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. Diagomanolin 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 standard pellet and given water ad libitum. All mice were kept under constant environmental conditions within a 12/12h light/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 LD$_{50}$ concentrations (i.p.) of cobalt chloride and copper chloride were 90 mg kg$^{-1}$bw [44] and 9.4 mg kg$^{-1}$bw [7], respectively. Cobalt chloride (45, 22.5, 11.25 mg kg$^{-1}$bw) and copper chloride (4.70, 2.35, 1.17 mg kg$^{-1}$bw) were injected i.p. to mice for 24 and 48 hours. The lowest concentrations were 1/8 of the LD$_{50}$ 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$^{-1}$bw 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 $\times$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$^{-1}$bw 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$^{-1}$bw 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 \(\text{mg kg}^{-1}\) | Total cell number mice \(5000/5\) | 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*     |                |         |
|                 | 11.2                                   | 5000/5                            | 1.10 ± 0.19       | 1.43 ± 0.10    | 2.35 ± 0.17***| 1.70 ± 0.33     |                |         |
| Cobalt chloride | 22.5                                   | 5000/5                            | 1.45 ± 0.25*      | 1.45 ± 0.08    | 1.90 ± 0.40** | 1.80 ± 0.39     |                |         |
|                 | 45                                     | 5000/5                            | 1.70 ± 0.02**     | 1.51 ± 0.01    | 2.55 ± 0.53***| 2.81 ± 0.38     |                |         |
| Copper chloride | 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. Bhuunya 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](image-url)
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.

REFERENCES

[1] Aaron, C.S., Sorg, R., & Zimmer D. (1989). The mouse bone marrow micronucleus test: evaluation of 21 drug candidates, *Mutation Research*, 223, 129–140.

[2] Agarwal, K., Sharma, A., & Talukder, G. (1990). Clastogenic effects of copper sulfate on the bone marrow chromosomes of mice in vivo, *Mutation Research*, 243 (1), 1–6.

[3] Beijer, K., & Jernelov, A. (1986). Sources, transport and transformation of metals in the environment, [in:] Friberg L, Nordberg GF, Vouk VB (eds) Handbook on the toxicology of metals (pp. 68–84). Elsevier, Amsterdam 1986.

[4] Beyersmann, D., & Hartwig, A. (2008). Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms, *Arch. Toxicol.*, 82, 493–512.
[5] Bhunya, S.P., & Pati, P.C. (1987). Genotoxicity of an inorganic pesticide, copper sulphate in mouse in vivo test system, *Cytologia*, 52, 801–808.

[6] Bhunya, S.P., & Jena, G.B. (1996). Clastogenic effect of copper sulphate in chick in vivo test system, *Mutation Research*, 367 (2), 57–63.

[7] Chemwatch. (2010). Material Safety Data Sheet, Section 11 – Toxicological Information, NC317TCP, 23–3153, 6.

[8] Cicchetti, R., Bari, M., & Argentin, G. (1999). Induction of micronuclei in bone marrow by two pesticides and their differentiation with CREST staining: an *in vivo* study in mice, *Mutation Research*, 439, 239–248.

[9] De Boeck, M., Kirsch-Volders, M., & Lison, D. (2003a). Cobalt and antimony: genotoxicity and carcinogenicity, *Mutation Research*, 533 (1–2), 135–152.

[10] De Boeck, M., Lombaert, N., De Backer, S., Finsy, R., Lison, D., & Kirsh-Volders, M. (2003b). In vitro effects of different combinations of cobalt and metallic carbide particles, *Mutagen*, 18, 177–186.

[11] Demerec, M., Bertani, G., & Flint, J. (1951). A survey of chemicals for mutagenic action on *E. coli*, *Am. Nat.*, 85, 119–136.

[12] Denizau, F., & Marion, M. (1989). Genotoxic effects of heavy metals in rat hepatocytes, *Cell Biol. Toxicol.*, 5, 15–25.

[13] Diagomanolin, V., Farhang, M., Ghazi-Khansari, M., & Jafarzadeh, N. (2004). Heavy metals (Ni, Cr, Cu) in the Karoon waterway river, *Iranc. Toxicol. Lett.*, 151 (1), 63–67.

[14] EPA. (1996). Health Effects Test Guidelines. OPPTS 870.5395 In Vivo Mammalian Cytogenetic Tests: Erythrocyte Micronucleus Assay. 712-C-96-226.

