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IMPACT OF COPPER TO THE CHELATION EFFECT OF BOVINE SERUM ALBUMIN AND SPERMATOZOA MOTILITY PARAMETER *IN VITRO**

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SUMMARY: The target of this in vitro study was to analyse the influence of copper on the spermatozoa motility in the presence of bovine serum albumin (BSA) as culture medium and to provide additional information on the interaction between serum albumin and copper (II) chloride ($CuCl_2$). The spermatozoa motility parameters was determined after exposure of $CuCl_2$ (3.9; 7.8; 15.6; 31.2; 62.5; 125; 250; 500; 1000 µmol/L) using the Sperm Vision™ CASA (Computer Assisted Semen Analyzer) system during different time periods (Time 0 h, 1 h, 2 h and 24). The culture medium containing 20% BSA, triladyl and 5% glucose increased the overall percentage of spermatozoa motility after 1 h of cultivation. The percentage of motility spermatozoa significantly ($P<0.001$) decreased after 2 h of cultivation at the concentrations ≥ 250 µmol/L of $CuCl_2$ in comparison with the control group (without $CuCl_2$ administration). The experimental administration at the doses ≤ 31.2 µmol/L of $CuCl_2$ stimulated the overall of motile spermatozoa (Time 24 h). Identical spermatozoa motility was detected also for the percentage of progressive motile spermatozoa during all time periods. Parameter of distance average path (DAP) showed increase in all $CuCl_2$ addition groups in comparison with the control group at Time 1 h. Concentration 125 µmol/L of $CuCl_2$ in various time periods of cultivation act stimulating on spermatozoa motility, but later (Time 24 h) inhibitory. Evaluation of velocity average path, showed similar results as for DAP. Measurement of the amplitude of lateral displacement (ALH) at Time 0 h as well as at Time 1 h was higher in all the experimental groups compared to the control group, but the differences were not significant ($P>0.05$). The experimental administration at the doses ≤ 62.5 µmol/L of $CuCl_2$ stimulated ALH during 24 h of cultivation. The results suggest that adding energy

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and protein substrate to the culture medium increases the spermatozoa motility parameters also the presence of high doses (≥ 125 µmol/L) of copper ions during short-term periods (Time 0 h, 1 h). Concurrently BSA maintained motility of spermatozoa (≤ 31.20 µmol/L of $CuCl_2$) during the long-term (Time 24 h) of cultivation, which confirms the protective effect of albumin binding to the copper ions.

Key words: *copper, bovine serum albumin, spermatozoa, motility.*

INTRODUCTION

Essential trace minerals (ETMs) are among important factors in maintaining and recovering health (Hostetler et al., 2003). The necessity of ETMs for support of life is largely unquestioned, however their requirement for reproduction has not been as extensively studied (Lu et al., 2009). 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; Tang, 2005). It is involved in numerous biological processes (Goyer, 1991; Massanyi et al., 2003) and it is a component of a number of metalloenzymes and metalloproteins, such as superoxide dismutase (Cu/Zn SOD) (Agarwal et al., 1990), catalase, peroxidases, cytochrome oxidase, lysine oxidase, dopamine-β-hydroxylase and ceruloplasmin, which are involved in energy and antioxidant metabolism (Haliwell and Gutteridge, 2000; Aydemir et al., 2006). Copper plays an important role in male and female reproduction system (Ebesh et al. 1999; Wong et al. 2001). The excessive Cu intake has a negative effect on the organs of reproduction (Jockenhovel al et., 1990; 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 and Massanyi, 2008; Roychoudhury et al., 2010; Knazicka et al., 2010a; 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 (Matsuoka et al., 2006) and bovine serum albumin (BSA) 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; Yan et al., 2003; 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 purpose of this *in vitro* study was to provide additional information on the interaction between serum albumin and copper (II) chloride on the spermatozoa motility parameters.

