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RELATIONSHIP BETWEEN COPPER IN DIFFERENT CULTURE MEDIA AND BOVINE SPERMATOZOA MOTILITY PARAMETERS *IN VITRO*

Zuzana Kňažická[1], Eva Tušimová[1], Jana Bezáková[1], Michal Miškeje[1], Tatiana Bojňanská[1], Norbert Lukáč[2]*

Address(es): MSc. Zuzana Kňažická, Ph.D.
[1]AgroBioTech Research Centre, Slovak University of Agriculture, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovak republic, phone number: + 421-37-6414903.
[2]Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Animal Physilogy, Tr. Andreja Hlinku 2, 949 76 Nitra, Slovak republic.

*Corresponding author: zuzanaknazicka25@gmail.com

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ABSTRACT

The target of this *in vitro* study was to investigate the dose- and time-dependent effects of copper ($CuSO_4.5H_2O$) in different culture media on the spermatozoa motility and to provide additional information on the interaction between serum albumin and copper ions. The spermatozoa motility was determined after exposure of $CuSO_4.5H_2O$ (3.90; 7.80; 15.60; 31.20; 62.50; 125; 250; 500; 1000 $\mu mol.dm^{-3}$) using the SpermVision™ CASA system (Computer Assisted Semen Analyzer) during different time periods (Time 0 h, 1 h, 2 h, 24 h). The initial percentage of motility spermatozoa in the presence $CuSO_4.5H_2O$ in physiological saline solution (PS) showed significantly ($P<0.001$) decreased values at high concentrations $\geq$ 250 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$. The low concentrations ($\leq$ 7.80 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$) maintained of spermatozoa motility (Time 2 h). The long-term cultivation significantly ($P<0.001$) reduced the average motility values in all experimental groups compared to the control group (medium without $CuSO_4.5H_2O$). The commercial medium (CM) containing triladyl, egg yolk and redistilled water increased the overall percentage of spermatozoa motility after exposure of high doses of $CuSO_4.5H_2O$, but only during short-time periods. The culture medium in composition of 20.0 % bovine serum albumin (BSA), triladyl, 5.0 % glucose and redistilled water maintained the spermatozoa motility in all experimental groups (Time 0 h, 1 h). Evaluation of the overall percentage of spermatozoa motility showed significant ($P<0.001$) decrease at high concentrations $\geq$ 500 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$ after 2 h and after 24 h of cultivation when exposed to doses $\geq$ 125 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$. The obtained data point out that copper at high doses acts as toxic element on the spermatozoa motility parameters. However, using a suitable culture medium containing an energy component- and protein-rich substrate, the spermatozoa motility could increase.

Keywords: copper, culture media, bovine spermatozoa, motility parameters

INTRODUCTION

Copper (Cu) is an important trace and essential element for the all organisms, because has a great positive role in physiological and regulatory processes **(Dobrzanski *et al.*, 1996; Tang, 2005).** Moreover, it is a component of a number of metalloenzymes and metalloproteins **(Agarwal *et al.*, 1990).** Copper plays an essential role in in spermatogenesis and fertility **(Wong *et al.*, 2001).** The excessive Cu intake has a negative effect on the organs of reproduction **(Katayose *et al.*, 2004).** The high concentrations of copper ions (Cu^{2+}) have a toxic effect on the epididymis **(Xu *et al.*, 1985),** testes, scrotum of mammals **(Skandhan, 1992; Eidi *et al.*, 2010),** which may ultimately lead to a reduced fertility **(Pesch *et al.*, 2006).** Several experimental studies demonstrated the adverse effects of Cu^{2+} on spermatozoa motility **(White and Rainbow, 1985; Viarengo *et al.*, 1996; Wong *et al.*, 2001; Machal *et al.*, 2002; Roychoudhury *et al.*, 2010; Sakhaee *et al.*, 2011).** This element reduces oxidative processes and glucose consumption **(Skandhan, 1992),** consequently it minimizes or disrupts spermatozoa motility **(Chen *et al.*, 1989).**
Spermatozoa are extremely sensible to *ex vivo* conditions and on the loss of exogenous energy sources therefore different culture media are used on the viability prolongation of spermatozoa. Semen culture media usually contain glucose or fructose as the dominant energy substrate, egg yolk as a protein supply and glycerol **(Matsuoka *et al.*, 2006).** However, preparation of a uniform semen cultivation media varies because of the quality of the egg yolk. Bovine serum albumin (BSA) has been used recently as a protein alternative to egg yolk **(Peters *et al.*, 1975).** Serum albumin is a multifunctional protein, which forms covalent adducts with various metals (Cu^{2+}, Ni^{2+}, Hg^{2+}, Ag^{2+}, Au^{+}) **(Stamler *et al.*, 1992; Simion *et al.*, 2009).** It provides a range of benefits including protection from oxidative damage, stabilization of other media components (i.e fatty acids, pyridoxal) and inactivating various toxic lipophilic metabolites (i.e bilirubin) **(Emerson, 1989).** Recently several researchers investigated interactions between Cu^{2+} with serum albumin, due to the importance of Cu for various biological and chemical processes **(Anzai *et al.*, 1996; Schwarz *et al.*, 2000; Pinto *et al.*, 2008).** However, in this field there is still a lack of information about the influence of BSA as a culture medium component on the general spermatozoa viability. Therefore, the target of this *in vitro* study was to investigate the dose- and time-dependent effects of copper ($CuSO_4.5H_2O$) in different culture media on the spermatozoa motility parameters and to provide additional information on the interaction between serum albumin and copper ions.

MATERIAL AND METHODS

Biological material

Bovine semen samples (n = 58) were obtained from 6 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic quality criteria given for the corresponding breed. Results are shown in the Table 1. 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 semen sample was diluted in physiological saline solution (PS) (sodium chloride 0.9 % w/v, Bieffe Medital, Grosotto, Italia), using a dilution ratio of 1:40, depending on the original spermatozoa concentration.

Table 1 The basic parameters of bovine semen samples (n = 58)

Parameters	x±S.D.
pH	6.56 ± 0.20
Spermatozoa concentration ($x10^9.mL^{-1}$)	3.15 ± 0.96
Semen volume (mL)	6.23 ± 1.69
Osmolarity ($mOsmol.kg^{-1}$)	297.50 ± 4.67
The overall of spermatozoa motility (MOT; %)	92.85 ± 3.86

Legend: x – arithmetic mean, S.D. – standard deviation

***In vitro* exposure**

Semen samples (i.e cell sediment and seminal plasma fraction) were cultivated in different culture media (Table 2) with various concentrations of copper (group I – 3.90; H – 7.80; G – 15.60; F – 31.20; E - 62.50; D - 125; C - 250; B - 500; A - 1000 $\mu mol.dm^{-3}$), in the form of copper sulphate ($CuSO_4.5H_2O$; Sigma-Aldrich, St. Louis, USA). Spermatozoa with $CuSO_4.5H_2O$ were incubated in the laboratory at room temperature (22-25°C) during different time periods (Time 0 h, 1 h, 2 h and 24 h). We compared the control (Ctrl) group (medium without $CuSO_4.5H_2O$) with the experimental groups (exposed to different concentrations of $CuSO_4.5H_2O$).

