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# GENOTOXIC EFFECTS OF COPPER SULFATE IN RABBITS

S. GEORGIEVA[1], B. POPOV[2] and V. PETROV[3]

[1] *Department of Genetics, Animal Breeding and Reproduction, Agricultural Faculty, Trakia University,* 6000 Stara Zagora, Bulgaria
[2] *Department of Molecular Biology, Immunology and Medical Genetics, Faculty of Medicine, Trakia University,* 6000 Stara Zagora, Bulgaria
[3] *Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University,* 6000 Stara Zagora, Bulgaria

*Abstract* - This study was carried out to determine the genotoxic effects of oral application of $CuSO_4$ in rabbits by the chromosome aberration (CA) and sister chromatid exchange (SCE) tests. Ten male New Zealand rabbits (5 months old, weighing 3.5-4.0 kg) were allocated into two groups. The first group received $CuSO_4$ ($5H_2O$) in drinking water for 6 consecutive days. The second group was used as a control. On the 7$^{th}$ day, blood samples were taken from the ear marginal vein and the SCE and CA tests in peripheral lymphocytes were used as genotoxicity and mutagenicity endpoints, respectively. Results showed a significant increase in the frequencies of the aberrant cells (7.4±0.24, P<0.001) and CA (chromatid fragments 3.2±0.37, chromosome fragments 4.2±0.37, P<0.001), and total aberrations (7.4±0.24, P<0.001) after the treatment with $CuSO_4$ when compared with the control group. The level of SCE per cell in the CuSO4-treated rabbits (9.66±0.062) was significantly higher than in rabbits from the control group. These findings show that copper exhibits a genotoxic and mutagenic potential in rabbits.

*Key words*: Copper sulfate, chromosome aberrations, sister chromatid exchange

## INTRODUCTION

Lack of specific information makes it difficult to reach firm conclusions about the hazards of dietary metals (Rojas et al., 1999), especially for copper, which is among the most important. Copper (Cu) is an essential trace element found in small amounts in a variety of cells and tissues, with the highest concentrations in the liver (Turnlund, 1998). It functions as a cofactor and is required for structural and catalytic properties of a variety of important enzymes and hormones (Gaetke and Chow, 2003; Turnlund, 1999; Uauy et al., 1998). The accumulation of large amounts of copper in cells and organs can be toxic. The toxicity of copper has been linked with reactive oxygen species (ROS) whose formation is catalyzed by free copper ions (or certain complexes) that can occur when the ability of the cells to store excess copper in a benign form has been exceeded (Linder, 2001). Copper acts as a catalyst in the formation of ROS and catalyzes the peroxidation of membrane lipids (Chan et al., 1982); it is known to be as effective or even more effective than iron in causing DNA damage (Ozawa et al., 1993), protein or peptide modification (Uchida and Kawakishi, 1990), and oxidation of low-density lipoproteins (Esterbauer et al., 1990).

The genotoxicity of copper compounds has been reported mainly *in vitro*. Positive results were reported for the Syrian hamster embryo by the cell trans-

formation/viral enhancement assay (Heidelberger et al., 1983), and in HL-60 cells (Ma et al., 1998). Guecheva et al. (2001) have shown *in vivo* genotoxicity in a freshwater planarian by the comet assay. However, negative results have also been observed by the recombinational assay in DNA repair-deficient *B. subtilis* (Leifer et al., 1981) in *Glycine max* by examining gene mutations (Vig, 1982), by studying mitotic recombination and gene conversion in *S. cerevisiae* (Zimmermann et al., 1984). An *in vivo* study by Saleha Banu et al. (2004) in mice showed that although copper genotoxicity demonstrated a clear dose-dependent response pattern, it gradually decreased from 48 h post-treatment, returning to control levels 2 weeks after treatment.

Since the genotoxicity of copper is controversial, further clarification is needed in order to address the potential toxicity of this compound. Therefore, the aim of the present work was to evaluate the genotoxicity and the mutagenicity of copper sulfate in rabbits *in vivo* by applying a subchronic exposure schedule of 6 days.

## MATERIALS AND METHODS

### *Animals*

Ten male New Zealand rabbits, purchased from the Animal House of the Agricultural Faculty, Trakia University, 5 months old, weighing 3.5-4.0 kg, were used for this study. The experimental protocol was approved by the Department of Animal Care and adhered to the European Community Guiding Principles for the Care and Use of Animals. The animals were fed on a standard rabbit diet, had access to water *ad libitum*, and were synchronized by maintaining controlled environmental conditions (light, temperature, feeding time, etc.) for at least two weeks prior to and throughout the experiments.

