

THE EFFECT OF COPPER SULPHATE ON INHIBITION OF MICROORGANISMS AND SPERM SURVIVAL IN SHORT-TERM BOAR SEMEN EXTENDER WITHOUT ANTIBIOTICS

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Abstract

The objective of this study was to investigate the effect of copper sulphate on inhibition of microorganisms and their influence on sperm survival in BTS extender without antibiotics. Six ejaculates from 3 healthy and fertile AI boars were used for this study. The amounts tested copper sulphate added to boar semen extender was not affected on initial sperm motility ($p < 0.05$) unlike higher amounts of copper sulphate. Tested amounts of copper sulphate CSA-0.015g/l and CSB-0.03g/l were added to BTS extender without antibiotics in dilution rate of 1+2, 1+4 and 1+8 and stored at 17°C up to 48h, for everyday evaluation. Sperm motility was evaluated according CASA program at 0h and with thermo-resistance survival test (TRT test). Sperm motility was affected dilution ratio and amount of copper sulphate ($p < 0.05$). Significant differences ($p < 0.05$) of total mean values of sperm motility in TRT test were found between samples BTS 59.11% and BTS+SCA 63.54% vs. BTS+SCB 41.33%. In progressive motility and in non-progressive motility were found differences between BTS+CSA and BTS+CSB by 21% and 14% ($p < 0.05$). Microorganisms were not significantly inhibited by any amounts of copper sulphate. In conclusion, the tested amount BTS+SCA increased sperm survival in the TRT test by 7.5% to BTS. Use of the tested amount of copper sulphate as a potential substitute for antibiotics in boar semen extender is not appropriate.

Key words: Boar semen, short extender, cooper sulphate, microorganisms, sperm motility

Artificial insemination using fresh diluted semen is used worldwide in the porcine industry (Boonkusol et al., 2010). Native boar semen is usually contaminated by a wide range of microorganisms, which endangering sperm quality and fertility sperm (Maes et al., 2008; Maroto et al., 2010). Recently many microorganisms have become resistant to the most antibiotic used in semen extenders (Schulze et al., 2015; Morrell and Wallgren, 2014) and now we are looking possible alternatives which could be used to reduce this resistance.

Copper sulphate is an inorganic compound that combines sulfur with copper. Copper is an antibacterial, antifungal and antiviral agent (Vincent et al., 2017). The toxicity

of copper sulphate depends on the copper content. Copper is the metal cofactor for a variety of enzymes—amine oxidase, copper-dependent superoxide dismutase, cytochrome oxidase and tyrosinase (Murray, 2006).

The objective of this study was to investigate the effect of copper sulphate on inhibition of microorganisms and their influence on sperm survival in BTS extender without antibiotics.

Material and Methods

Six sperm rich ejaculate fraction with motility $\geq 80\%$ and number of morphologically abnormal spermatozoa $\leq 25\%$ from three fertile AI

boars of Přeštice black-pied pig aged 3.5 to 5 years were collected using the gloved-hand technique. The boars were kept in the same housing, feeding and breeding conditions.

The following parameters were evaluated in the fresh native boar semen: semen volume, sperm motility, sperm concentration, morphologically abnormal spermatozoa (MAS), pH and osmolality. The sperm motility was assessed subjectively using phase contrast microscopy with a heating stage (38°C) at 200× magnification. Sperm concentration was measured with Chroma Colorimeter 254 (Sherwood Scientific Ltd., Cambridge, England). Morphologically abnormal spermatozoa (MAS) were assessed according to the staining method of Čerovský (1976) and evaluated microscopically under oil immersion and 1500× magnification. The pH was assessed using the Hanna precision pH meter at 20°C (Sigma-Aldrich, Czech Republic) and osmolality (mOsmol/kg) with the Marcel Osmometr OS 300 (2THETA ASE, Czech Republic).

