

ASSESSMENT OF COPPER CONTENT IN SEMEN AND ITS EFFECT ON THE SPERMATOZOA MOTILITY*

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SUMMARY: The general objective of this in vitro study was at first to evaluate copper (Cu) content in the cell sediment and seminal plasma fraction and compare their relationship with basic motility characteristics and secondly expand the knowledge of its impact on the fertilization potential of the spermatozoa. Semen samples were collected from 12 breeding bulls. The motility analysis was carried out using the Computer Assisted Semen Analysis (CASA) system. The mean value for the percentage of motile spermatozoa (MOT) was $91.24 \pm 5.17\%$ and the progressive motility of the spermatozoa (PROG) as $88.58 \pm 5.85\%$. Subsequently, the samples were centrifuged to obtain the seminal plasma fraction and cell sediment. The seminal plasma Cu concentrations were analyzed by UV/VIS spectrophotometry. The total Cu concentration of the seminal plasma was $0.23 \mu\text{g/mL}$. The analysis by the flame atomic absorption spectrophotometry (FAAS) showed that the average cell sediment Cu concentration was $0.0014 \mu\text{g/mL}$. The correlation analysis revealed a moderate positive correlations between MOT and seminal plasma Cu concentration ($r_p = 0.504$; $P > 0.05$) as well as between PROG and Cu content in the seminal plasma ($r_p = 0.410$; $P > 0.05$). Copper in the cell sediment positively affected both MOT ($r_p = 0.265$) and PROG ($r_p = 0.227$), however, no significant differences were found ($P > 0.05$). Conclusions of this study clearly indicated that Cu is important for the preservation of spermatozoa motility. The obtained results proved higher level of Cu in the seminal plasma, which seems representative of cumulative exposures of this trace element. Based on these results, we can conclude that the evaluation of total content of Cu in the

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whole semen is an important factor for determining the fertilization potential of spermatozoa.

Key words: *copper, bovine spermatozoa, semen parameters, cell sediment, seminal plasma, motility parameters.*

INTRODUCTION

Exposure to heavy metals is a risk factor in the assessment of spermatogenesis (Semczuk and Kurpisz, 2006; Kabata-Pendias and Mukherjee, 2007), while certain trace elements have been shown to be essential for testicular development and spermatogenesis (Eidi et al., 2010; Akinloye et al., 2011). Mammalian semen is known to contain a big variety of chemical elements (Marzec-Wroblewska et al., 2012), whose influence on spermatozoa viability has been extensively studied in animals as well as in humans (Kanwal et al., 2000; Massanyi et al., 2003a, b; Eghbali et al., 2008; Atig et al., 2012). A large number of studies have proven that chemical elements are an essential component in the preservation of the fertilization capacity of spermatozoa (Tyrdá et al., 2012). Some of them are crucial for a proper sperm cell function (e. g. sodium, magnesium, calcium, potassium), others are required in relatively narrow limits (zinc, manganese, copper, iron, cobalt, selenium) (Massanyi et al., 2003a, b; 2004).

The possible influence of metals ions, and especially copper (Cu), on the male infertility is a matter of great interest. Copper is an important trace and essential element for the all organisms (Craig et al., 2009), 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), which are involved in energy and antioxidant metabolism (Haliwell and Gutteridge, 2000; Aydemir et al., 2006). Copper is a normal constituent of semen bound to the tail midpiece of spermatozoa (Manu, 1974) and present in seminal plasma, ampullar and seminal vesicular fluids and also released by other structures of the reproductive system (i.e. epididymis) (Valsa et al., 1994). Copper deficiency affects the development of sperm cells (Van Niekerk and Van Niekerk, 1989; Leonhard-Marek, 2000). On the other hand, the high doses 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). Increased levels of metal ions in semen (Umeyama et al., 1986) or seminal plasma (Stanwell-Smith et al., 1983) appear to be significantly and positively correlated with male infertility. 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 (Skandhan, 1992; Huang et al., 2000; Massanyi et al., 2004; Yuyan et al., 2007). Several experimental studies demonstrated the adverse effects of Cu^{2+} on spermatozoa motility (Wong et al., 2001; Machal et al., 2002; Roychoudhury and Massanyi, 2008; Roychoudhury et al., 2010; Knazicka et al., 2012a, c; Sakhaee et al., 2011). The incubation of spermatozoa in the presence of Cu had a negative effect on some of motility parameters (distance and velocity) examined by Computer Assisted Semen Analysis (CASA) system in studies by Roychoudhury et al. (2008). Our previous *in vitro* studies evaluated the negative effects