Table 2 Culture media with a different composition used for the experiment

Culture medium	Composition
1. PS	physiological saline solution (sodium chloride 0.9 % w/v, Bieffe Medital, Grosotto, Italia)
2. CM	commercial medium - triladyl (MiniTüb, Tiefenbach, Germany), egg yolk and redistilled water
3. BSA	20.0 % BSA (bovine serum albumin, Sigma-Aldrich, St. Louis, USA), triladyl (MiniTüb, Tiefenbach, Germany), 5.0 % glucose (D-glukosamonohydratp.a, Penta, Chrudim, Czech Republic) and redistilled water

Spermatozoa motility analysis

The motility analysis was carried out using a CASA (Computer Assisted Semen Analyzer) system – SpermVision™ program (MiniTüb, Tiefenbach, Germany) with the Olympus BX 51 microscope (Olympus, Tokyo, Japan). Each sample was placed into the Makler Counting Chamber (deph 10 µm, Sefi-Medical Instruments, Haifa, Israel) and the following parameters were evaluated: percentage of motile spermatozoa (motility > 5 $\mu m.s^{-1}$; MOT); percentage of progressive motile spermatozoa (motility > 20 $\mu m.s^{-1}$; PROG); distance average path (DAP; µm) and velocity average path (VAP; $\mu m.s^{-1}$).

Statistical analysis

Statistical analysis of the results was carried out using the statistical program GraphPad Prism 3.02 (GraphPad Software Incorporated, San Diego, California, USA). Descriptive statistical characteristics (arithmetic 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 ^{A} ($P<0.001$); ^{B} ($P<0.01$); ^{C} ($P<0.05$).

RESULTS

Evaluation of spermatozoa motility exposed of copper in PS culture medium

The initial spermatozoa motility (Time 0 h) showed significantly ($P<0.001$) decreased values at concentrations ≥ 250 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$ in comparison with the control group. After 1 h of cultivation we proved that the average motility values significantly ($P<0.001$) reduced at the concentrations ≥ 62.50 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$ (Figure 1). The lowest spermatozoa motility was recorded ($P<0.001$) in the group A (34.33 ± 8.29 %) using the highest concentrations (1000 $\mu mol.dm^{-3}$) of $CuSO_4.5H_2O$. On the other hand, the low concentrations (≤ 7.80 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$) maintained of spermatozoa motility (Time 2 h), but the differences were not significant ($P>0.05$). The long-term cultivation (Time 24 h) significantly ($P<0.001$) reduced the average motility values in all experimental groups. Identical spermatozoa motility was detected also for the percentage of progressive motile spermatozoa (PROG; > 20 $\mu m.s^{-1}$) during all time periods (Table 3). Parameter of velocity average path (VAP; $\mu m.s^{-1}$) revealed that concentrations ≤ 7.80 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$ in short-term periods act stimulating on spermatozoa motility, but later (Time 24 h) inhibiting of selected parameter (Table 4). Evaluation of distance average path (DAP; µm), showed similar results as for VAP (Table 5).

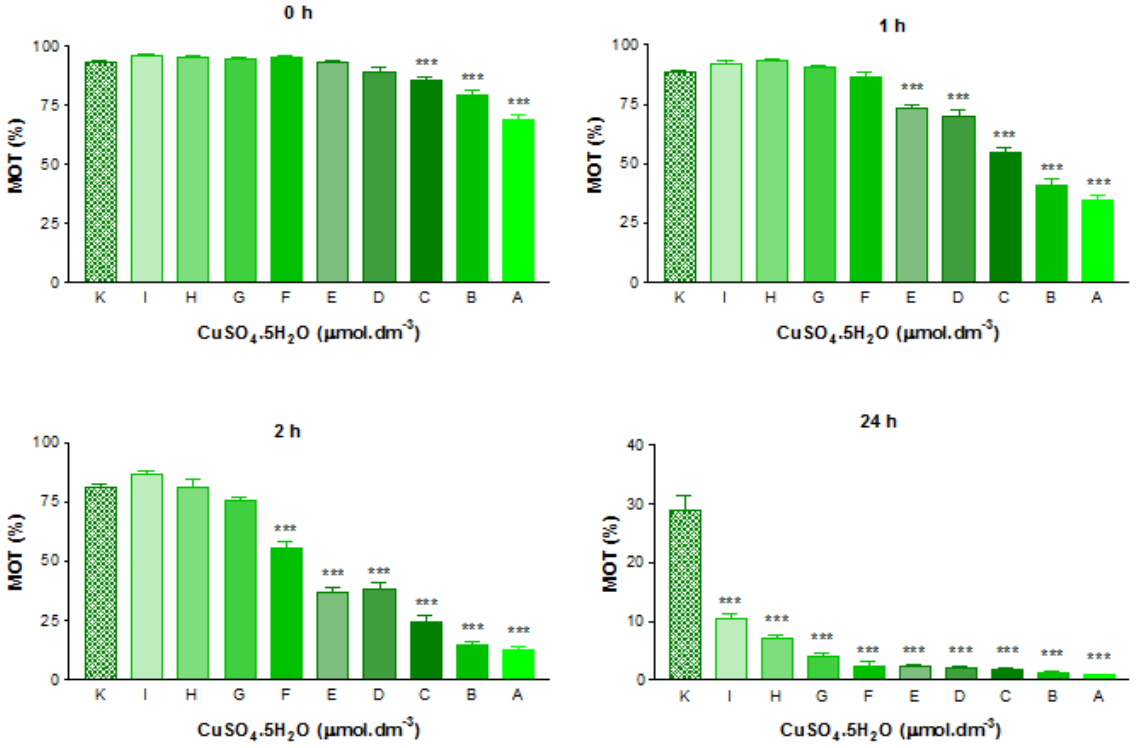

Figure 1 Spermatozoa motility (MOT; %) exposed to copper ($CuSO_4.5H_2O$) in PS culture medium during different time periods.

Legend: This study was performed in five replicates at each concentration (n = 8). The control group received a culture medium without $CuSO_4.5H_2O$ administration. Group A – 1000 $\mu mol.dm^{-3}$; group B - 500 $\mu mol.dm^{-3}$; group C – 250 $\mu mol.dm^{-3}$; group D - 125 $\mu mol.dm^{-3}$; group E – 62.50 $\mu mol.dm^{-3}$; group F – 31.20 $\mu mol.dm^{-3}$; group G – 15.60 $\mu mol.dm^{-3}$; group H – 7.80 $\mu mol.dm^{-3}$; group I - 3.90 $\mu mol.dm^{-3}$ of $CuSO_4.5H_2O$. $^{***}P<0.001$; $^{**}P<0.01$;$^{*}P<0.05$. Statistical difference between the values of the control group and treated spermatozoa in indicated by asterisks (Dunnett´s multiple comparison test).