[15] Fahmy, M.A., & Aly, F.A.E. (2000). *In vivo* and *in vitro* studies on the genotoxicity of cadmium chloride in mice, *J. App.Toxic.*, 20, 231–238.

[16] Feng, S., Wang, X., Wei, G., Peng, P., Yang, Y., & Cao, Z. (2007). Leachates of municipal solid waste incineration bottom ash from Macao: Heavy metal concentrations and genotoxicity, *Chem.*, 67 (6), 1133–1137.

[17] Gabbianelli, R., Lupidi, G., Villarini, M., & Falcioni, G. (2003). DNA Damage Induced by Copper on Erythrocytes of Gilthead Sea Bream *Sparus aurata* and Mollusk *Scapharca inaequivalvis*, *Arch. Environ. Contam. Toxicol.*, 45, 350–356.

[18] Garrett, N.E., & Lewtas, J. (1983). Cellular toxicity in Chinese hamster ovary cells culture I, *Environ. Res.*, 32, 455–465.

[19] Guecheva, T., Henriques, J.A.P., & Erdtmann, B. (2001). Genotoxic effects of copper sulphate in freshwater planarian *in vivo*, studied with single-cell gell test (comet assay), *Mutation Research*, 497, 19–27.

[20] Hall, J.L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance, *J. Exp. Bot.*, 53, 1–11.

[21] Hartmann, A., & Speit, G. (1994). Comparative investigations of the genotoxic effects of metals in the single cell gell (SCG) assay and the sister chromatid exchange (SCE) test, *Environ. Mol. Mutagen.*, 23, 299–305.

[22] Hartwig, A. (1995). Current aspects in metal genotoxicity, *Bio. Metals*, 8, 3–11.

[23] Heidelberger, C., Freeman, A.E., Pienta, R.J., Sivak, A., Bertram, J.S., Casto, B.C., Dunkel, V.C., Francis, M.W., Kakunaga, T., Little, J.B., & Schechtman, L.M. (1983). Cell transformation by chemical agents – a review and analysis of the literature: a report of the US, Environmental Protection Agency Gene-Tox Program. *Mutation Research*, 114 (3), 283–385.

[24] International agency for research on cancer (IARC). (1991). Chlorinated drinking water; chlorination by products; some other halogenated compounds; cobalt and cobalt compounds. IARC monographs on the evaluation of carcinogenic risks to humans, 52 (pp. 363–472), IARC, Lyon 1991.

[25] International agency for research on cancer (IARC). (2003). Cobalt in hard metals and cobalt sulfate, gallium arsenide, indium phosphate and vanadium pentoxide. IARC monographs on the evaluation of carcinogenic risks to humans, 86 (pp. 119–237) IARC, Lyon 2006.

[26] Katnoria, J.K., Arora, S., & Nagpal, A. (2008). Genotoxic potential of agricultural soils of amritsars, *Asian J. Sci. Res.*, 1 (2), 122–129.

[27] Knasmuller, S., Gottmann, E., Steinckelner, H., Fomin, A., Pickl, C., Paschki, A., God, R., & Kundi, M. (1998). Detection of genotoxic effects of heavy metal contaminated soils with plant bioassays, *Mutation Research*, 420, 37–48.
[28] Kovalchuk, O., Titov, V., Hohn, B., & Kovalchuk, I. (2001). A sensitive transgenic plant system to detect toxic inorganic compounds in the environment, *Nat. Biotech.*, 19, 568–572.

[29] Kuo, H.W., & Wu, M.L. (2002). Effects of chromic acid exposure on immunological parameters among electroplating workers, *Int. Arch. Occup. Environ. Health*, 75, 186–190.

[30] Law, L.W. (1938). The effects of chemicals on the lethal mutation rate in *Drosophila melanogaster*, *Proc. Natl. Acad. Sci. USA.*, 24, 546–550.

[31] Maksymiec, W. (1997). Effect of copper on cellular processes in higher plants, *Photosyn.*, 34 (3), 321–342.

[32] Marzin, D.R., & Phi, H.V. (1985). Study of the mutagenicity of metal derivatives with *Salmonella typhimurium*, *Mutation Research*, 155, 49–51.