of a wide range of concentrations of Cu as a risk factor of environment on the motility of spermatozoa and subsequent pointed out a cytotoxic effect of Cu on the mitochondrial complex (Knazicka et al., 2012a, b).

Since, that Cu plays an essential role in spermatogenesis and fertility; this study was conducted to investigate the copper content in cell sediment and seminal plasma fraction and compare their relationship with basic motility characteristics. Furthermore, also expand the knowledge of its impact on the fertilization potential of the spermatozoa.

MATERIAL AND METHODS

Biological material. Bovine semen samples were obtained from 12 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) and basic measurements were performed – volume (mL), pH, concentration ($\times 10^9/\text{mL}$) and osmolality (mOsmol/kg) (Table 1). Each 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.

Spermatozoa motility analysis. The spermatozoa motility was carried out using the Computer Assisted Semen Analysis (CASA) system – SpermVision™ program (MiniTüb, Tiefenbach, Germany) with the Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan) equipped with heating plate (37°C). Each sample was placed into the Makler Counting Chamber (depth 10 μm , Sefi-Medical Instruments, Haifa, Israel) and the following parameters were evaluated: MOT - percentage of motile spermatozoa (%; motility > 5 $\mu\text{m/s}$); PROG - percentage of progressive motile spermatozoa (%; motility > 20 $\mu\text{m/s}$); DAP - distance average path (μm); DCL - distance curved line (μm); DSL - distance straight line (μm); VAP - velocity average path ($\mu\text{m/s}$); VCL - velocity curved line ($\mu\text{m/s}$); VSL - velocity straight line ($\mu\text{m/s}$); STR – straightness (VSL:VAP); LIN – linearity (VSL:VCL); WOB – wobble (VAP:VCL); ALH - amplitude of lateral head displacement (μm) and BCF – beat cross-frequency (H_z). 1000-1500 cells were examined for each sample.

Samples processing. After measurements the samples were centrifuged (10 min, 9500 rpm, 4 °C) to obtain the cell sediment and seminal plasma fraction (supernatant). The fractions were separated and transferred into 1.5 mL tubes and kept frozen (-80°C) for further analysis.

Analysis of cell sediment Cu concentration. The cell sediment fractions were mineralized by adding 1 mL of HNO_3 (65%; Sigma-Aldrich, St. Louis, MO, USA). The resulting solution was diluted to 3 mL with demineralised water. The Cu contents in sediment were determined by direct aspiration of the acidic sample into the flame atomic absorption spectrophotometry (FAAS). This complies with the specification for standardized FAAS quick procedure for metals when using the BUCK Model 200A atomic absorption spectrophotometer (Cole-Parmer International, Court Vernon Hills, Illinois, USA) equipped with a hollow cathode lamp. All measurements were performed at wavelength 324.80 nm. The quantification limit for Cu was 0.096 mg/L and for the detection limit 0.29 mg/L. Calibration Cu was delineated using suitable standard con-

centrations (0.125, 0.25, 0.50, 1.0 and 10.0 µg/g) by diluting standard (0.50% HNO₃). Concentrations were converted to µg/mL.

