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REGULAR ARTICLE

IN VITRO EFFECTS OF COPPER ON THE MOTILITY AND VIABILITY OF SPERMATOZOA

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ABSTRACT

Copper (Cu) is an environmental risk factor which has various effects on the animal and human organism. The target of this study was to investigate the effects of Cu on motility and viability of spermatozoa *in vitro*. Specifically, we examined the dose- and time-dependent effect of copper (II) chloride ($CuCl_2$) on the survival of spermatozoa during different time periods (Time 0 h, 1 h, 2 h). The percentage of motile spermatozoa was determined after exposure to concentrations of 3.9; 7.8; 15.6; 31.2; 62.5; 125; 250; 500; 1000 $\mu mol.dm^{-3}$ of $CuCl_2$ using the Sperm Vision^{TM} CASA (Computer Assisted Semen Analyzer) system. The cell viability was measured by the MTT (metabolic activity) assay. The initial spermatozoa motility showed slightly increased values at doses < 31.20 $\mu mol.dm^{-3}$ of $CuCl_2$ compared to the control group. In this time, the lowest spermatozoa motility was recorded significantly ($P<0.001$) in the group A using the highest dose of $CuCl_2$ (1000 $\mu mol.dm^{-3}$). After 1 h of cultivation we proved that the average motility values decreased proportionally to the increasing concentration of $CuCl_2$. The low doses (< 7.80 $\mu mol.dm^{-3}$) of $CuCl_2$ increased the spermatozoa motility and concurrently of mitochondrial activity (Time 2 h). The obtained data confirm that Cu (in the form $CuCl_2$) at high doses acts as a toxic element on the

spermatozoa motility and it has a destructive effect on the mitochondrial complex, which is necessary for their life processes. The low concentrations (< 7.80 μmol.dm^{-3}) of $CuCl_2$ stimulated the mitochondrial activity of cells and maintained of spermatozoa motility during the short-term of cultivation.

Keywords: copper chloride, bovine spermatozoa, motility, viability

INTRODUCTION

Copper (Cu) is an important microelement for the animal and human organism, because it has a great positive role in physiological and regulatory processes **(Dobrzanski *et al.*, 1996).** It is involved in numerous biological processes, especially as an integral part of enzymes (ferroxidases, tyrosinase, lysyloxidase) **(Massányi *et al.*, 2003)** and thus it functions like a cellular respiration (cytochrome-c-oxidase) and antioxidant defence (Cu/Zn superoxide dismutase) in the body **(Agarwal *et al.*, 1990)**. Its deficiency and toxicity may lead to physiological abnormalities and Cu concentration in the body is connected to its concentration in food and environment **(Kabata-Pendias and Pendias, 1993).**

Spermatozoa are extremely sensitive to different effects which may lightly disturb spermatogenesis and consequently lead to a decrease in spermatozoa quality and production. Copper in ionic form rapidly becomes toxic to a variety of cells **(Eidi *et al.*, 2010),** including human spermatozoa **(Wong *et al.*, 2001).** High concentration of Cu usually has a harmful consequence on the reproductive system, which is connected with the structure of testes and ovaries as well as spermatozoa function **(Máchal *et al.*, 2002)**. The negative effect on the reproductive organs of males and females may ultimately lead to a reduced fertility **(Pesch *et al.*, 2006)**. Copper chelation complexes suppress spermatogenesis **(Oster and Salgo, 1979)** and their high concentrations have a toxic effect on the spermatozoa motility **(White and Rainbow, 1985; Viarengo *et al.*, 1996).** The aim of the present study was to evaluate the dose- and time-dependent effects of this essential metal on the motility and viability of spermatozoa during different time periods. Furthermore, it was also necessary to examine the possible cytotoxicity effect of copper (II) chloride ($CuCl_2$) on these cells.