Table 3 Progressive spermatozoa motility (PROG; %) exposed to copper ($CuSO_4.5H_2O$) in PS culture medium during different time periods.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500	1000
	Ctrl	I	H	G	F	E	D	C	B	A
					$CuSO_4.5H_2O$ (µmol.dm^{-3})					
Time 0 h										
x	90.13	93.08	92.66	90.84	92.36	89.20	86.32	81.95^{A}	72.83^{A}	62.20^{A}
minimum	81.35	88.57	85.07	84.33	87.06	77.94	60.24	69.04	60.58	44.08
maximum	95.38	99.65	98.00	95.80	94.63	96.33	97.70	92.37	81.45	76.56
S.D.	3.46	2.51	3.52	3.22	1.82	4.74	8.87	7.24	6.87	9.20
CV (%)	3.84	2.70	3.79	3.54	1.97	5.31	10.28	8.83	9.44	14.79
Time 1 h										
x	84.60	88.59	90.17	86.78	84.77	70.07^{A}	67.16^{A}	53.28^{A}	38.07^{A}	31.30^{A}
minimum	76.47	80.76	85.00	72.32	64.61	56.04	46.68	39.79	23.22	23.40
maximum	94.78	96.72	97.70	94.87	92.56	88.44	84.73	68.63	57.31	42.96
S.D.	4.23	5.23	3.58	6.40	6.53	9.81	14.21	10.69	11.51	7.07
CV (%)	4.99	5.90	3.97	7.38	7.70	14.01	21.16	20.06	30.24	22.58
Time 2 h										
x	80.13	84.36	79.58	72.54^{C}	52.25^{A}	30.53^{A}	36.13^{A}	22.42^{A}	9.86^{A}	10.56^{A}
minimum	75.00	72.40	48.48	63.24	40.84	20.22	26.82	18.86	7.48	7.94
maximum	87.38	91.24	89.92	86.47	66.84	41.19	42.85	31.19	15.60	14.28
S.D.	3.91	5.30	14.65	5.82	8.22	7.68	5.78	5.17	2.33	2.52
CV (%)	4.88	6.28	18.41	8.02	15.74	25.16	16.01	23.06	23.62	23.88
Time 24 h										
x	24.42	7.14^{A}	4.56^{A}	3.34^{A}	2.37^{A}	2.22^{A}	2.03^{A}	1.15^{A}	1.06^{A}	0.83^{A}
minimum	16.73	4.34	1.26	1.69	1.07	0.97	1.38	0.57	0.22	0.00
maximum	35.84	9.23	6.41	4.34	4.34	4.16	2.69	2.06	1.70	1.56
S.D.	5.71	2.02	1.72	1.17	1.34	1.20	0.66	0.42	0.76	0.65
CV (%)	23.39	28.17	37.73	34.96	56.72	53.97	32.33	36.34	71.70	77.72

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Table 4 Velocity average path (VAP; µm.s^{-1}) exposed to copper ($CuSO_4.5H_2O$) in PS culture medium during different time periods.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500	1000
	Ctrl	I	H	G	F	E	D	C	B	A
					$CuSO_4.5H_2O$ (µmol.dm^{-3})					
Time 0 h										
x	92.29	94.91^{C}	93.05^{C}	83.62	86.58	81.33^{A}	80.26^{A}	82.22^{A}	69.55^{A}	61.90^{A}
minimum	85.66	83.83	80.06	76.11	75.44	70.93	74.99	70.12	63.37	48.60
maximum	97.02	105.90	110.20	100.60	103.80	97.88	85.85	100.60	73.81	68.94
S.D.	4.08	6.25	9.26	6.37	8.20	5.69	3.41	9.65	3.53	5.50
CV (%)	4.42	6.59	9.95	7.62	9.47	7.00	4.25	11.74	5.07	8.89
Time 1 h										
x	81.03	89.76^{A}	85.44	79.65	71.40^{A}	59.08^{A}	58.84^{A}	45.37^{A}	32.11^{A}	26.54^{A}
minimum	63.61	76.54	80.17	71.44	50.27	46.59	40.61	37.24	16.75	14.03
maximum	89.81	105.80	92.49	87.94	84.92	76.06	75.64	54.57	48.88	43.31
S.D.	6.79	7.81	3.97	4.38	8.87	11.91	14.22	5.71	9.61	9.84
CV (%)	8.37	8.71	4.65	5.50	12.43	20.17	24.17	12.59	29.94	37.06
Time 2 h										
x	76.96	86.07	75.07	55.49^{A}	48.53^{A}	24.31^{A}	27.50^{A}	21.29^{A}	10.28^{A}	8.51^{A}
minimum	68.60	71.72	64.49	39.79	31.91	15.42	21.99	16.21	6.81	5.84
maximum	84.10	95.61	82.93	69.41	68.77	36.97	33.62	25.84	15.22	11.86
S.D.	5.48	8.23	5.13	11.87	11.34	7.57	5.84	4.84	3.51	3.07
CV (%)	7.12	9.56	6.83	21.40	23.38	31.15	21.24	22.72	34.17	36.01
Time 24 h										
x	26.76	14.23^{A}	9.98^{A}	5.80^{A}	1.35^{A}	2.32^{A}	1.47^{A}	0.00^{A}	0.00^{A}	0.00^{A}
minimum	16.44	13.22	7.47	4.18	0.89	0.49	1.05	0.00	0.00	0.00
maximum	33.38	16.08	15.73	8.08	2.15	4.47	2.46	0.00	0.00	0.00
S.D.	5.20	1.17	3.23	2.03	0.70	1.24	0.62	0.00	0.00	0.00
CV (%)	19.43	8.21	32.38	34.99	51.82	53.22	42.06	0.00	0.00	0.00

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Table 5 Distance average path (DAP; µm) exposed to copper ($CuSO_4.5H_2O$) in PS culture medium during different time periods.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500	1000
	Ctrl	I	H	G	F	E	D	C	B	A
					$CuSO_4.5H_2O$ (µmol.dm^{-3})					
Time 0 h										
x	35.04	38.65^{A}	36.30	35.15	34.79	33.41	31.32^{A}	31.91^{A}	28.63^{A}	22.45^{A}
minimum	28.44	34.99	33.04	31.43	29.56	25.63	26.90	25.48	27.44	14.12
maximum	41.07	43.52	42.66	43.50	42.23	41.94	40.72	41.30	29.81	27.73
S.D.	3.35	3.02	3.21	3.01	3.04	3.50	2.93	4.48	0.69	4.90
CV (%)	9.57	7.82	8.83	8.57	8.73	10.49	9.36	14.04	2.41	21.83
Time 1 h										
x	33.57	37.29^{A}	35.81	32.05	31.30	29.04^{A}	28.23^{A}	26.73^{A}	16.12^{A}	12.53^{A}
minimum	28.68	30.73	33.02	24.51	23.57	23.69	18.30	16.56	8.53	6.65
maximum	36.07	43.95	41.95	37.10	36.28	31.82	32.83	30.86	20.95	17.59
S.D.	2.04	3.27	2.61	3.74	3.22	2.46	5.96	5.25	4.27	3.63
CV (%)	6.07	8.78	7.29	11.67	10.30	8.47	21.11	19.63	26.51	28.95
Time 2 h										
x	31.07	32.25	28.75	28.00	22.88^{A}	14.09^{A}	17.32^{A}	10.21^{A}	6.04^{A}	2.15^{A}
minimum	20.81	19.07	18.34	12.83	12.61	8.43	10.38	7.83	2.40	1.33
maximum	35.74	38.39	35.51	34.20	27.24	18.82	24.49	12.62	8.37	2.89