Analysis of seminal plasma Cu concentratio. The analysis of Cu in the seminal plasma was performed BioLa Test commercial kit (PLIVA-Lachema, Brno, Czech Republic). The measurement was based on a colorimetric reaction between Cu(I) ions and bathocuproine (BCP) forming a stable orange coloured complex (Landers and Zak, 1958), which was easy to detect photometrically at 480 nm (Genesys 10 spectrophotometer, Thermo Fisher Scientific Inc., Madison, USA). Concentrations were expressed as µg/mL.

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. Pearson's correlation coefficient (two tailed) test was used to examine correlations between analyzed parameters of the semen. The level of significance was set at ^A (P<0.001); ^B (P<0.01); ^C (P<0.05).

RESULTS

The results summarized in Table 1 indicate that the concentration of spermatozoa in semen was $3.15 \pm 0.96 \times 10^9$ per mL. The volume of the collected semen of bulls was 6.23 ± 1.69 mL. The total Cu concentration of the seminal plasma was in the range 0.09-0.39 µg/mL with the average value 0.23 µg/mL. Results of the semen evaluation show, that the average cell sediment Cu concentration measured by the FAAS method was 0.0014 µg/mL.

Table 1 The basic parameters of analyzed bovine semen samples (n=12).

Parameters	x±S.D.
pH	6.56±0.20
Spermatozoa concentration (x10 ⁹ /mL)	3.15±0.96
Semen volume (mL)	6.23±1.69
Osmolarity (mOsmol/kg)	297.50±4.67
Seminal plasma copper concentration (µg/mL)	0.23
Cell sediment copper concentration (µg/mL)	0.0014

x – arithmetic mean; S.D. – standard deviation

The mean value for the percentage of motile spermatozoa was $91.24 \pm 5.17\%$. The CASA analysis showed $88.58 \pm 5.85\%$ of progressive motile spermatozoa, the average path distance was 37.81 ± 6.79 µm and the average path velocity was 89.53 ± 16.79 µm/s. The other motility parameters with statistical differences are shown in the Table 2.

Table 2 The average values of spermatozoa motility parameters of analyzed bovine semen samples.

Parameters Motility	X	Minimum	Maximum	S.D.	Cv (%)
MOT (%)	91.24	76.08	99.39	5.17	5.67
PROG (%)	88.58	72.15	96.66	5.85	6.60
DAP (µm)	37.81	25.39	69.03	6.79	17.96
DCL (µm)	60.90	42.64	96.92	10.02	16.45
DSL (µm)	31.72	21.42	64.80	6.71	21.16
VAP (µm/s)	89.53	60.19	159.70	16.79	18.75
VCL (µm/s)	143.80	104.00	223.90	24.10	16.76
VSL (µm/s)	75.46	49.36	150.60	16.50	21.87
STR	0.83	0.74	0.96	0.04	4.88
LIN	0.52	0.38	0.68	0.06	12.45
WOB	0.62	0.46	0.74	0.06	9.36
ALH (µm)	4.72	2.99	6.14	0.72	15.33
BCF (Hz)	34.99	27.41	48.14	3.70	10.57

MOT – percentage of motile spermatozoa (%); PROG - percentage of progressive motile spermatozoa (%); DAP- distance average path (µm); DCL – distance curved line (µm); DSL – distance straight line (µm); VAP- velocity average path (µm/s); VCL – velocity curved line (µm/s); VSL – velocity straight line (µm/s); STR – straightness (VSL:VAP); LIN – linearity (VSL:VCL); WOB – wobble (VAP:VCL); ALH - amplitude of lateral head displacement (µm) and BCF – beat cross-frequency (Hz).

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

In our study, the correlation analysis revealed a moderate positive correlations between percentage of motile spermatozoa and seminal plasma Cu concentration ($r_p=0.504$; $P>0.05$) as well as between progressive of motile spermatozoa and Cu content in the seminal plasma ($r_p=0.410$; $P>0.05$). Copper in the cell sediment positively affected both motility ($r_p=0.265$) and progressive motility ($r_p=0.227$), however, no significant differences were found ($P>0.05$). Results of correlation analysis are listed in Table 3, 4.