MATERIAL AND METHODS

Semen samples and *in vitro* culture

Bull semen samples were obtained from 3 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic criteria given for the corresponding breed. The semen was obtained on a regular collection schedule using an artificial vagina. After collecting the samples, they were stored in the laboratory at room temperature (22-25°C). Each sample was diluted in physiological saline solution (PS) (sodium chloride 0.9% w/v, Bieffe Medital, Italia), using a dilution ratio of 1:40, depending on the original spermatozoa concentration. The cells were incubated with various concentrations of Cu (group I – 3.9; H – 7.8; G – 15.6; F – 31.2; E - 62.5; D - 125; C - 250; B - 500; A - 1000 $\mu mol.dm^{-3}$), in the form of copper (II) chloride ($CuCl_2$; Sigma-Aldrich, St. Louis, USA). Spermatozoa were cultivated in the laboratory at room temperature (22-25°C). We compared the control (Ctrl) group (medium without $CuCl_2$) with the experimental groups (exposed to different concentrations of $CuCl_2$).

Spermatozoa motility

The motility analysis was carried out using a CASA (Computer Assisted Semen Analyzer) system – SpermVision^{TM} program (MiniTűb, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, Japan) at cultivation Times 0 h, 1 h and 2 h. Each sample was placed into the Makler Counting Chamber (deph 10 µm, Sefi-Medical Instruments, Israel) and was evaluated percentage of motile spermatozoa (motility > 5 $\mu m.s^{-1}$; MOT). This study was performed in three replicates at each concentration (n = 8).

Cytotoxicity evaluation

The viability of the cells exposed to Cu *in vitro* was evaluated by the metabolic activity (MTT) assay **(Mosmann, 1983; Byun *et al.*, 2008)**. This colorimetric assay measures the conversion of a yellow tetrazolium salt (3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) to purple formazan particles by mitochondrial succinate dehydrogenase (mitochondrial reductase) of intact mitochondria of living cells (Figure 1). Formazan can then be measured spectrophotometerically. In brief, the cultured

$3.52x10^9$cells.mL^{-1} (in 200 µL medium per well) in 96-well plates (MTP, Greiner, Germany) were stained with MTT tetrazolium salt (Sigma, St. Louis, USA). MTT was dissolved in PBS (Dulbecco´s Phosphate Buffer Saline, Sigma, St. Louis, USA) at 5 mg.mL^{-1} and added to the cells (in 20 µL per well). After Time 0 h, 1 h and 2 h incubation (37 ºC), the cells and the formazan crystals were dissolved in 80 µL of isopropanol (2-propanol, p.a. CentralChem, Bratislava). The optical density was determined at a measuring wavelength of 570 nm against 620 nm as reference by a microplate ELISA reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were expressed in percentage of control (i.e., optical density of formazan from cells not exposed to $CuCl_2$). Results from the analysis were collected during two repeated experiments at each concentration (n = 8) **(Kňažická *et al.*, 2012).**

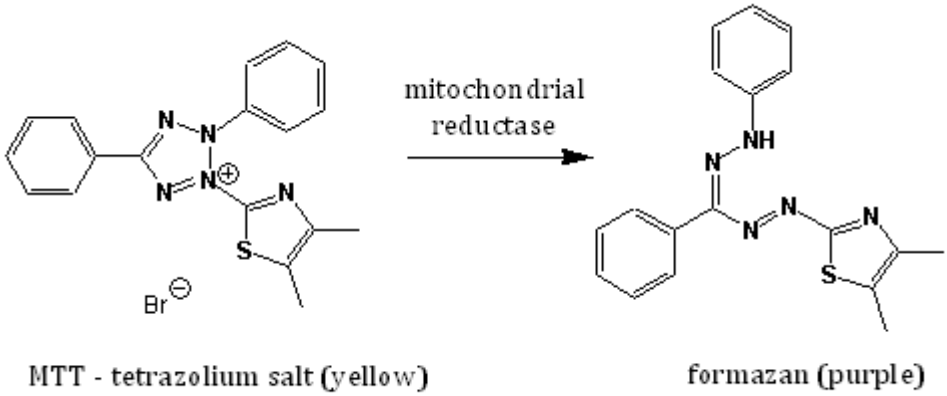

Figure 1 Reduction of the tetrazolium salt (3-(4,5-dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) to the formazan.

Statistical analysis

Obtained data were statistically analyzed by PC program GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical characteristics (mean, minimum, maximum, standard deviation and coefficient of variation) were evaluated. One-way analysis of variance (ANOVA) and the Dunnett´s multiple comparison test were used for statistical evaluations. The level of significance was set at *** (P<0.001); ** (P<0.01); * (P<0.05).