The boar semen was diluted in dilution rate 1+2, 1+4 and 1+8 in extender BTS without antibiotics - control samples (Minitüb, Germany). Copper sulphate CSA-0.015g/l, CSB-0.03 g/l, (Lachema Brno, Czech Republic) was added to in extender BTS without antibiotics in the dilution rate 1+2, 1+4, 1+8. This selected amount of substances added to boar semen extender was not affected on initial sperm motility ($p < 0.05$) unlike higher amount of substances. Samples were stored at a temperature of 17°C up to 48h.

Sperm motility was evaluated according Computer Assisted Semen Analysis (CASA) program (0h) and with thermo-resistance survival test (TRT test) after storage time 24h and 48h. For this study, value of sperm motility 0h was expressed according CASA program as a total motility (M), progressive sperm motility $VCL > 25 \mu\text{m/s}$ and $STR \geq 45\%$ (PM), non-progressive motility $VCL > 25 \mu\text{m/s}$ and $STR < 45\%$ (NP) and immotile (IM). The TRT test was performed on 3 ml samples kept at 38°C in water bath after storage time 24h and 48h and motility of

spermatozoa was evaluated at the 1h, 3h and 5 h during the incubation.

The assessment antibacterial activity of copper sulphate was in a microbiological laboratory. Each sample was diluted 100× in physiological saline solution (Penta s.r.o., Czech Republic) and then 100µl of the sample was inoculated on blood agar with 5% defibrinated ram blood (HiMedia Laboratories, USA). Samples were incubated for 48h at 37°C in a biological thermostat BT 120MR (EKOM s.r.o., Czech Republic). The number of colonies was determined by colony counter STC 1000 (VWR, Switzerland) and the total number of microorganisms was determined according to the formula and expressed in colony-forming unit (CFU/ml).

Basic statistical characteristics of the results of arithmetic means, standard deviations (SD) and significance (p) were calculated by the QC Expert program (TriloBite Statistical Software s.r.o., Pardubice, Czech Republic). The data were analysed by statistical analysis of variance (ANOVA) followed by the Fisher test ($p < 0.05$). Statistical significance was determined using a two-way ANOVA to assess the effect of diluent, dilution ratio, evaluation day, evaluation hour and interactions of these factors, always in combination with extender on sperm motility.

Results and Discussion

The initial quality of native semen was as follows: semen volume 226.00 ± 57.27 ml, sperm motility $81.00 \pm 4.18\%$, sperm concentration $366.00 \pm 81.74 \times 10^3/\text{mm}^3$, MAS $24.36 \pm 1.45\%$, pH 7.93 ± 0.34 and osmolality 317.40 ± 10.41 mOsmol/kg. Values of pH and osmolality extenders are presented in the Table 1.

Comparison of total values of sperm motility 0h (%) according CASA program in tested samples are presented in the Figure 1.

In progressive motility and in non-progressive motility were found differences between BTS+CSA and BTS+CSB by 21% and 14% ($p < 0.05$). Sperm motility was affected by

dilution ratio and amounts of copper sulphate ($p < 0.05$). Significant differences ($p < 0.05$) of total mean values of sperm motility in TRT test were found between samples BTS 59.11% and BTS+SCA 63.54% vs. BTS+SCB 41.33% (Figure 2).

The effect dilution rate on sperm motility in TRT test are noted in the Figure 3. There were found differences in dilution rate in BTS and BTS+SCA vs. BTS+SCB and in the different dilution rates in the same extender ($p < 0.05$).

The effect hours of evaluation on sperm motility in TRT test are shown in the Figure 4. There were found differences at 3h of evaluation in BTS vs. BTS+SCA and BTS+SCB and at 5h in BTS and BTS+SCA vs. BTS+SCB and in hours of evaluation in the same extender ($p < 0.05$).

We found that sperm motility was reduced higher amounts of copper sulphate BTS+SCB to

BTS+SCA by 22% in TRT test. However, excess copper can oxidize proteins and lipids, bind to nucleic acids and enhance the production of free radicals and reduces the oxidative processes and glucose consumption, which reduces or abolishes sperm motility (Abdul-Rasheed, 2010). Eidi et al. (2010) found that excess copper in seminal plasma was detrimental for male reproductive capacity by reducing sperm count, motility, vitality and morphology. On the other hand, Tabassomi et al. (2013) found that copper additive at the rate of 0.032 mg/l gives a better protection of sperms through the process of dilution, equilibration and freeze-thawing than that in control and other Cu concentrations, while 0.064 mg/l CuSO_4 had deleterious effect on the water buffalo's sperm.