Table 3 The Pearson's coefficient of correlations (r_p -values) for relationship between seminal plasma Cu concentration and selected spermatozoa motility parameters.

	Cu	MOT	PROG
Cu	1		
MOT	0.504	1	
PROG	0.410	0.963 ^A	1

The correlation analysis was based on the value of the correlation coefficient: ± 0.111 to ± 0.333 : *low correlation*; ± 0.334 to ± 0.666 : *moderate correlation*; ± 0.667 to ± 0.999 : *high correlation*.

MOT – percentage of motile spermatozoa (%); PROG - percentage of progressive motile spermatozoa (%); ^A $P<0.001$; ^B $P<0.01$; ^C $P<0.05$

Table 4 The Pearson's coefficient of correlations (r_p -values) for relationship between cell sediment Cu concentration and selected spermatozoa motility parameters.

	Cu	MOT	PROG
Cu	1		
MOT	0.265	1	
PROG	0.227	0.586	1

The correlation analysis was based on the value of the correlation coefficient: ± 0.111 to ± 0.333 : *low correlation*; ± 0.334 to ± 0.666 : *moderate correlation*; ± 0.667 to ± 0.999 : *high correlation*.

MOT – percentage of motile spermatozoa (%); PROG - percentage of progressive motile spermatozoa (%); ^AP<0.001; ^BP<0.01; ^CP<0.05

DISCUSSION

Essential trace minerals (ETMs) are among important factors in maintaining and recovering health (Hostetler et al., 2003); however their requirement for reproduction has not been as extensively studied (Lu et al., 2009). Despite the complex relationship between semen analysis and ETMs, we have attempted evaluate a content of trace element - Cu in the whole semen (i.e. seminal plasma versus cell sediment/fraction) and expand the knowledge concerning its relationship with spermatozoa motility.

Semen volume, pH, concentration, viability and motility of spermatozoa as well as composition of the seminal plasma are common parameters to assess spermatozoa quality (Alavi and Cosson, 2006). These factors are directly related to the fertilization success (Bozkut et al., 2009). Our results of basic semen parameters showed that all observed characteristics were at physiological rates. The semen has a very high buffering capacity, much higher than that of most other fluids in the body (Meacham, 2002). The bovine semen maintains a slightly acidic pH (Gamcik, 1992), which was in accordance with our results (pH of 6.56). The semen is notable also for its high osmolarity, which is substantially higher than that of blood plasma. The osmolarity of semen depends greatly on the concentration of sugars and other organics concentrations as well as ionic salt concentrations (Mandal and Bhattacharyya, 1987; Owen and Katz, 2005). In our experiment, semen has a target osmolarity of 297.50 mOsmol/kg. Some researchers have noted that osmolarity increases measurably with semen aging (Velazquez et al., 1977).

Currently, there are little studies dealing with issue of analysis of trace elements (Cu) in the cell sediment or only in the seminal plasma alone. Therefore, this study is the first, which completely evaluate total content of Cu in the whole semen. Our observation showed higher level of Cu in the seminal plasma (0.23 $\mu\text{g/mL}$) in comparison with the cell sediment (0.0014 $\mu\text{g/mL}$). Besides, we found a weak positive correlations between cell sediment Cu concentration and the percentage of motile spermatozoa ($r_p=0.265$; $P>0.05$) as well as progressive motility of the spermatozoa ($r_p=0.227$; $P>0.05$).