S.D.	4.60	6.44	5.92	5.82	5.02	3.63	5.15	2.40	2.61	0,67
CV (%)	14.81	19.97	20.59	20.79	21.94	25.74	29.73	23.47	43.09	31.21
Time 24 h										
x	17.70	8.49^{A}	3.88^{A}	2.48^{A}	0.99^{A}	0.71^{A}	1.44^{A}	0.00^{A}	0.00^{A}	0.00^{A}
minimum	10.63	6.22	1.13	1.59	0.23	0.00	0.00	0.00	0.00	0.00
maximum	25.82	10.84	5.46	3.54	2.00	1.21	2.41	0.00	0.00	0.00
S.D.	4.73	1.96	1.89	0.84	0.74	0.48	0.92	0.00	0.00	0.00
CV (%)	26.73	23.06	48.78	33.81	75.03	68.38	63.87	0.00	0.00	0.00

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$;$^{C}P<0.05$

Evaluation of spermatozoa motility exposed of copper in CM culture medium

The commercial medium increased the overall percentage of spermatozoa motility after exposure of high doses of $CuSO_4.5H_2O$, but only during short-time periods (Figure 2). The percentage of motile spermatozoa decreased slowly after 2 h of cultivation at the concentrations ≥ 125 µmol.dm^{-3} of $CuSO_4.5H_2O$ ($P>0.05$). The lowest average motility values were detected after 24 h. The progressive spermatozoa motility showed similar results as for MOT (%) during all time periods (Table 6). Evaluation of the velocity average path (VAP) showed decrease of selected parameter (Time 2 h) at the concentrations 500 µmol.dm^{-3} ($P<0.05$) a 1000 ($P<0.001$) µmol.dm^{-3} of $CuSO_4.5H_2O$. In this time, the highest spermatozoa motility was recorded ($P<0.05$) in the group I (62.28 ± 6.01 %) (Table 7). Other dates were not significant in comparison with the control group. Similar results were detected also for the parameter of distance average path (DAP) (Table 8).

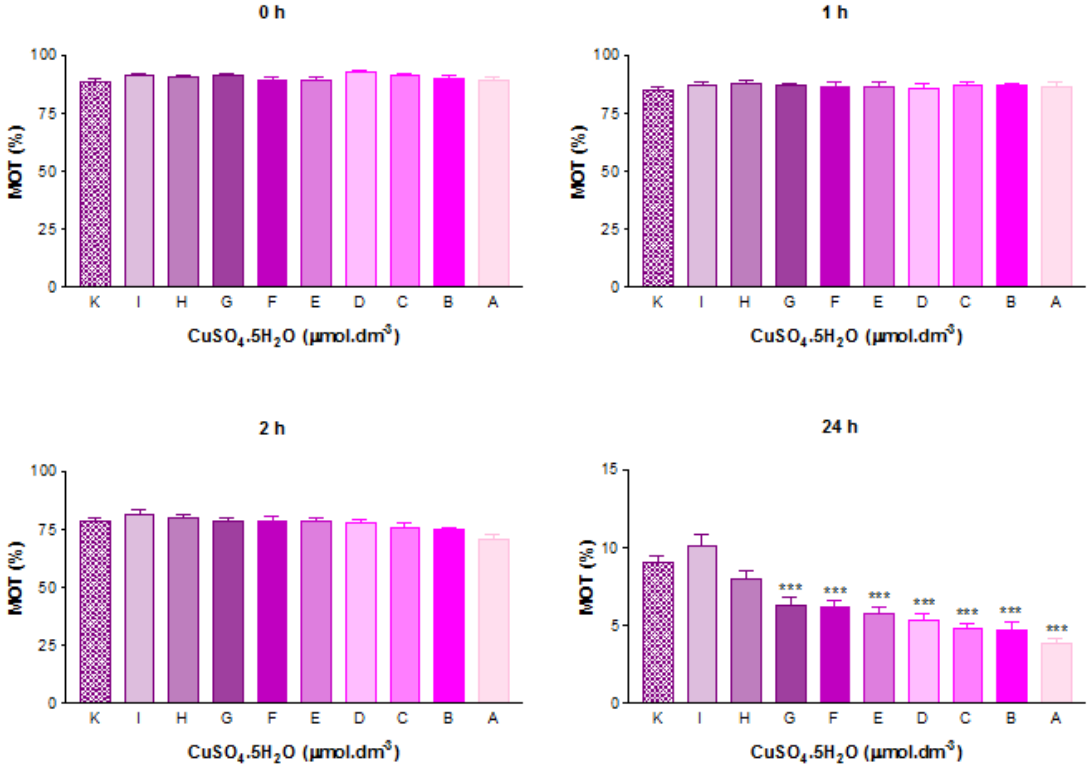

Figure 2 Spermatozoa motility (MOT; %) exposed to copper ($CuSO_4.5H_2O$) in CM culture medium during different time periods.
Legend: This study was performed in four replicates at each concentration (n = 8). The control group received a culture medium without $CuSO_4.5H_2O$ administration. Group A – 1000 µmol.dm^{-3}; group B - 500 µmol.dm^{-3}; group C – 250 µmol.dm^{-3}; group D - 125 µmol.dm^{-3}; group E – 62.50 µmol.dm^{-3}; group F – 31.20 µmol.dm^{-3}; group G – 15.60 µmol.dm^{-3}; group H – 7.80 µmol.dm^{-3}; group I - 3.90 µmol.dm^{-3} of $CuSO_4.5H_2O$. $^{***}P<0.001$; $^{**}P<0.01$;$^{*}P<0.05$. Statistical difference between the values of the control group and treated spermatozoa in indicated by asterisks (Dunnett´s multiple comparison test).