MATERIAL AND METHODS

Bovine semen samples were obtained from 4 adult breeding bulls (Slovak Biological Services, Nitra, Slovak Republic). The samples had to accomplish the basic quality 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 culture medium of bovine serum albumin (BSA; final concentration of 20%; Sigma-Aldrich, USA) was prepared by dissolving protein into buffer (Phosphate Buffer Saline - PBS; Sigma, St. Louis, USA), triladyl® (MiniTüb; Tiefenbach Germany), glucose (5% D-glukosa monohydrate p.a; Penta Chrudim, Czech Republic) and redistilled water. Semen samples were added to the culture medium and cultivated 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 µmol/L), in the form of copper (II) chloride ($CuCl_2$; Sigma-Aldrich, St. Louis, USA). Spermatozoa with $CuCl_2$ were incubated in the laboratory at room temperature (22-25°C) for 24 h. We compared the control (Ctrl) group (medium without $CuCl_2$) with the experimental groups (exposed to different concentrations of $CuCl_2$).

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, Japan) at cultivation Times 0 h, 1 h, 2 h and 24 h. Each sample was placed into the Makler Counting Chamber (deph 10 µm, Sefi-Medical Instruments, Izrael) and the following parameters were evaluated: percentage of motile spermatozoa (motility > 5 µm/s; MOT); percentage of progressive motile spermatozoa (motility > 20 µm/s; PROG); distance average path (DAP; µm); velocity average path (VAP; µm/s) and amplitude of lateral head displacement (ALH; µm) This study was performed in three replicates at each concentration (n = 8).

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 (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

The initial (Time 0 h) spermatozoa motility showed slightly increased values at doses ≥ 31.20 µmol/L of $CuCl_2$ but no significant differences ($P>0.05$) were found between these groups and the control group (without $CuCl_2$ administration). The percentage of motile spermatozoa decreased slowly after 1 h of cultivation compared to Time 0. The average motility values significantly ($P<0.001$) decreased during 2 h of cultivation

at the concentrations ≥ 250 µmol/L of $CuCl_2$ (Table 1) in comparison with the control group. However, the other concentrations stimulated the percentage of spermatozoa motility. The lowest spermatozoa motility was significantly ($P<0.001$) detected in the groups with the highest doses (≥ 125 µmol/L) of $CuCl_2$ (Time 24 h). The low concentrations increased the average motility values, especially in the groups H and I compared to the control group (69.65% and 70.04% versus 64.15%).

Table. 1. Spermatozoa motility (MOT; %) exposed to copper ($CuCl_2$) in BSA during different time periods.

Groups	Control	1000	500	250	125	62.5	31.2	15.6	7.8	3.9
	Ctrl	A	B	C	D	E	F	G	H	I
CuCl2 (µmol/L)										
Time 0 h										
x	87.53	90.38	91.53	90.36	88.32	87.71	88.40	85.58	86.75	85.67
minimum	80.43	84.50	88.65	85.36	81.35	76.59	80.85	76.19	75.75	74.19
maximum	100.0	97.95	93.33	95.23	97.05	96.55	96.06	94.68	96.29	94.73
S.D.	5.50	4.12	1.47	2.98	5.34	5.84	3.97	5.91	6.08	7.33
CV (%)	6.29	4.56	1.61	3.29	6.04	6.66	4.49	6.90	7.01	8.56
Time 1 h										
x	81.32	85.55	85.72	82.45	85.84	84.96	82.60	82.37	83.65C	83.53C
minimum	70.00	75.47	75.00	77.77	73.07	70.52	70.58	69.23	69.23	70.00
maximum	88.52	93.54	93.57	90.41	96.03	96.10	93.13	92.39	96.93	94.73
S.D.	6.05	5.30	5.42	4.12	5.72	7.68	6.49	7.34	9.50	7.85
CV (%)	7.44	6.19	6.32	4.99	6.66	9.04	7.85	8.91	11.36	9.40
Time 2 h										
x	75.87	70.05A	73.88A	74.58A	76.07	76.78	79.50	79.27	80.90	79.62C
minimum	61.11	56.66	55.17	51.35	61.64	54.54	67.92	60.00	65.11	70.07
maximum	90.00	82.81	86.20	92.17	89.58	92.72	88.65	94.59	91.52	90.74
S.D.	9.24	7.61	11.62	13.26	7.45	10.53	7.00	9.95	6.05	6.55
CV (%)	12.17	10.86	15.73	17.78	9.79	13.72	8.80	12.55	7.47	8.23
Time 24 h										
x	64.15	2.78A	6.03A	11.38A	38.86A	58.97	66.47	68.45	69.65	70.04
minimum	41.17	1.07	2.63	5.00	30.76	27.27	30.43	47.05	60.56	50.00
maximum	79.74	5.49	9.25	17.33	41.38	67.30	78.37	79.72	77.61	86.66
S.D.	12.04	1.51	2.08	4.80	4.06	10.44	13.15	9.45	6.19	11.06
CV (%)	18.76	54.28	34.51	42.21	10.45	17.71	19.79	13.81	8.88	15.79