RESULTS AND DISCUSSION

Male fertility can be impaired by various toxicants. Effects may be at different stages of the cell cycle such as during meiotic disjunction and such abnormalities can have deleterious effects on reproductive system. Exposure to metals is long associated with low spermatozoa motility and density, increased morphological anomalies can cause male infertility. The toxic effects of different metals depend on dose, duration, route of administration and animal species **(Mathur *et al.*, 2010).** Therefore, our experiment evaluated dose- and time-dependent effects of $CuCl_2$ on the motility and viability of spermatozoa.

The initial spermatozoa motility showed slightly increased values at doses < 31.20 $\mu mol.dm^{-3}$ of $CuCl_2$ compared to the control group. Results are shown in the Table 1. The lowest spermatozoa motility was recorded in the group A ($P<0.001$) using the highest dose of $CuCl_2$ (25.20±7.50%). After 1 h of cultivation we proved that the selected parameter decreased proportionally to the increasing concentration of this trace element. In this time, the experimental administration at the doses < 15.60 $\mu mol.dm^{-3}$ of $CuCl_2$ stimulated the percentage of motile spermatozoa. The low concentrations increased the average motility values also during Time 2 h, especially in the groups H and I compared to control group (85.69% and 89.17% versus 82.77%). However, the other concentrations (> 31.20 $\mu mol.dm^{-3}$ of $CuCl_2$) significantly ($P<0.001$) decreased the overall percentage of spermatozoa motility. The highest inhibitory effect on the selected parameter after 2 h was observed in groups A (2.54±1.44%) and B (2.35±0.96%), which was probably caused by the toxicity of these concentrations.

Table 1 Spermatozoa motility (%) exposed to $CuCl_2$ in different time periods

Groups	**Control Ctrl**	**1000 A**	**500 B**	**250 C**	**125 D**	**62.50 E**	**31.20 F**	**15.60 G**	**7.80 H**	**3.90 I**
					$CuCl_2$ (μmol.dm^{-3})					
0 h										
x	90.50	25.20^{A}	65.63^{A}	77.73^{A}	79.51^{A}	86.24	91.66	92.83	93.08	92.33
minimum	76.08	20.06	58.92	74.41	58.92	82.60	82.55	86.44	91.00	85.05
maximum	98.09	38.00	68.96	83.33	88.43	89.69	98.07	97.61	95.87	98.14
S.D.	6.29	7.50	4.28	3.01	9.95	3.37	3.66	2.70	1.84	3.46
CV (%)	6.95	29.75	6.53	3.87	12.51	3.87	3.99	2.90	1.98	3.74
1 h										
x	89.90	10.00^{A}	20.89^{A}	37.86^{A}	69.99^{A}	70.31^{A}	80.26^{C}	90.25	90.38	91.78
minimum	86.50	5.76	10.34	31.25	57.57	44.18	49.12	78.26	83.33	81.81
maximum	93.94	15.21	38.18	47.94	82.60	88.67	93.51	100.0	96.26	100.0
S.D.	2.05	3.17	9.50	5.19	8.56	17.67	14.57	4.71	3.52	3.76
CV (%)	2.28	31.78	45.49	13.70	12.23	25.14	18.15	5.17	3.90	4.10
2 h										
x	82.77^{A}	2.54^{A}	2.35^{A}	26.11^{A}	35.23^{A}	47.63^{A}	61.47^{A}	76.54^{C}	85.69	89.17
minimum	72.46	1.03	1.44	17.39	26.47	44.18	43.47	72.22	78.00	75.55
maximum	88.23	5.55	3.57	30.23	45.10	50.00	77.77	82.92	92.98	95.23
S.D.	5.104	1.44	0.96	4.97	7.71	2.19	9.04	3.55	5.00	4.87
CV (%)	6.17	56.72	40.71	19.03	21.89	4.59	14.70	4.63	5.84	5.46