Regarding of seminal plasma makes up about 95% of the total volume of whole semen (Gamcik, 1992) and contains a variety of biochemical components, some of which are relatively specific for the regulation of spermatozoa function (Asadpour, 2012). Besides, the seminal plasma is a reliable biological marker for evaluating vitality, sperm metabolism, motility and others relevant semen parameters (Maxwell et al., 1996; Asadpour, 2012). Several studies have shown its relationships with volume, pH,

concentration, vitality and morphology (Massanyi et al., 2005; Shinohara et al., 2005; Akinloye et al., 2011). The experimental study by Machal et al. (2002) confirmed a positive coefficient of correlation ($r_p=0.360$; $P<0.01$) between the Cu concentration in seminal plasma and the mean volume of bulls semen. A positive significant ($P<0.05$) correlation between seminal plasma Cu and semen volume was reported also in the human study by Akinloye et al. (2011). Shinohara et al. (2005) found significant correlations between Cu concentration in semen and sperm concentration, semen volume and abnormal morphology. Eidi et al. (2010) examined seminal plasma levels of Cu and its relationship with human semen parameters. Their study demonstrated significant negative correlation between seminal plasma Cu concentration and pH ($r_p=-0.173$; $P<0.05$) as well as sperm concentration ($r_p=-0.114$; $P<0.05$) and motility ($r_p=-0.399$; $P<0.01$). Subsequently, they confirmed that high concentration of Cu is related to lowering pH of seminal plasma, acidic pH, with changing condition of seminal plasma due to decrease motile or alive percent of spermatozoa. The excess Cu in seminal plasma is detrimental for male reproductive capacity by reducing spermatozoa count, motility, vitality and morphology. Katayose et al. (2004) demonstrated that higher concentrations of Cu had significant adverse effects on spermatozoa motility. Equally, Rebrelo et al. (1996) confirm that the high concentration of this essential element in seminal plasma is correlated with reduced spermatozoa motility. In our case, the mean value for the percentage of motile spermatozoa (quantity of movement) was $91.24\pm5.17\%$ and the progressive motility of the spermatozoa (quality of movement) as $88.58\pm5.85\%$. The correlation analysis revealed a moderate positive correlation between percentage of motile spermatozoa and seminal plasma Cu concentration ($r_p=0.504$; $P>0.05$) as well as between progressive of motile spermatozoa and Cu content in the seminal plasma ($r_p=0.410$; $P>0.05$), which is in agreement with the report of Tvrdá et al. (2012). Eghbali et al. (2008) recorded, that spermatozoa motility was $92.24\pm0.51\%$ in excellent group, $81.66\pm0.62\%$ in good group and moderate group $71.66\pm1.05\%$, which were significantly different. In this study demonstrated a positive correlation between seminal plasma Cu concentration and bovine (*Bubalus bubalis*) spermatozoa motility with viability. Machal et al. (2002) reported a statistically significant ($P<0.05$) positive coefficients of correlation between the Cu concentration in seminal plasma and spermatozoa motility ($r_p=0.330$) and the total number of sperm cells with progressive motility ($r_p=0.280$). These their results correspond with the studies of Dhami et al. (1994) and Leonhard-Marek (2000). Wong et al (2001) also recorded a weak but significant positive correlation between blood Cu concentrations and spermatozoa motility. In a similar study, Jockenhövel et al. (1990) showed significant correlation between seminal plasma Cu concentrations and spermatozoa count, motility and normal morphology. The findings of other authors (Eidi et al., 2010; Akinloye et al., 2011) are however controversial in the comparison with our results.

The differences in opinion concerning the Cu content in seminal plasma of different species of animals were detected. The mean total Cu value of the buffalo seminal plasma in the study of Eghbali et al. (2008) was recorded as 2.51 ± 0.04 mg/kg wet weight. Comparing these results with other authors we found out that according to Massanyi et al., (2003a, b) the seminal plasma Cu concentration was significantly higher ($P<0.01$) in the rams (2.49 ± 0.18 mg/kg), fox (2.16 ± 0.53 mg/kg) than that in the bulls (1.64 ± 0.21 mg/kg), boars (1.64 ± 0.28 mg/kg) and stallions (0.86 ± 0.10 mg/kg). The concentration of Cu in rabbit semen was assessed Lukac et al., (2009) on the level 20.10 ± 4.09 mg/kg wet weight, while rabbit semen is characterized by very high Cu concentration. Machal et