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

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

Identical spermatozoa motility was detected also for the percentage of progressive motile spermatozoa (> 20 µm/s) during all time periods (Table 2). A significant ($P<0.001$) decrease of progressive motility at the concentrations ≥ 62.50 µmol/L of $CuCl_2$ was detected during the long-term cultivation (Time 24 h). However, the experimental administration at the doses ≤ 31.20 µmol/L of $CuCl_2$ stimulated ($P<0.001$) the overall of progressive motile spermatozoa.

Table. 2. Progressive spermatozoa motility (PROG; %) exposed to copper ($CuCl_2$) in BSA during different time periods.

Groups	Control	1000	500	250	125	62.5	31.2	15.6	7.8	3.9
	Ctrl	A	B	C	D	E	F	G	H	I
$CuCl_2$ (µmol/L)										
Time 0 h										
x	84.69	86.72	88.49	87.56	85.41	85.36	86.84	83.70	84.00	82.87
minimum	79.06	78.66	86.48	82.85	76.31	75.75	79.04	73.07	66.78	74.19
maximum	97.82	97.95	89.53	90.47	97.05	93.84	92.41	93.13	93.82	91.89
S.D.	5.47	5.82	1.06	2.35	6.70	5.71	3.62	5.65	7.75	6.05
CV (%)	6.46	6.71	1.19	2.68	7.84	6.69	4.17	6.75	9.22	7.30
Time 1 h										
x	79.36	82.04	81.84	79.70	82.56	80.35	79.77	79.97	80.16	81.33
minimum	66.66	73.33	72.22	71.26	67.85	70.83	72.91	69.23	65.38	67.64
maximum	88.00	92.30	92.66	87.67	93.06	88.70	90.81	90.19	93.12	91.76
S.D.	7.04	5.77	5.31	4.58	7.16	5.78	5.35	5.50	8.70	7.88
CV (%)	8.87	7.03	6.48	5.75	8.67	7.20	6.71	6.88	10.85	9.68
Time 2 h										
x	70.54	66.52	69.26	70.55	70.87	71.19	74.96	75.61	75.28^{C}	75.24^{C}
minimum	58.91	48.48	45.71	48.58	58.90	50.90	64.58	63.63	62.79	68.42
maximum	84.00	83.63	87.01	86.95	85.41	85.45	84.44	89.18	84.84	85.18
S.D.	7.65	9.24	14.01	12.37	7.74	10.32	5.45	7.79	5.36	5.24
CV (%)	10.85	13.89	20.23	17.54	10.92	14.50	7.27	10.31	7.12	6.96
Time 24 h										
x	59.87	1.20^{A}	2.63^{A}	3.02^{A}	22.48^{A}	46.84^{A}	61.39^{A}	62.90^{A}	62.15^{A}	66.92^{A}
minimum	40.00	0.29	0.33	1.07	18.36	37.50	36.36	47.05	37.50	43.75
maximum	75.38	2.19	5.88	6.15	28.57	56.16	66.66	71.87	73.33	80.95
S.D.	11.78	1.06	2.30	1.64	3.10	6.22	9.23	8.06	12.32	10.20
CV (%)	19.68	87.77	87.39	54.19	13.77	13.28	15.04	12.81	19.83	15.25

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

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

Parameter of distance average path (DAP) showed increase in all $CuCl_2$ addition groups in comparison with the control group during 1 h of cultivation (Table 3). The DAP analysis revealed significant differences ($P<0.001$) at the concentrations $\geq$ 250 µmol/L of $CuCl_2$ in comparison to the control group at Time 2 h. Other data are not significant in comparison with the control group. Interestingly, concentration 125 µmol/L of $CuCl_2$ in short-term periods of cultivation act stimulating on the spermatozoa motility, but later (Time 24 h) inhibiting of selected parameter.