Table 1. Mean values and standard deviation (SD) of pH and osmolality of extender BTS, BTS+CSA and BTS+SCB.

Extender	pH	Osmolality (mOsmol/kg)
BTS	8.14±0.10	331.50±8.34
BTS+SCA	8.11±0.31	340.00±3.46
BTS+SCB	7.76±0.01	333.50±3.54

Figure 1. Comparison of total sperm motility (%) 0h according CASA program in BTS, BTS+CSA and BTS+SCB. (M-total motility, PR-progressive motility, NP-non-progressive motility and IM-immotile).

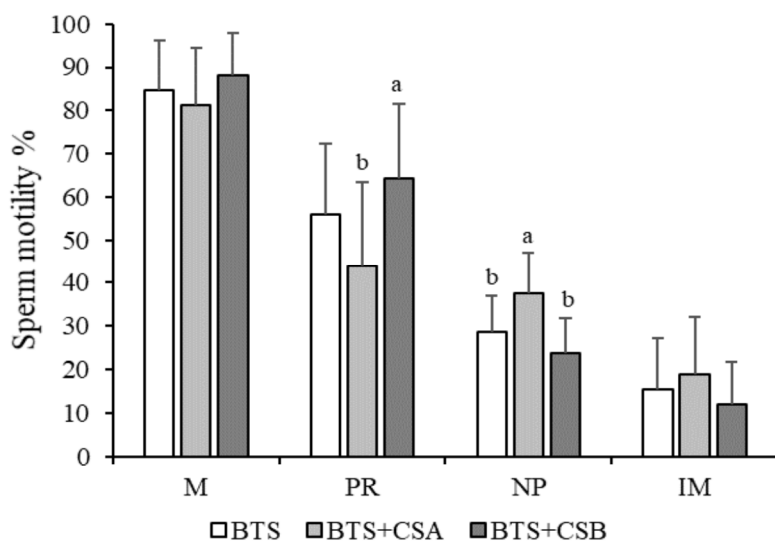
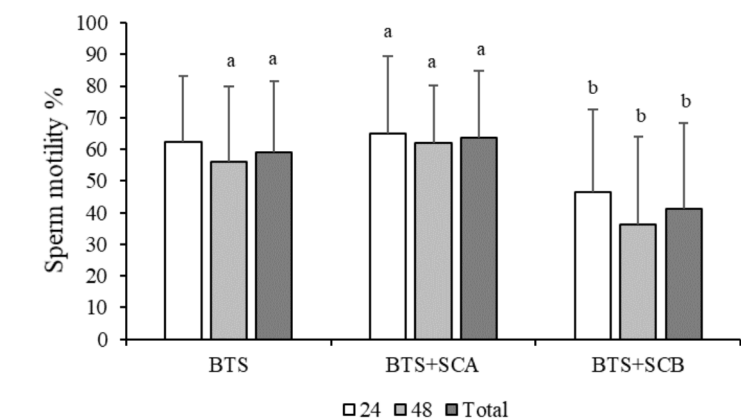
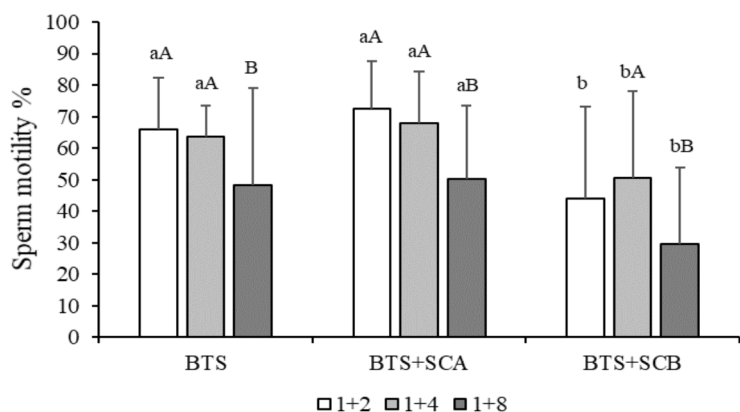


Figure 2. Total sperm motility (%) and during 24h a 48h storage time in TRT test.



