

Several studies have shown that metal genotoxicity was caused by indirect mechanisms. Gabbianelli et al. [17] reported that copper genotoxicity is induced by oxidative stress and production of DNA damaging reactive oxygen species [36]. Beyersmann and Hartwig [4] also indicated that indirect mechanisms such as induction of oxidative stress, its interference with DNA repair and deregulation of cell proliferation cause metal genotoxicity.

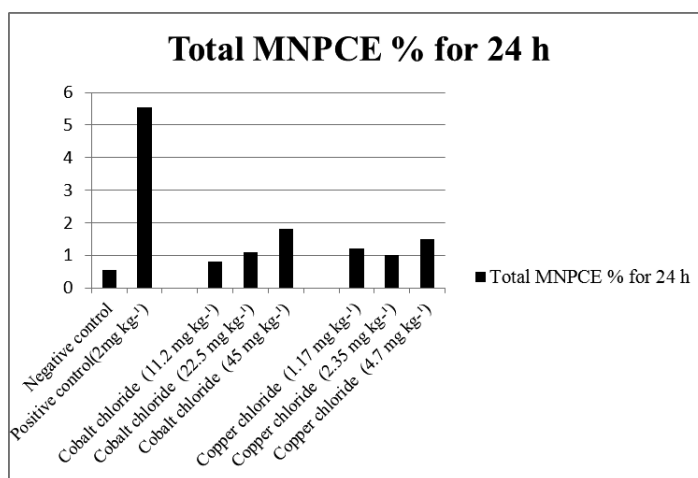


Fig. 1. The increase in the frequency of micronucleated PCE observed in mice treated with cobalt chloride and copper chloride for 24 hours

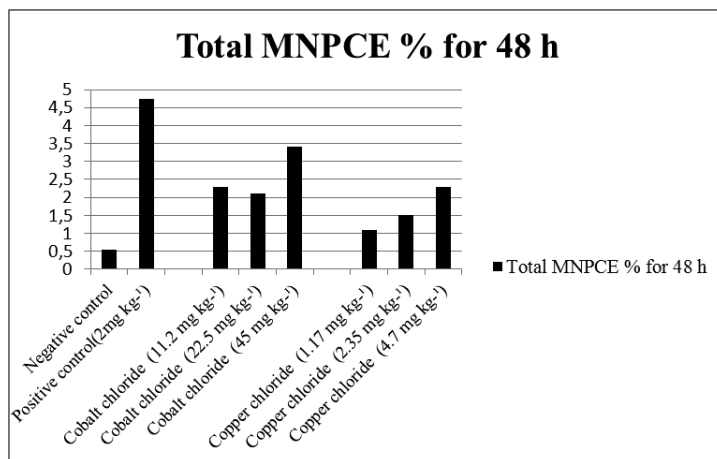


Fig. 1. The increase in the frequency of micronucleated PCE observed in mice treated with cobalt chloride and copper chloride for 48 hours

PCE/NCE ratio is indicative of bone marrow cytotoxicity. A significant decrease of PCE/NCE ratio in treated animals provides evidence of an erythropoiesis depression, with reduced proliferation of nucleated erythrocyte precursor cells [8]. Cobalt chloride decreased PCE/NCE ratio in mice at concentrations tested when compared with the control group. Contrary to this, copper chloride decreased PCE/NCE ratio at all concentrations in mice for 24 and 48 hours. So it can be accepted as a cytotoxic agent.

CONCLUSIONS

The formation of MN observed at 24 and 48 hour sampling times indicated that both cobalt and copper chloride showed their genotoxic effects. In addition, copper chloride has cytotoxic effects in mice because it decreases PCE/NCE ratio. The *in vivo* micronucleus assay used in this study was a very sensitive and reliable method to evaluate the genotoxic effect in mammalian cells exposed to chemical substances. According to these results, cobalt chloride and copper chloride seem to potentiate genotoxic effect in mice bone marrow. Our observations may indicate that environment polluted with cobalt chloride and copper chloride may lead to severe damage of human health.

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