Table. 3. Distance average path (DAP; μm) exposed to copper ($CuCl_2$) in BSA during different time periods.

Groups	Control	1000	500	250	125	62.5	31.2	15.6	7.8	3.9
	Ctrl	A	B	C	D	E	F	G	H	I
CuCl2 (μmol/L)										
Time 0 h										
x	41.29	43.17	43.47C	43.07	41.85	41.67	41.55	38.13A	37.69A	39.56
minimum	39.15	39.17	40.07	40.28	38.90	38.53	38.93	34.64	35.62	37.17
maximum	43.83	45.52	46.55	48.65	46.98	45.02	45.67	45.02	39.15	41.01
S.D.	1.46	1.58	1.83	2.77	2.87	1.87	2.50	3.81	0.99	1.16
CV (%)	3.52	3.66	4.20	6.43	6.87	4.48	6.01	9.98	2.62	2.94
Time 1 h										
x	34.83	38.13A	38.2A	35.09A	38.42A	37.32C	35.24	35.47	35.70	35.44
minimum	30.30	35.53	33.52	31.55	36.08	33.19	30.18	30.39	30.54	30.43
maximum	38.55	41.86	41.86	39.87	41.52	41.46	39.47	40.79	38.74	39.95
S.D.	2.47	1.38	2.59	2.43	1.67	2.17	3.42	2.92	2.52	2.62
CV (%)	7.10	3.63	6.77	6.93	4.35	5.80	9.71	8.24	7.07	7.39
Time 2 h										
x	30.30	28.78A	29.06A	29.16A	30.59	31.19	32.76	32.19	33.28	33.10
minimum	25.25	25.20	22.87	27.68	25.62	26.09	28.28	28.81	31.06	29.21
maximum	33.21	31.96	32.78	31.59	36.86	37.78	38.85	37.48	37.56	39.56
S.D.	2.33	2.63	2.84	1.46	3.09	3.57	2.30	2.46	2.31	3.33
CV (%)	7.68	9.14	9.76	5.01	10.09	11.43	10.07	7.65	6.93	10.06
Time 24 h										
x	20.11	3.88A	6.05A	11.57A	13.69A	20.14	22.77	24.70C	25.46A	29.45A
minimum	16.92	2.45	3.00	9.43	11.78	17.93	16.64	14.17	15.62	23.53
maximum	23.47	5.60	8.40	12.89	14.94	22.66	27.68	28.37	31.23	34.10
S.D.	1.84	1.21	2.34	1.87	1.11	1.68	4.25	5.06	5.25	3.05
CV (%)	9.16	31.10	38.59	16.18	8.09	8.34	18.67	20.50	20.60	10.36

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

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

Evaluation of velocity average path (VAP), showed similar results as for DAP (Time 0 h, 1 h). After 2 h of cultivation we proved that the experimental administration at the highest dose (1000 μmol/L) of $CuCl_2$ significantly ($P<0.001$) decreased of selected parameter. Parameter of velocity average path detected that spermatozoa exposed to low copper concentrations (≤ 31.20 μmol/L of $CuCl_2$) after 24 h of cultivation ($P<0.001$) are more active as those in control group, but in relation to higher copper concentration (≥ 500 μmol/L of $CuCl_2$) significant ($P<0.001$) decrease was observed.

Table. 4. Velocity average path (VAP; μm/s) exposed to copper ($CuCl_2$) in BSA during different time periods.