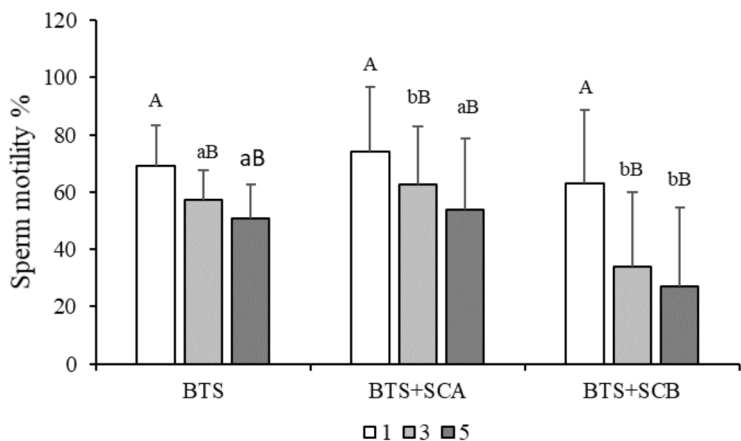
^{a,b}p<0.05

Figure 3. The effect dilution rate 1+2, 1+4 and 1+8 in BTS, BTS+SCA and BTS+SCB on boar sperm motility in TRT test



Different letters indicate significant differences between BTS, BTS+SCA and BTS+SCB in the same dilution rate ^{a,b}p<0.05 and in the different dilution rates in the same extender ^{A,B}p<0.05.

Figure 4. The effect hours of evaluation in BTS, BTS+SCA and BTS+SCB on boar sperm motility in TRT test.



Different letters indicate significant differences between BTS, BTS+SCA and BTS+SCB in the same hour of evaluation ^{a,b}p<0.05 and in hour of evaluation in the same extender ^{A,B}p<0.05.

The most common microorganisms found in boar semen samples: *E. coli* (especially in native semen), *Proteus sp.*, *Staphylococcus aureus*, *Staphylococcus cohnii* subsp. *Cohnii*, *Staphylococcus simulans*, *Staphylococcus cohnii* subsp. *urealyticum*, *Staphylococcus capitis* subsp. *capitis*, *Staphylococcus haemolyticus*, *Corynebacterium sp.*, *Bacillus sp.*, *Moraxella canis*, *Chryseobacterium gleum*. A similar

representation of microorganisms was reported by Bresciani et al. (2014) and Gaczarzewicz et al. (2016). The mean value of microorganisms in native boar semen was 3.0×10^3 CFU/ml. Typical bacterial concentrations in the boar semen are presented range from 10^3 to 10^5 CFU/ml (Morrell and Wallgren, 2011; Stojanov et al. 2020). BTS+SCA and BTS+SCB did not inhibit microorganisms in samples of diluted semen $p > 0.05$ (Figure 5, 6 and 7).

Figure 5. Determination of the colony-forming unit (CFU/ml) of microorganisms in days of evaluation at native semen, in BTS extender, BTS+CSA and BTS+CSB at a dilution rate 1+2.