al. (2002) state that the mean Cu level in seminal plasma of bulls was 38.17 $\mu\text{M/L}$, which in comparison with our results is too high a concentration. Skandhan and Mazundar (1979) stressed that an enhanced seminal plasma concentration of Cu may be one of the direct factors attributed to oligoasthenospermia and asthenospermia. Excess levels of monovalent and divalent Cu ions in solution should result in lipid peroxidation in sperm plasma membrane, an effect that may render spermatozoa immotile (Rebrelo et al., 1996; Wong et al., 2001). It is reported that Cu acts as a catalyst in the formation of reactive oxygen species (ROS) that can lead to oxidative stress development (Stohs and Bagchi, 1995). Therefore, it is important to systematically evaluate its content in tissues and body fluids in relation to antioxidant capacity, in order to prevent complications resulting from its bilateral role in the organism (Tvrdá et al., 2012).

CONCLUSION

The data obtained from this *in vitro* study proved that the copper is necessary component of bovine semen and is needed for a proper spermatozoa function. Conclusions of this study clearly indicated that there is a relationship between a copper concentration in the semen and basic motility characteristics (motility, progressive motility), which also are affected by many factors. The obtained results proved higher level of copper in the seminal plasma, which seems representative of cumulative exposures of this trace element. Additionally, we can conclude that the evaluation of total content of copper in the whole semen (i.e. seminal plasma and cell sediment) is an important factor for determining the fertilization potential of spermatozoa.

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ODREĐIVANJE KONCENTRACIJE BAKRA U SPERMI I NJEGOV EFEKT NA POKRETLJIVOST SPERMATOZOIDA

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Izvod

Cilj ovog istraživanja je bio da se, *in vitro*, odredi sadržaj Bakra (Cu) u sedimentu frakciju ćelija i semene plazme i uporedit ovaj odnos sa osnovnim karakteristikama motiliteta spermatozoida i, drugo, proširi znanje o njegovom uticaju na oplodni potencijala spermatozoida. Uzorci semen su sakupljeni od 12 priplodnih bikova. Analiza motilitet je izveden korišćenjem računarskog sistema Assisted Semen Analiza (CASA). Srednja vrednost za procenat pokretnih spermatozoida (MOT) je 91.24 ± 5.17 %, progresivna pokretljivost (PROG) 88.58 ± 5.85 %. Nakon toga, uzorci su centrifugirani da se odvoji frakcija spermatozoida i frakcija plazme. Koncentracije Cu u plazmi je analizirani pomoću UV/VIS spektrofotometrije. Ukupna Cu koncentracija u semenoj plazmi bila je 0.23 ug / ml. Analiza plamena atomske apsorpcione spektrofotometrije (FAAS) pokazala je da prosečna koncentracija Cu u ćelijama sedimenta iznosi 0.0014 ug/mL. Ustanovljena je umerena pozitivna korelacija između MOT i koncentracije Cu u semenoj plazmi ($RP = 0.504$; $p > 0.05$), kao i između PROG i sadržaja Cu u semenoj plazmi ($RP = 0.410$; $p > 0.05$). Prisustvo Cu u ćelijama sedimenta, pozitivno je uticao na MOT ($rp = 0.265$) i PROG ($rp = 0.227$), ali pve razlike nisu statistički značajne ($p > 0.05$). Rezultati ove studije jasno pokazuju da je Cu važan za očuvanje motiliteta spermatozoida. Na osnovu dobijenih rezultata, može se zaključiti da je procena ukupnog sadržaja Cu u celom ejakulatu, važan faktor za određivanje stepena oplodnog potencijal spermatozoida.

Ključne reči: bakar, spermatozoidi bika, parametri sperme, ćelijski sediment, semena plazma, parametri pokretljivosti.

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