Table 6 Progressive spermatozoa motility (PROG; %) exposed to copper ($CuSO_4.5H_2O$) in CM culture medium during different time periods.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500	1000
	Ctrl	I	H	G	F	E	D	C	B	A
				$CuSO_4.5H_2O$ (µmol.dm^{-3})						
Time 0 h										
x	84.14	85.90	85.87	85.55	85.21	85.20	87.04	86.16	86.33	85.23
minimum	68.42	64.15	70.93	71.42	69.20	69.29	65.00	76.98	64.56	62.83
maximum	91.46	92.10	93.12	95.09	95.12	93.00	92.72	91.89	94.50	93.79
S.D.	5.92	5.56	4.59	6.03	6.33	7.05	5.71	3.94	6.37	8.88
CV (%)	7.03	6.48	5.35	7.05	7.43	8.28	6.55	4.57	7.38	10.42
Time 1 h										
x	78.48	81.61	82.74	81.47	83.00	82.93	81.83	80.78	80.12	80.05
minimum	67.69	61.40	60.00	68.18	64.15	61.34	59.16	65.38	60.19	60.71
maximum	90.06	96.20	92.12	92.18	95.19	93.43	91.86	92.10	90.00	89.20
S.D.	6.96	10.22	8.20	7.20	9.64	9.58	7.53	6.23	7.49	10.16
CV (%)	8.87	12.52	9.91	8.83	11.61	11.55	9.20	7.71	9.35	12.69
Time 2 h										
x	72.65	74.87	74.32	73.94	73.62	73.04	70.94	68.24	67.12^{C}	66.97^{C}
minimum	59.09	43.18	59.52	54.43	52.38	52.00	51.56	53.96	57.74	49.85
maximum	85.71	96.34	92.93	95.40	92.85	89.84	87.82	85.23	79.76	75.19
S.D.	8.20	16.93	9.97	13.19	13.66	12.69	12.48	10.36	6.57	7.99
CV (%)	11.29	22.62	13.42	17.84	18.55	17.37	17.59	15.18	9.78	11.94
Time 24 h										

x	8.18	9.11	6.56	5.93^{C}	5.86^{C}	3.91^{A}	3.63^{A}	2.94^{A}	2.10^{A}	2.00^{A}
minimum	5.20	6.14	4.13	4.07	3.25	2.51	1.17	1.73	1.37	0.81
maximum	10.20	11.97	9.78	9.33	7.69	5.63	5.35	4.26	2.98	2.70
S.D.	2.09	2.09	1.94	1.44	1.52	1.14	1.35	1.07	0.52	0.62
CV (%)	25.50	22.97	29.53	24.20	25.94	29.04	37.18	36.42	24.71	31.08

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Table 7 Velocity average path (VAP; μm.s^{-1}) exposed to copper ($CuSO_4.5H_2O$) in CM culture medium during different time periods.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500	1000
	Ctrl	I	H	G	F	E	D	C	B	A
				$CuSO_4.5H_2O$ (μmol.dm^{-3})						
Time 0 h										
x	78.67	80.91	80.42	80.08	79.14	79.20	78.99	78.14	77.70	77.83
minimum	70.71	70.14	70.67	73.02	70.12	68.16	70.99	71.18	70.46	68.97
maximum	91.11	93.27	89.57	89.53	89.91	88.98	92.15	89.57	89.34	84.63
S.D.	5.09	6.52	6.10	6.02	5.69	7.59	6.41	6.42	6.06	4.89
CV (%)	6.47	8.05	7.58	7.52	7.19	9.58	8.12	8.22	7.80	6.28
Time 1 h										
x	72.52	74.95	74.71	73.41	73.24	73.17	72.94	73.52	73.37	73.29
minimum	62.07	63.91	64.94	63.67	62.52	64.21	67.48	69.57	67.43	66.78
maximum	77.50	90.27	87.60	82.15	82.83	81.19	82.15	78.54	77.41	80.21
S.D.	4.68	7.39	6.37	6.39	6.17	6.11	4.51	2.71	3.16	4.32
CV (%)	6.45	9.86	8.53	8.70	8.42	8.35	6.18	3.68	4.30	5.89
Time 2 h										
x	57.93	62.28^{C}	59.48	59.36	59.08	59.34	56.22	54.17	53.50^{C}	51.52^{A}
minimum	50.65	50.71	51.27	48.56	50.25	50.42	49.61	46.46	40.95	41.11
maximum	64.86	72.95	71.12	70.10	70.10	69.12	60.49	61.49	59.32	58.40
S.D.	3.98	6.01	6.45	6.24	5.84	6.62	3.00	3.38	4.26	4.86
CV (%)	6.87	9.65	10.84	10.52	9.88	11.16	5.33	6.24	7.96	9.42
Time 24 h										
x	16.51	16.96	14.43	13.58	12.02^{A}	11.58^{A}	11.28^{A}	10.69^{A}	8.26^{A}	5.72^{A}
minimum	10.09	12.64	10.06	9.01	8.15	7.18	8.13	7.12	6.63	3.38
maximum	26.66	20.82	19.61	19.87	17.80	17.47	17.42	16.30	9.34	8.80
S.D.	4.88	2.88	3.62	3.49	3.37	3.84	3.49	2.82	0.98	1.89
CV (%)	29.55	16.97	25.07	25.69	28.05	33.15	30.95	26.36	11.88	32.95

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Table 8 Distance average path (DAP; μm) exposed to copper ($CuSO_4.5H_2O$) in CM culture medium during different time periods.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500	1000
	Ctrl	I	H	G	F	E	D	C	B	A
				$CuSO_4.5H_2O$ (μmol.dm^{-3})						
Time 0 h										
x	33.29	34.44	34.68	34.71	34.02	33.60	33.39	33.12	33.11	33.16
minimum	28.84	30.24	29.29	28.11	29.27	29.13	29.02	29.71	29.22	29.12
maximum	37.33	40.12	40.12	42.20	40.64	39.93	40.71	38.52	37.98	37.36
S.D.	1.86	3.23	3.21	4.22	3.33	2.89	3.34	2.99	2.45	2.53
CV (%)	5.59	9.39	9.25	12.16	9.77	8.59	10.01	9.02	7.40	7.64
Time 1 h										
x	30.04	32.14	32.49	31.37	31.06	30.98	30.79	30.64	30.46	30.91
minimum	25.14	27.87	28.97	27.22	26.73	28.03	27.84	28.43	28.04	26.11
maximum	32.90	38.78	36.12	36.20	35.19	35.69	35.97	34.12	34.78	38.24
S.D.	2.40	3.30	2.60	3.07	2.40	2.43	2.09	1.54	2.01	3.72
CV (%)	7.98	10.26	8.00	9.79	7.73	7.84	6.77	5.03	6.61	12.03
Time 2 h										
x	26.31	27.73	27.70	26.92	26.59	26.35	25.25	24.48	24.32	23.99^{A}
minimum	22.87	23.16	23.25	22.70	23.13	21.10	20.22	21.32	19.75	19.65
maximum	32.90	33.20	33.62	35.13	32.96	32.02	30.96	30.12	28.52	27.84
S.D.	2.57	2.84	3.14	3.85	2.93	3.58	3.13	2.43	2.01	2.02
CV (%)	9.78	10.22	11.34	14.31	11.00	13.60	12.41	9.93	8.26	8.41
Time 24 h										
x	10.45	10.73	9.98	8.98	8.23^{C}	7.75^{A}	7.67^{A}	6.96^{A}	4.02^{A}	2.81^{A}
minimum	8.16	9.49	8.10	7.44	5.10	5.32	5.97	5.39	1.33	1.10
maximum	12.98	11.76	12.74	10.56	11.53	9.63	10.81	8.92	5.15	4.87
S.D.	1.56	0.80	1.47	1.13	2.24	1.59	1.45	1.48	1.55	1.22
CV (%)	14.96	7.44	14.75	12.64	27.20	20.46	18.87	21.20	38.50	43.28