[33] National Toxicology Program (NTP). (1998). Toxicology and carcinogenesis studies of cobalt sulfate heptahydrate in F334/N rats and B6C3F1 mice (inhilation studies), NTP Technical Report 507, USA: Research Triangle Park, NC, 1998.

[34] NICNAS. (2003). Existing Chemicals Information Sheet, GPO Box 58, Australia: Sidney NSW, 2003.

[35] Nishioka, H. (1975). Mutagenic activities of metal compounds in bacteria, *Mutation Research*, 31, 185–189.

[36] Obiakor, M.O., Okonkwo, J.V., Ezeonyejiaku, C.D., & Ezenwelu, C.O. (2010). Genotoxicity: Single and joint action of copper and zinc to Synodontis claris and Tilapia nilotica, *J. Appl. Sci. Environ. Manage.*, 14 (3), 59–64.

[37] Olivier, P., & Marzin, D. (1987). Study of the genotoxic potential of 48 inorganic derivatives with the SOS chromotest, *Mutation Research*, 189, 263–269.

[38] Pra, D., Franke, S.I.R., Giulian, R., Yoneama, M.L., Dias, J.F., Erdtmann, B., & Henriques, J.A.P. (2008). A sensitive transgenic plant system to detect mutagens of carcinogens in *Saccharomyces cerevisiae*, *Mutation Research*, 117, 149–152.

[39] Saleha, B.B., Ishaq, M., Danadevi, K., Padmavathi, P., & Ahuja, Y.R. (2004). DNA damage in leukocytes of mice treated with copper sulfate, *Food Chem. Toxicol.*, 42 (12), 1931–1936.

[40] Schmid, W. (1975). The micronucleus test, *Mutation Research*, 31, 9–15.

[41] Schutzendubel, A., & Polle, A. (2002). Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization, *J. Exp. Bot.*, 53, 1351–1365.

[42] Sideris, E.G., Charalambous, S.C., Tsolomyty, A., & Katsaros, N. (1988). Mutagenesis, carcinogenesis and the metal elements – DNA interaction, *Prog. Clin. Biol. Res.*, 259, 13–25.

[43] Sigma-Aldrich. (2006). Material Safety Data Sheet, 2006, www.sigma-aldrich.com (29/Aug/2006).

[44] Singh, I. (1983). Induction of reverse mutation and mitotic gene conversion by some metal compounds in *Saccharomyces cerevisiae*, *Mutation Research*, 117, 149–152.

[45] Sipczuk-Walczak, H., Matczak, W., Raźewska, G., & Szymczak, M. (2005). Neurologic and neurophysiologic examinations of workers occupationally exposed to aluminium, *Med. Pr.*, 56, 9–17.

[46] Sirover, M.A., & Loeb, L.A. (1976). In *Drosophila melanogaster* and *Vicia faba* induced in vitro mutagens of carcinogens, *Sci.*, 94, 1434–1436.

[47] Sora, S., Carbone, M.L.A., Pacciariini, M., & Magni, G.E. (1986). Disomic and diploid meiotic products induced in *Saccharomyces cerevisiae* by the salts of 27 elements, *Mutagen.*, 1 (1), 21–28.

[48] Souguir, D., Ferjani, E., Ledoigt, G., & Goupil, P. (2008). Exposure of *Vicia faba* and *Pisum sativum* to copper-induced genotoxicity, *Protoplas.*, 233, 203–207.

[49] Tinwell, H., & Ashby J. (1990). Inactivity of copper sulphate in a mouse bone-marrow micronucleus assay, *Mutation Research*, 245 (3), 233–236.

[50] Tsiashala, M.D., Kabengele, K., & Lumu, B.M. (1990). Trace element determination in scalp hair of people working at a copper smelter, *Biol. Trace Elem. Res.*, 26–27, 287–294.

[51] Tso, W.W., & Fung, W.P. (1981). Mutagenicity of heavy metals, *Bull. Environ. Contam. Toxicol.*, 40, 597–603.

[52] Yildiz, M., Cigerci, I.H., Konuk, M., Fidan, A.F., & Terzi, H. (2009). Determination of genotoxic effects of copper sulphate and cobalt chloride in *Allium cepa* root cells by chromosome aberration and comet assays, *Chem.*, 75 (7), 934–938.

[53] Yruela, I. (2005). *Copper in plants*, *Braz. J. Plant Physiol.*, 17 (1), 145–156.