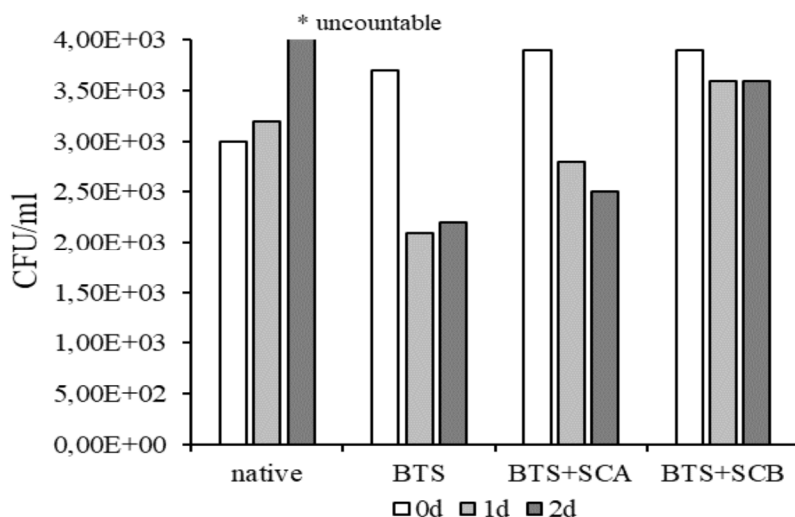


Figure 6. Determination of the colony-forming unit (CFU/ml) of microorganisms in days of evaluation at native semen, in BTS extender, BTS+CSA and BTS+CSB at a dilution rate 1+4.

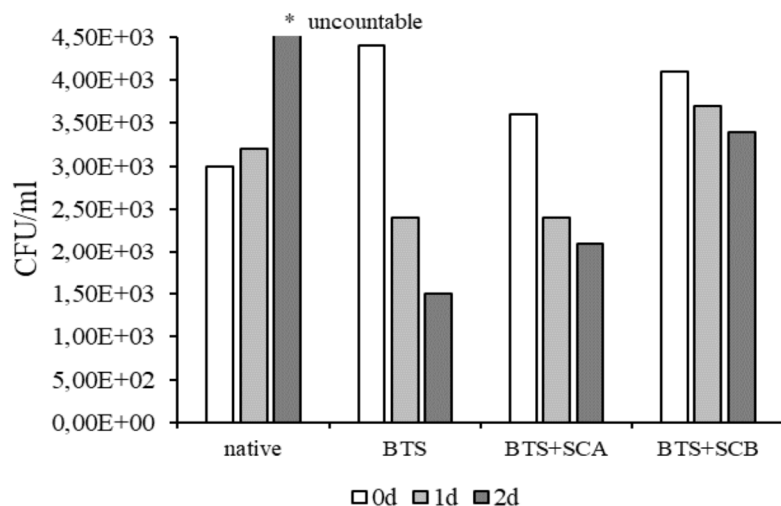
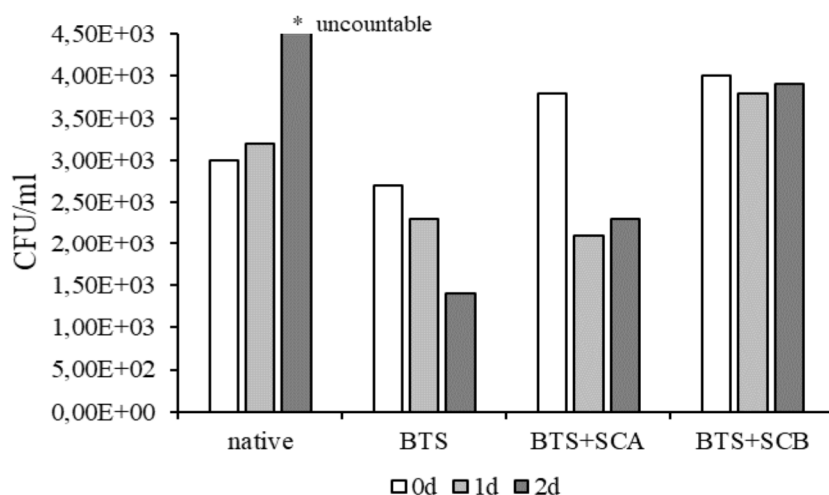


Figure 7. Determination of the colony-forming unit (CFU/ml) of microorganisms in days of evaluation at native semen, in BTS extender, BTS+CSA and BTS+CSB at a dilution rate 1+8.



Conclusion

The tested amount BTS+SCA increased sperm survival in the TRT test by 7.5% to BTS as control sample. Utilization of the tested amount of copper sulphate as a potential substitute for antibiotics in boar semen extender was not appropriate because their low activity in the reduction of microorganisms was found. Therefore, it is necessary to research other potential substances for a possible replacement for antibiotics in boar semen extender.

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