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Evaluation of spermatozoa motility exposed of copper in BSA culture medium

The culture medium BSA maintained the overall percentage of spermatozoa motility in all experimental groups during short-term periods (Time 0 h, 1 h). Evaluation of the percentage of motile spermatozoa showed significant ($P<0.001$) decrease at high concentrations ≥ 500 μmol.dm^{-3}of $CuSO_4.5H_2O$ after 2 h and after 24 h of cultivation when exposed to doses ≥ 125 μmol.dm^{-3}of $CuSO_4.5H_2O$, in comparison to control group. The other experimental groups stimulated of selected parameter(Figure 3).Similar spermatozoa motility was detected for the percentage of progressively motile spermatozoa during all time periods, too (Table 9). The lowest concentrations (≤ 7.80 μmol.dm^{-3}of $CuSO_4.5H_2O$) significantly ($P<0.001$) increased the average motility values (Time 2 h).A significant ($P<0.001$) decrease of progressive motility at the concentrations ≥ 125 μmol.dm^{-3}of $CuSO_4.5H_2O$ was detected during the long-term cultivation (Time 24 h). However, the experimental administration at the doses ≤ 15.60 μmol.dm^{-3}of $CuSO_4.5H_2O$ stimulated ($P<0.001$) the overall of progressive motile spermatozoa. Parameter of velocity average path revealed that concentrations ≤ 62.50 μmol.dm^{-3}of $CuSO_4.5H_2O$ act stimulating on spermatozoa motility during all time periods (Table 10). Evaluation of distance average path showed similar results as for VAP (Table 11).

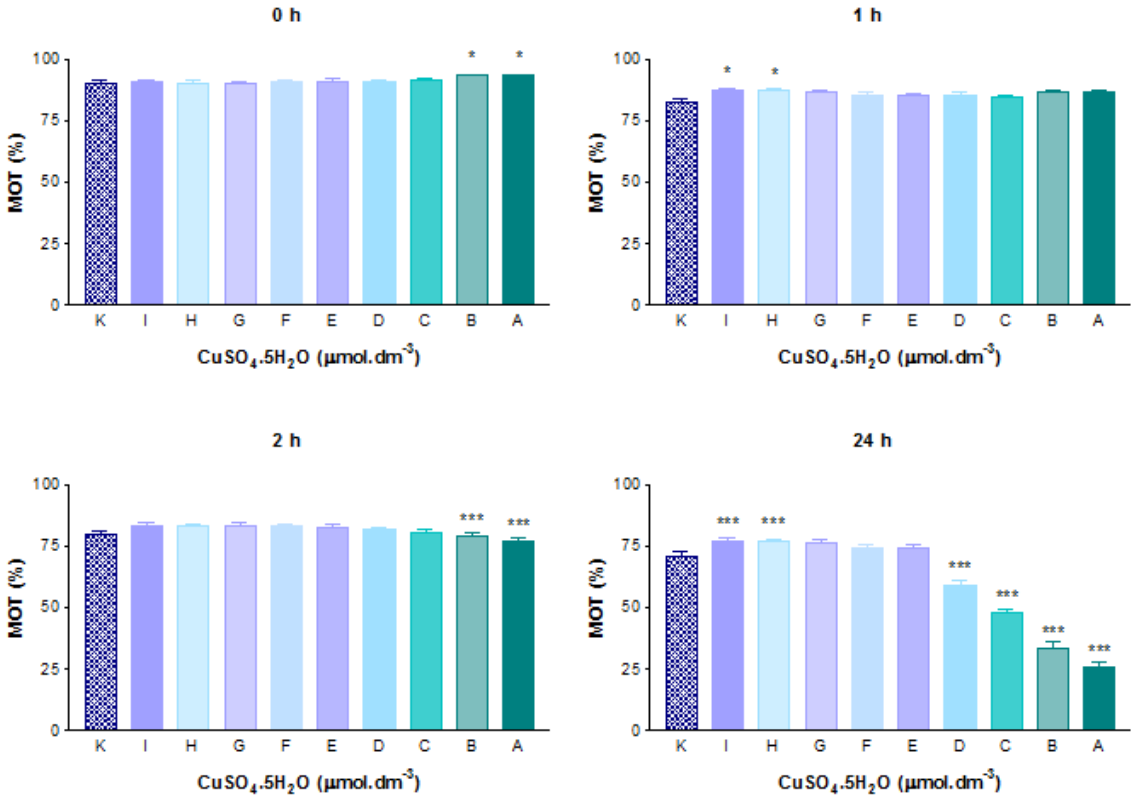

Figure 3 Spermatozoa motility (MOT; %) exposed to copper ($CuSO_4.5H_2O$) in BSA culture medium during different time periods.
Legend: This study was performed in five replicates at each concentration (n = 8). The control group received a culture medium without $CuSO_4.5H_2O$ administration. Group A – 1000 µmol.dm^{-3}; group B - 500 µmol.dm^{-3}; group C – 250 µmol.dm^{-3}; group D - 125 µmol.dm^{-3}; group E – 62.50 µmol.dm^{-3}; group F – 31.20 µmol.dm^{-3}; group G – 15.60 µmol.dm^{-3}; group H – 7.80 µmol.dm^{-3}; group I - 3.90 µmol.dm^{-3} of $CuSO_4.5H_2O$. $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$. Statistical difference between the values of the control group and treated spermatozoa in indicated by asterisks (Dunnett´s multiple comparison test).