Legend: x – mean, S.D. – standard deviation, CV (%) – coefficient of variation

$^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Roychoudhury and Massányi (2008) examined parameters of spermatozoa motility during three time periods (0 h, 1 h, 2 h*) in vitro*. Observed data from their study demonstrated negative influence of $CuSO_4$ on semen motility and subsequently confirmed changes in male reproductive functions. Our experiment indicates similar results and also confirms the idea that Cu (in the form of $CuCl_2$) is a toxic element on spermatozoa motility at high. However, the low concentrations (< 15.60 μmol.dm^{-3} of $CuCl_2$) have a positive effect on spermatozoa motility during the short-term *in vitro* cultivation. Similar results were also observed in our previous study with copper sulphate on the bovine spermatozoa motility **(Kňažická *et al.*, 2010; 2012)**. **Rebrelo et al. (1996)** studied the effect of Cu^{2+} on the motility, viability, acrosome reaction and fertilization capacity of human spermatozoa *in vitro*. Motility, viability and acrosome reaction in spermatozoa incubated for 5 h were significantly affected by Cu^{2+} at a concentration of 100 μg.mL^{-1}, but not at lower concentrations. Incubation for 24 h did not affect the motility and viability of spermatozoa incubated in the presence of Cu^{2+} ranging from 10 ng.mL^{-1} to 10 μg.mL^{-1}, but the concentration of 100 μg.mL^{-1} caused a significant decrease of both parameters.

Motility of spermatozoa depends on mitochondrial activity **(Aziz *et al.*, 2005; Aziz, 2006).** The viability of cells after being exposed to Cu^{2+} we assessed by the MTT assay, which is reduced by the mitochondrial enzyme succinate dehydrogenase **(Mosmann, 1983)**,

resulting in purple formazan crystals which selectively accumulate in viable cells (Figure 2). **Slater *et al.* (1963)** described how this enzyme is involved in the reduction of MTT. They have shown that MTT is reduced by the dehydrogenase via the NADH-cytochrome oxidase systems.

Results of the present study indicated a significant (P<0.001) decrease of spermatozoa viability in experimental groups from A to F (at doses > 31.20 μmol.dm^{-3} of $CuCl_2$) in Time 0 h (Figure 2). These concentrations decreased the mitochondrial activity or enzymatic complex, which includes the production of ATP. Subsequently the cells were not able to utilize energy, which is necessary for their functions. The highest survival of cells was determined at low doses of $CuCl_2$ (< 15.60 μmol.dm^{-3}), while these concentrations significantly (P<0.001) supported spermatozoa mitochondrial activity. The mitochondrial ATP synthesis with using of the ATPase complex transport energy into the cells. This metabolic energy is located in the connecting section of spermatozoon flagellum and in the seminal plasma **(Katayose *et al.*, 2004)**, and is required for a wide range of spermatozoa functions, particularly for the motility support **(Breuer and Wells, 1977; Miki, 2007)**. After 1 h and 2 h of cultivation we proved that the survival of cells decreased proportionally to the increasing concentration of $CuCl_2$. The experimental administration at the doses < 7.80 μmol.dm^{-3} stimulated mitochondrial activity and concurrently maintained motility of spermatozoa during the short-term *in vitro* of cultivation. Spermatozoa are very active cells and rich in mitochondria, therefore, the reduction of MTT by spermatozoa is faster that other cells **(Byun *et al.*, 2008)**. The sperm viability is positively related to sperm quality parameters like acrosome integrity, mitochondrial activity and even these parameters also correlate positively with fertility **(Garner *et al.*, 1997)**.