Groups	Control	1000	500	250	125	62.5	31.2	15.6	7.8	3.9
	Ctrl	A	B	C	D	E	F	G	H	I
$CuCl_2$ (μmol/L)										
Time 0 h										
x	97.98	101.20	103.00	100.10	99.29	98.04	99.27	92.69	95.55	93.61
minimum	91.44	93.41	92.25	90.95	91.64	85.72	91.05	76.39	78.43	85.44
maximum	102.50	111.40	119.10	105.50	107.00	116.20	109.90	108.60	113.60	101.80
S.D.	3.70	4.51	8.77	4.22	5.77	8.99	6.28	10.79	10.70	5.82
CV (%)	3.78	4.46	8.51	4.22	5.81	9.17	6.32	11.64	11.20	6.21
Time 1 h										
x	80.63	89.34^{A}	89.16^{C}	83.34	90.02^{A}	85.68	83.59	83.86	85.09	85.12
minimum	72.47	79.16	76.29	70.20	73.85	75.20	71.44	76.61	65.07	70.18
maximum	88.46	99.12	99.63	100.60	107.40	98.98	99.05	98.05	97.92	99.63
S.D.	5.70	5.93	7.35	10.22	10.16	7.04	9.43	6.37	10.35	9.04
CV (%)	7.07	6.64	8.25	12.26	11.28	8.22	11.28	7.59	12.17	10.62
Time 2 h										
x	74.19	66.41^{A}	67.82^{C}	68.50	72.19	74.66	75.05	75.20	76.98	76.54
minimum	58.64	51.56	53.36	60.23	54.77	62.91	65.27	66.45	70.24	61.41
maximum	88.34	79.97	78.53	88.34	83.06	81.99	86.66	89.90	92.12	94.27
S.D.	8.01	8.80	8.20	7.98	7.39	5.32	6.99	7.22	6.14	7.69
CV (%)	10.80	13.37	12.09	11.65	10.24	7.12	9.31	9.60	7.98	10.05
Time 24 h										
x	41.87	10.79^{A}	18.51^{A}	25.15	31.56	45.61	54.75^{A}	56.19^{A}	57.30^{A}	65.56^{A}
minimum	36.50	7.89	9.68	19.51	27.02	38.65	31.65	29.41	33.43	50.28
maximum	48.95	14.78	27.67	28.63	38.70	59.84	67.94	67.88	69.74	76.90
S.D.	3.57	2.83	7.14	4.93	3.86	6.21	12.24	13.35	12.71	7.59
CV (%)	8.52	26.22	38.57	19.60	12.21	13.63	22.36	23.76	22.18	11.58

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

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

Measurement of the amplitude of lateral displacement (ALH) at Time 0 h as well as at Time 1 h was higher in all the experimental groups compared to the control group but the differences were not significant ($P>0.05$). The experimental administration at the doses ≤ 250 μmol/L of $CuCl_2$ stimulated ALH during 2 h of cultivation. Similar results were observed after 24 h, with the exception of groups A ($P<0.05$), B, C and D with the high copper concentrations, which decreased of ALH (Table 5).

Table. 5. Amplitude of lateral head displacement (ALH; μm/s) exposed to copper ($CuCl_2$) in BSA during different time periods.

Groups	Control	1000	500	250	125	62.5	31.2	15.6	7.8	3.9
	Ctrl	A	B	C	D	E	F	G	H	I
CuCl2 (μmol/L)										
Time 0 h										
x	4.68	5.02	5.09	5.07	5.03	4.84	4.88	4.76	4.79	4.64
minimum	3.66	4.20	4.18	3.95	3.48	3.56	3.72	3.49	3.15	3.19
maximum	5.89	5.70	6.25	6.25	6.21	6.21	6.04	6.36	6.02	6.21
S.D.	0.76	0.43	0.74	0.79	0.78	0.93	0.81	0.90	0.77	0.85
CV (%)	16.20	8.55	14.61	15.67	15.55	19.33	16.55	18.96	16.02	18.20
Time 1 h										
x	5.10	5.56	5.49	5.22	5.64	5.42	5.30	5.37	5.28	5.28
minimum	4.58	4.80	3.88	3.98	5.22	4.05	4.10	3.59	4.15	4.15
maximum	5.62	7.91	8.01	6.30	7.01	8.40	6.36	6.65	6.81	5.80
S.D.	0.39	0.74	1.02	0.65	0.51	1.02	0.66	0.78	0.75	0.38
CV (%)	7.67	13.39	18.55	12.49	9.01	18.79	12.93	14.57	14.12	7.20
Time 2 h										
x	4.87	4.80	4.85	4.90	5.03	5.09	5.11	5.17	5.19	5.11
minimum	4.29	4.22	3.58	3.59	3.09	4.69	4.23	3.93	4.50	4.74
maximum	5.75	5.43	5.91	6.00	6.57	6.34	7.07	6.38	7.08	5.76
S.D.	0.58	0.42	0.74	0.72	0.95	0.54	0.80	0.69	0.89	0.30
CV (%)	11.95	8.85	15.30	14.64	18.82	10.64	15.57	13.42	17.18	5.95
Time 24 h										
x	2.94	1.81C	1.84	2.02	2.60	3.58	4.08C	4.18A	4.39A	4.85A
minimum	1.85	1.12	1.00	1.56	2.37	2.19	2.80	1.78	2.61	3.86
maximum	3.85	2.28	2.85	2.36	3.07	4.36	4.96	5.46	5.44	5.57
S.D.	0.81	0.47	0.67	0.30	0.22	0.65	0.77	1.05	0.96	0.53
CV (%)	27.45	25.74	36.56	14.76	8.38	18.06	18.79	25.08	21.91	10.88