Table 9 Progressive spermatozoa motility (PROG; %) exposed to copper ($CuSO_4.5H_2O$) in BSA culture medium during different time periods.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500	1000
	Ctrl	I	H	G	F	E	D	C	B	A
				$CuSO_4.5H_2O$ (µmol.dm^{-3})						
Time 0 h										
x	85.83	85.64	85.46	85.18	86.50	86.80	86.37	87.05	88.31	87.63
minimum	79.06	78.04	76.74	78.28	80.41	73.23	72.22	75.00	80.92	79.72
maximum	90.82	90.21	93.61	92.22	94.53	94.59	93.00	92.30	93.47	92.85
S.D.	3.30	3.88	5.07	3.78	4.17	5.37	5.12	3.82	2.87	3.31
CV (%)	3.85	4.53	5.93	4.44	4.82	6.19	5.93	4.39	3.25	3.77
Time 1 h										
x	74.11	80.36^{A}	79.61^{A}	78.53	76.09	76.87	77.94	76.54	78.77	78.29
minimum	60.71	69.95	63.15	66.98	65.07	69.28	67.90	64.91	69.09	63.07
maximum	87.27	91.66	89.91	94.01	91.20	86.53	91.37	93.87	89.31	87.27
S.D.	7.05	6.20	8.12	7.54	6.82	4.51	7.31	7.36	5.53	7.07
CV (%)	9.52	7.72	10.20	9.60	8.97	5.86	9.37	9.62	7.02	9.03
Time 2 h										
x	71.89	78.61^{A}	77.75^{A}	76.27	74.32	73.65	73.34	72.37	69.82	68.66
minimum	50.00	64.15	70.37	68.75	57.74	63.79	63.72	62.85	59.25	56.92
maximum	86.88	87.83	83.46	89.53	80.95	81.29	79.78	79.12	79.20	75.40
S.D.	11.02	5.87	3.81	6.53	6.17	5.14	4.92	4.52	5.80	4.23
CV (%)	15.33	7.46	4.90	8.56	8.31	6.98	6.71	6.25	8.30	6.16
Time 24 h										
x	61.89	71.76^{A}	70.99^{A}	70.67^{A}	68.57	66.60	51.27^{A}	35.13^{A}	28.72^{A}	19.24^{A}
minimum	43.47	50.98	62.19	52.38	57.26	55.93	43.02	26.31	19.11	10.75
maximum	68.96	86.36	77.65	82.22	80.45	76.82	58.57	50.72	48.48	25.37
S.D.	8.48	8.31	4.31	6.98	6.12	6.18	4.12	7.01	9.76	5.37
CV (%)	13.71	11.57	6.08	9.88	8.93	9.28	8.03	19.94	33.98	27.89

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Table 10 Velocity average path (VAP; µm.s^{-1}) exposed to copper ($CuSO_4.5H_2O$) in BSA culture medium during different time periods.

Groups	Control	3.90	7.80	15.60	31.20	62.50	125	250	500	1000
	Ctrl	I	H	G	F	E	D	C	B	A
				$CuSO_4.5H_2O$ (µmol.dm^{-3})						
Time 0 h										
x	87,76	90,24	89,66	89,54	88,93	91,36	90,18	90,10	92,59	91,31
minimum	80,61	74,20	76,79	74,59	80,93	72,00	81,95	78,74	81,70	80,84
maximum	100,60	104,70	107,10	102,70	99,77	110,00	98,17	108,40	105,10	104,70
S.D.	7,83	8,05	8,55	8,92	4,56	11,51	5,51	7,44	7,26	6,85
CV (%)	8,93	8,92	9,54	9,96	5,12	12,60	6,11	8,25	7,84	7,50

Time 1 h										
x	80,64	85,67	84,54	83,40	86,43	85,11	82,37	82,09	83,00	82,62
minimum	73,16	71,71	73,22	73,69	75,69	70,04	75,07	71,36	75,49	71,27
maximum	97,91	101,50	93,12	94,58	95,91	100,10	91,00	94,58	97,52	92,25
S.D.	6,03	9,60	5,58	7,13	6,53	10,24	4,51	7,30	6,81	6,52
CV (%)	7,47	11,20	6,60	8,55	7,56	12,03	5,47	8,89	8,20	7,89
Time 2 h										
x	74,33	78,65	76,60	76,48	76,38	75,57	73,27	70,55	67,13^{A}	65,43^{A}
minimum	63,66	70,09	63,73	74,11	69,42	69,31	66,01	66,38	59,19	57,16
maximum	81,03	86,23	91,96	89,43	84,50	84,54	78,47	77,95	76,42	71,92
S.D.	6,54	4,25	8,17	5,25	5,10	5,18	3,74	2,96	3,91	3,66
CV (%)	8,80	5,40	10,67	6,86	6,67	6,86	5,10	4,19	5,83	5,60
Time 24 h										
x	53,54	66,60^{A}	61,16^{A}	62,24^{A}	59,79^{A}	59,92^{A}	43,99^{A}	42,58^{A}	35,57^{A}	26,80^{A}
minimum	44,62	55,04	51,44	49,69	47,87	49,99	35,41	30,12	25,82	21,49
maximum	60,17	76,88	72,28	70,51	77,86	69,12	53,46	53,13	45,08	37,17
S.D.	4,85	5,67	6,52	4,96	6,90	5,26	6,42	7,49	5,41	5,04
CV (%)	9,07	8,51	10,66	7,97	11,55	8,78	14,58	17,58	15,22	18,81

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

Table 11 Distance average path (DAP; µm) exposed to copper ($CuSO_4.5H_2O$) in BSA culture medium during different time periods.

Groups	Control Ctrl	3.90 I	7.80 H	15.60 G	31.20 F	62.50 E	125 D	250 C	500 B	1000 A
				$CuSO_4.5H_2O$ (µmol.dm^{-3})						
Time 0 h										
x	37.86	39.63	39.37	39.30	39.28	39.07	38.73	38.25	41.44	40.15
minimum	34.00	32.32	30.71	32.88	33.89	29.70	32.27	34.30	36.63	31.00
maximum	47.41	47.22	49.21	44.34	47.61	48.65	49.52	45.30	47.75	47.88
S.D.	4.36	4.41	6.48	3.78	4.40	5.02	5.29	3.50	3.27	4.72
CV (%)	11.51	11.13	16.45	9.62	11.19	12.86	13.65	9.14	7.88	11.76
Time 1 h										
x	33.64	36.86^{A}	36.85^{A}	36.82^{C}	35.45	34.76	34.64	33.99	36.40	36.67
minimum	26.28	32.19	29.50	31.79	29.98	30.07	28.09	31.18	32.12	33.81
maximum	38.89	43.81	45.30	41.94	40.75	42.52	39.15	36.64	43.45	40.07
S.D.	2.62	3.14	4.33	3.48	3.07	4.68	3.20	1.62	3.53	1.82
CV (%)	7.79	8.51	11.75	9.44	8.65	13.47	9.23	4.77	9.68	5.00
Time 2 h										
x	30.92	32.81	32.02	31.14	31.99	31.19	29.22	28.81	28.63	27.95^{C}
minimum	25.25	29.33	28.75	28.49	26.81	26.09	21.17	26.43	20.93	23.93
maximum	36.94	37.67	37.56	37.48	37.81	37.78	36.86	34.94	33.69	32.83
S.D.	2.83	2.64	2.47	2.62	3.34	3.57	3.99	2.25	3.41	2.98
CV (%)	9.15	8.04	7.72	8.41	10.43	11.43	13.65	7.80	11.93	10.67
Time 24 h										
x	24,07	27,71^{A}	26,10	25,93	25,83	25,29	18,84^{A}	15,31^{A}	14,11^{A}	12,06^{A}
minimum	17,71	21,64	20,59	20,54	21,54	20,30	10,81	11,69	10,59	8,27
maximum	32,18	30,74	30,82	29,95	29,53	28,47	22,99	24,97	18,88	15,54
S.D.	3,87	2,60	3,14	2,88	2,36	2,31	4,11	3,38	2,57	1,80
CV (%)	16,09	9,39	12,02	11,09	9,12	9,13	21,80	22,05	18,21	14,91