### *Treatment and blood sampling*

Two groups of five rabbits were used in the experiment. Each group was gavaged (10 ml/1000 g of body weight) with a single daily dose of either water (control group) or CuSO4 ($5H_2O$), (purity >98%); 7.5 mg Cu/kg body weight (experimental group), for six consecutive days. The dose of copper was about 12% (Canton et al., 1989) of the rabbit oral $LD_{50}$. The substance was prepared just before treatment and protected from light. For cytogenetic analyses, blood (4 ml) was obtained on the $7^{th}$ day after the beginning of the experiment from the ear marginal vein in sterile tubes with heparin (30 U/ml).

### *Culture set up for chromosome aberrations*

The method of Evans (1984) with a modification for rabbits was applied. Briefly, 0.5 ml of experimental and control whole heparinized blood were incubated in 7.0 ml (RPMI-1640) medium containing 3.0 ml heat-inactivated fetal calf serum, 0.2 ml reconstituted phytohemagglutinin-M (PHA-M), 100 units/ml penicillin, and 50 μg/ml gentamicin. The cultivation flasks were placed in a thermostat in the dark at 39°C. Each sample group included two cultivation flasks from each donor. All cultures were incubated for 72 h. Colcemid at a final concentration of 0.2 μg/ml was added to the cultures for the final 2 h. At the end of the 72 h from the beginning of lymphocyte incubation, chromosomal preparations for the detection of chromosomal aberrations were established. The slides were stained with 10% Giemsa (Merck), and mounted. The number of aberrations was observed under an Olympus microscope using a 100x oil immersion objective. A cell was considered aberrant if it had one or more CA in complete metaphase. In each group a total of 500 cells (100 cells from each donor) were scored. The results are presented as the number of chromosome aberrations/100 cells.

### *Culture set up for sister chromatid exchanges (SCE)*

For sister chromatid exchanges, 5-bromodeoxyuridine (10 μg/ml, Sigma) was added 24 h after setting up the cultures. Cells were harvested after 72 h. Slides were prepared by the air drying method and stained with Hoechst 33258 and 4% Giemsa, following the method of Perry and Wolff (1974). For calculating the frequency of SCE per cell, 60 or 30 metaphases were analyzed as per international practice.

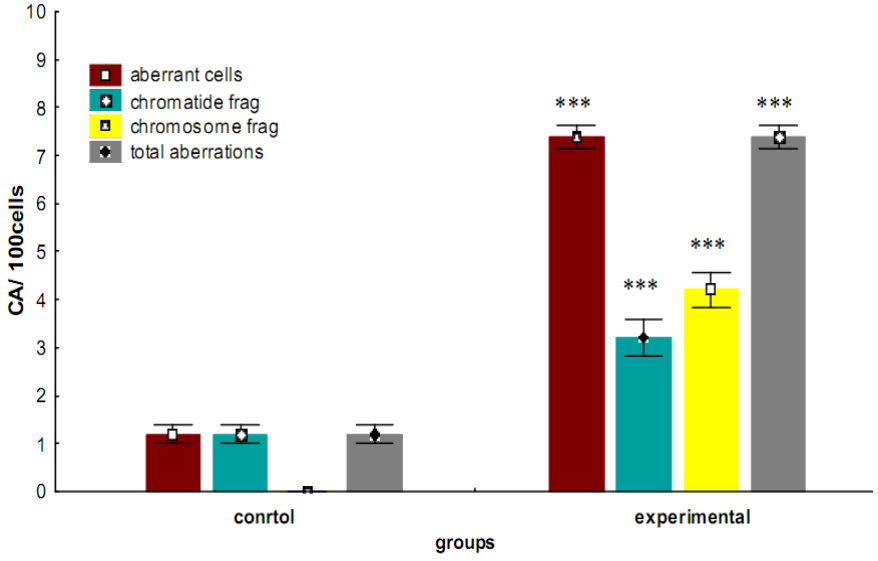


**Fig. 1.** Frequencies of chromosome aberrations (CA) in rabbit blood lymphocytes in control and experimental (treated with $CuSO_4$) groups. Data are presented as mean ±SD; n=5; (100 metaphases per rabbit). *** p<0.001 in comparison with control group.

All chemicals were obtained from Sigma-Aldrich Inc., (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

### *Statistical analysis*

The results were expressed as mean ± standard deviation (SD) for five animals in each group. Differences between the groups were assessed by Student's t- test. P-values <0.05 were considered significant.