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

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

DISCUSION

In the present study we evaluated the spermatozoa motility in the presence of $CuCl_2$ with culture medium addition in composition of 20% BSA, triladyl and 5% 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 (≥ 125 μmol/L) of $CuCl_2$. 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). 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 doses $\leq$ 31.20 μmol/L of $CuCl_2$ 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. From our study, the highest sensitivity of spermatozoa to Cu was found when activating in BSA containing the highest doses (500 μM; 1000 μM) of $CuCl_2$.

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; 1982). An appropriate energy substrate (Knazicka et al., 2010b), 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.

Copper in ionic form rapidly becomes toxic to a variety of cells (Eidi et al., 2010), including human spermatozoa (Holland and White, 1998; Wong et al., 2001). Rebrelo 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, 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 to 10 μg/mL, but the concentration of 100 μg/mL 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 sulphate ($CuSO_4$) on the bovine spermatozoa motility (Knazicka et

al., 2010a). 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).

CONCLUSION

The data obtained from this *in vitro* study proved that adding energy and protein substrate to the culture medium increases the spermatozoa motility parameters also the presence of high doses ($\geq$ 125 μmol/L) of copper ions during short-term periods (Time 0 h, 1 h). Therefore we may assume that 20% BSA stimulating the spermatozoa metabolism. Concurrently BSA maintained motility of spermatozoa ($\leq$ 31.20 μmol/L of $CuCl_2$) during the long-term (Time 24 h) of cultivation, confirms 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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UTICAJ BAKRA NA HELACIONI EFEKTA GOVEĐIH SERUMSKIH ALBUMINA I PARAMETRE POKRETLJIVOSTI SEPERMATOZOIDA *IN VITRO*

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Izvod

Cilj ovog in vitro istraživanja je bio da se analizira uticaj bakra na pokretljivost spermatozoida u prisustvu bovinog serum albumina (BSA), kao medijuma za kultivaciju, kao i da se dobiju dodatne informacije u vezi sa interakcijom između serum albumina i bakar (II) hlorida ($CuCl_2$). Parametri pokretljivosti spermatozoida su utvrđeni posle izlaganja delovanju $CuCl_2$ (3.9; 7.8; 15.6; 31.2; 62.5; 125; 250; 500; 1000 µmol/L), upotrebom Sperm Vision™ CASA (Computer Assisted Semen Analyzer) system, tokom različitih vremenskih perioda (0 h, 1 h, 2 h and 24). Medijum za kultivaciju je sadržao 20% BSA, triladyl i 5% glukoze. Procent pokretnih spermatozoida je signifikantno ($P<0.001$) opao posle 2h kultivacije, kada je koncentracija $CuCl_2$ iznosila ≥ 250 µmol/L, u poređenju sa ontrolnom grupom (bez dodatka $CuCl_2$). Dodatak ≤ 31.2 µmol/L of $CuCl_2$ je stimulisao pokretljivost spermatozoida, posle 24h.
Identična pokretljivost spermatozoida je ustanovljena tokom svih ispitivanih perioda. Ovi rezultati pokazuju da dodavanje energije i proteina, kao i prisustvo visokih doza bakarnih jona*(≥ 125 µmol/L) tokom kratkog vremenskog perioda (oh i 1h)* u kultivacione medijume povećavaju pokretljivost spermatozoida. Dodavanje BSA održava pokretljivost spermatozoida tokom dužeg vremena kultivacije (24h), što potvrđuje zaštitni efekt albumina vezanih za jone bakra.

Ključne reči: bakar, goveđi serum albumin, spermatozoidi, pokretljivost.

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