Legend: x – arithmetic mean, S.D. – standard deviation, CV (%) – coefficient of variation, $^{A}P<0.001$; $^{B}P<0.01$; $^{C}P<0.05$

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).**

Roychoudhury and Massanyi (2008) examined parameters of rabbit spermatozoa motility during three time periods (0 h, 1 h, 2 h) *in vitro*. Observed data from their study demonstrated negative influence of $CuSO_4.5H_2O$ on semen motility and subsequently confirmed changes in male reproductive functions. Our experiment indicates similar results and also confirms that copper (in the form of $CuSO_4.5H_2O$ in PS culture medium) is a toxic element on spermatozoa motility at high doses. However, the low concentrations (≤7.80 µmol.dm^{-3} of $CuSO_4.5H_2O$) have a positive effect on spermatozoa motility parameters during the short-term *in vitro* cultivation. **Robrelo *et al.* (1996)** observed 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. **Dhami *et al.* (1994)** stressed the impact of Cu on spermatozoa motility. **Katayose *et al.* (2004)** claimed that higher concentrations of Cu had significant adverse effects on the spermatozoa motility. Similar results were also observed in our previous study with copper ($CuSO_4.5H_2O$; $CuCl_2$) on the bovine spermatozoa motility **(Knazicka *et al.*, 2012a, b).** A significant ($P<0.05$) decrease in spermatozoa concentration, motility and viability after experimentally induced $CuSO_4$ poisoning in male rats was seen the study of **Sakhaee *et al.* (2011).** The data obtained their study show that $CuSO_4$ at a dose of 200 mg/kg/day caused testicular atrophy and induced structural abnormalities in spermatozoa. The authors stated that the spermicidal effect of $CuSO_4$ may be responsible for these effects. **Meeker *et al.* (2008)** found evidence of an inverse association between high Cu levels and semen quality, which is consistent with a number of animal and human studies **(Battersby *et al.*, 1982; Skandhan, 1992; Huang *et al.*, 2000; Massanyi *et al.*, 2004; Yuyan *et al.*, 2007).**

The commercial medium containing triladyl, egg yolk and redistilled water increased the overall percentage of spermatozoa motility after exposure of high doses of $CuSO_4.5H_2O$, but only during short-time periods. This observation could be explained by an originally low concentration of energetic and protein substrate in the medium. Egg yolk is relatively unstable for extended periods of time because of high content of fatty acids sensitive to degradation. Therefore, it seems that removal of chicken egg yolk from semen diluents produces several advantages, such as improvement of consistency in the components of semen diluents and elimination of various pathogens **(Matsuoka *et al.*, 2006; Muller-Schlosser *et al.*, 1995).** BSA could be a good protein alternative because of its stability, good amino acid profile andprotective functions. There are several authors who had been studying the possible effects of BSA on the spermatozoa viability of different animal species **(Bakst and Cecil, 1992).** In the present study we evaluated the spermatozoa motility in the presence of $CuSO_4.5H_2O$ with culture medium addition in composition of 20.0 % BSA, triladyl and 5.0 % glucose. This culture medium increased the bovine spermatozoa motility during the short-term of cultivation (Time 0 h, 1 h) in spite of the presence of high doses $CuSO_4.5H_2O$. This observation could be explained binding of copper ions to albumin, which confirms previous experimental studies **(Bradshaw *et al.*, 1968; Masuoka and Saltman, 1994).** The role of albumin is protective as a result of its ability to trap toxic substances in the culture media **(Yamane *et al.*, 1976; Fox**

and Flynn, 2003; Knazicka *et al.*, 2012c). The binding of metals to proteins is a defense to reduce toxicity by preventing availability of the metals **(Davidson *et al.*, 2007)** and it is vital to our understanding of the relationship between its structure and function **(Masuoka *et al.*, 1993; Masuoka and Saltman, 1994).** Several authors consider bovine serum albumin as a suitable protein supplement for the long-term spermatozoa cultivation, because it has protective functions **(Yamane *et al.*, 1976)** and in addition a good essential amino acid profile **(Peters *et al.*, 1975).** Regarding our results we can confirm, that the overall percentage of motile spermatozoa at concentrations ≤ 62.50 µmol.dm^{-3} of $CuSO_4.5H_2O$ was maintained during the long-term (Time 24 h) of *in vitro* cultivation. It could be explained by a high concentration of energy and protein substrates in the medium. Several investigators have found that the incorporation of BSA in semen diluents can protect and stimulate the spermatozoa of many species. **Klem *et al.* (1986)** confirmed that BSA increases of equine the spermatozoa motility. It is likely that higher values of bovine spermatozoa motility characteristics obtained in our study may be at attributed to the stimulating effect of BSA. Equally, **Bakst and Cecil (1992**) reported the possible effects of BSA on the turkey spermatozoa viability. Similar observations have been recorded with spermatozoa of different animal species **(Harrison *et al.*, 1978; 1983).** An appropriate energy substrate **(Knazicka *et al.*, 2010)**, protein supply, as well as optimal laboratory conditions are important factors for a successful *in vitro* spermatozoa motility and viability **(Tvrda *et al.*, 2010).**There are still questions about the optimal BSA concentration for spermatozoa cultivation, since high concentrations of any substance may be toxic **(Tvrda *et al.*, 2010).** The aim of the investigation of **Serniene *et al.* (2001)** was to study the effect on semen quality caused by the addition of BSA to boar semen and to determine the optimal dose of the BSA. The analysis revealed that addition of BSA, spermatozoa storage time and their interaction had significant effect only on the agglutination rate. In their conclusion addition of 0.5 g BSA to the insemination dose significantly decreased the agglutination rate of spermatozoa and did not significantly affect the motility, vigor rate and a number of viable/non damaged spermatozoa per ejaculation. **El-Kon (2011)** conducted to test the post-thaw spermatozoa characteristics through addition of different concentrations BSA to buffalo semen. Observed data from this study demonstrated that spermatozoa motility (58.20 ± 4.60 % and 59.40 ± 4.80 %) and viability (69.30 ± 4.10 % and 69.20 ± 4.20 %) were significantly (P<0.05) higher in the 10 % and 15 % BSA groups than in the tris-egg yolk control group and other samples (0.5; 1.0 and 5.0 % BSA) containing BSA. These findings are in agreement with the previous results by **Matsuoka *et al.* (2006),** which studied the effects of different BSA concentrations (0; 0.3; 1; 5; 10 and 15 %) on the post-thaw viability of ram spermatozoa. Our own results argue in favour of 20 % BSA which has a stimulating function on the spermatozoa motility.

CONCLUSION

The obtained data from this *in vitro* study confirm the dose- and time-dependent effect of copper on the spermatozoa motility parameters in the presence of different culture media. Our results point out that copper at high concentrations acts as toxic element on the spermatozoa motility. However, using a suitable culture medium containing an energy component- and protein-rich substrate, the spermatozoa motility could increase. The results confirm the protective effect of albumin binding to the copper ions. Findings of the present study demonstrated the importance metal-protein interactions.

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