## RESULTS

The scoring of chromosome aberrations allows a direct assessment of the mutagenicity of various physical and chemical agents. The results of determining the frequencies of chromosome aberrations (CA) in blood lymphocytes from control and experimental (CuSO4-treated rabbits) groups are presented in Fig.1. A significant increase in the frequencies of aberrant cells (7.4±0.24, P<0.001) and CA (chromatid fragments 3.2±0.37, chromosome fragments 4.2±0.37) and total aberrations (7.4±0.24) was observed after treatment with CuSO4 in comparison with the control group.

The results from the determination of sister chromatid exchange analysis (SCE) are presented in Table 1. SCE is a rapid method of observing reciprocal exchanges between sister chromatids and it is one of the methods to evaluate genotoxicity in the environment. The level of SCE per cell in the CuSO4-treated rabbits (9.66±0.062) was significantly higher than in rabbits from the control group. The frequencies of SCE/cell induced by $CuSO_4$ treatment exceed by 85% the frequencies of SCE/cell in the control group.

The presented results show subchronic exposure of rabbits to copper was genotoxic and mutagenic.

## DISCUSSION

One of the most accepted explanations for copper-induced cellular toxicity comes from the assumption that copper ions are prone to participate in the formation of ROS (Gaetke and Chow, 2003). Cupric and cuprous copper ions can act in oxidation and reduction reactions. The cupric ion (Cu(II)), in the presence of biological reductants such as ascorbic acid or GSH, can be reduced to cuprous ion (Cu(I)),

**Table 1.** SCE in rabbit blood lymphocytes from control and experimental (treated with $CuSO_4$) groups.

| Groups/ No of rabbits | Observed methaphases | SCE total | SCE per cell |
|---|---|---|---|
| control | | | |
| 1 | 60 | 352 | 5.41 |
| 2 | 60 | 303 | 5.05 |
| 3 | 60 | 321 | 5.35 |
| 4 | 60 | 311 | 5.18 |
| 5 | 60 | 309 | 5.15 |
| mean±SE | | 309.2±8.69 | 5.22±0.066 |
| Experimental | | | |
| 6 | 30 | 285 | 9,5 |
| 7 | 30 | 286 | 9,53 |
| 8 | 30 | 293 | 9,76 |
| 9 | 30 | 292 | 9,73 |
| 19 | 30 | 294 | 9,8 |
| mean±SE | | 290.0±1.87 | 9.66±0..62*** |

***P < 0.001 in comparison with control group (Student's t-test)

which is capable of catalyzing the formation of reactive hydroxyl radicals (•OH) through decomposition of hydrogen peroxide via the Fenton reaction (Lloyd et al., 1997b). The hydroxyl radical is very reactive and can further react with practically any biological molecules in the near vicinity via hydrogen abstraction, leaving behind a carbon-centered radical, as observed during the formation of a lipid radical from unsaturated fatty acids (Buettner and Jurkiewicz, 1996). Copper is also capable of inducing DNA strand breaks and the oxidation of bases via oxygen free radicals (Brezova et al., 2003). Copper in both oxidation states (cupric or cuprous) was more active than iron in enhancing DNA breakage induced by the genotoxic benzene metabolite 1,2,4-benzenetriol. DNA damage occurred mainly by a site-specific Fenton reaction (Brezova et al., 2003). The copper system also generated significant levels of DNA lesions. Given the lack of correlation between DNA strand breaks and bulky adducts, the authors concluded that it was likely that the lesions arise via two different mechanisms, perhaps involving the association of transition metal ions with different regions of the double helix and different moieties of individual nucleotides (Lloyd et al. 1997a).

The ingestion of trace nutrients increases as a result of the consumption of enriched foods, as well as multiple vitamin-mineral supplement tablets and the consumption of high level of nutrients as supplements in not unusual (Troppmann et al., 2002). Some ideas about the physiology of metals have being questioned and there is growing evidence that copper might be associated to DNA through *in situ* reactions, leading to genome damage (Tkeshelashvili et al., 1991; Meneghini, 1997). Further studies on the physiology of copper are still needed before we can make more definitive statements.

## CONCLUSION

The results from our experiment with rabbits show significant *in vivo* genotoxicity and mutagenicity of copper at the high dose tested. It remains to be examined whether similar effects would be observed at lower doses than those tested. It is essential to elucidate the correct dosages of copper to improve health without leading to noxious effects under various metabolic situations.

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