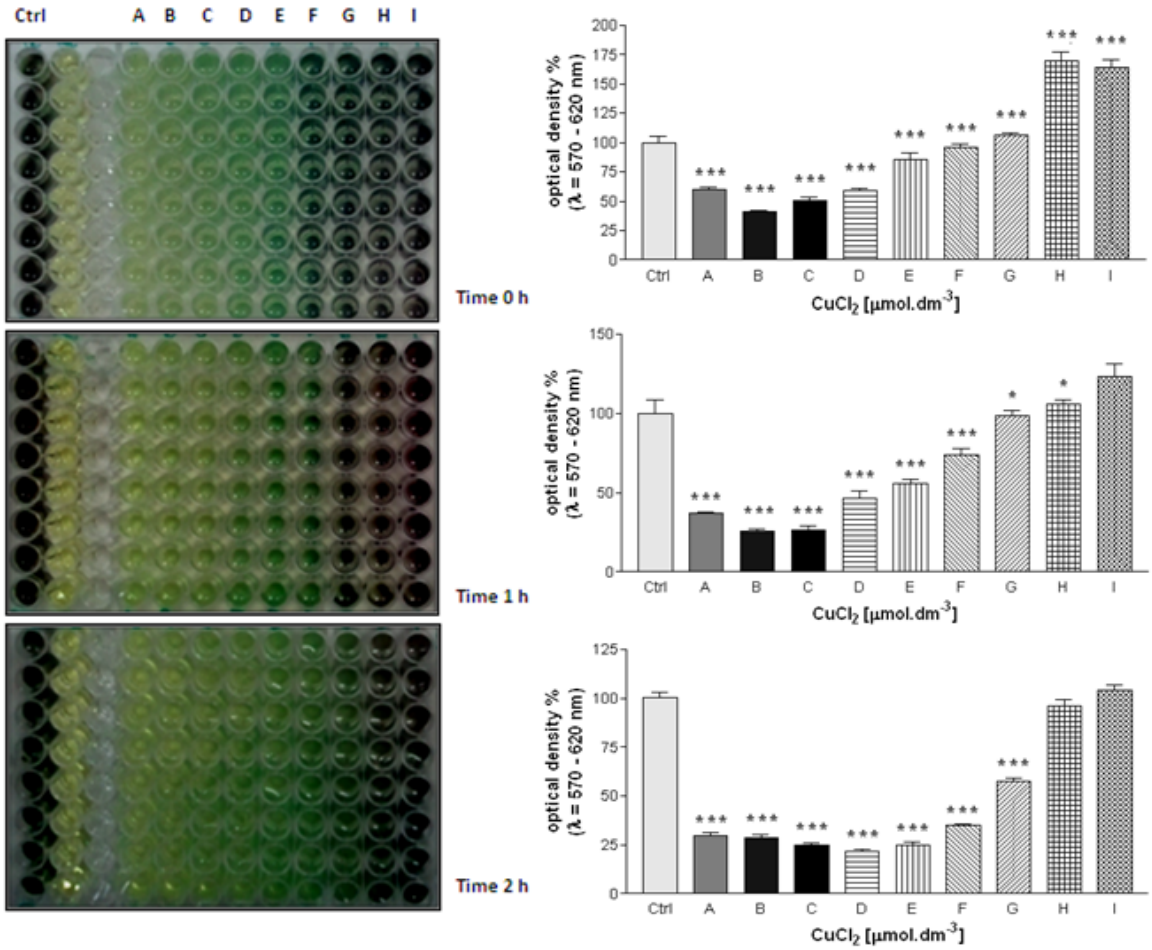

Figure 2 Effect of various doses of copper ($CuCl_2$) on the viability of spermatozoa during different time periods.

Legend: Each bar represent the mean (±SD) optical density as percent of controls (n = 8), which represented 100%. The control group (Ctrl) received a culture medium without $CuCl_2$ administration. Group A – 1000 µmol.dm^{-3}; group B - 500 µmol.dm^{-3}; group C – 250 µmol.dm^{-3}; group D - 125 µmol.dm^{-3}; group E – 62.5 µmol.dm^{-3}; group F – 31.2 µmol.dm^{-3}; group G – 15.6 µmol.dm^{-3}; group H – 7.8 µmol.dm^{-3}; group I - 3.9 µmol.dm^{-3} of $CuCl_2$. Statistical difference between the values of the control and treated spermatozoa in indicated by asterisks (Dunnett´s multiple comparison test). $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$. MTT assay.

CONCLUSION

The obtained data from this *in vitro* study confirm the dose- and time-dependent effect of copper on the motility and viability of spermatozoa. Our results point out that copper (in the form $CuCl_2$) at high doses (> 250 µmol.dm^{-3} of $CuCl_2$) clearly damages the mitochondrial complex and concurrently decrease of the spermatozoa motility. Additionally, we found that the low concentrations (< 7.80 µmol.dm^{-3} of $CuCl_2$) stimulated the mitochondrial activity of

cells (confirmed by the MTT assay) and maintained of spermatozoa motility during the short-term of cultivation. Based on these results, we can concluded that the copper at the low concentrations significantly involved in the stimulated of cytochrome oxidase system. Copper at low doses can use in biotechnological processes in fertilization techniques (IVF, ICSI).

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