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# THE EFFECT OF TREHALOSE, CAFFEINE AND GLUTATHIONE ON BOVINE SPERMATOZOA: 2. MORPHOLOGY AND OXIDATIVE STATUS *IN VITRO**

PETER MASSÁNYI, EVA TVRDÁ, MARTINA RAFAJOVÁ, NORBERT LUKÁČ[1]

*SUMMARY: The aim of this study was to examine the effects of glutathione, trehalose and caffeine on selected bovine spermatozoa morphology parameters, as well as lipid peroxidation following an in vitro cultivation at different temperatures and time periods. Morphological analysis showed similar occurrence of morphological abnormalities in all group with no significant differences ($p>0.05$). The TBARS assay revealed that the selected concentrations of trehalose, caffeine and glutathione did not have a significant effect ($p>0.05$) on lipid peroxidation as a process leading to oxidative stress development in spermatozoa.*

***Key words:*** *trehalose, caffeine, glutathione, spermatozoa, bulls, morphology, MDA.*

## INTRODUCTION

Artificial insemination (AI) has become one of the most important pillars in animal biotechnology. Especially in the cattle AI, bull semen quality is highly important to ensure a good biological material for breeding as well as a certain biodiversity protection (Ibrahim et al., 2000).

Progress in the use of AI has been related to search for semen extenders with a potential ability to stimulate the viability and to enhance the fertilizing ability of animal and human spermatozoa (Pivko et al., 2009; Spalekova et al., 2011). A special attention is dedicated to substances with antioxidant properties, as sperm cell membranes contain high concentrations of polyunsaturated fatty acids susceptible to lipid peroxidation. Inversely, the seminal plasma possesses a wide antioxidant system to prevent the

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[1]Peter Massányi, MVD, PhD, professor, Eva Tvrdá, MSc, Martina Rafajová MSc, PhD, Norbert Lukáč, MSc, PhD, associated professor, Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak Universtity of Agriculture, Nitra, Slovakia.
Corresponding author: Eva Tvrdá, e-mail:evina.tvrda@gmail.com, tel:+421 37 641 4288.

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oxidative cellular damage (Kefer et al., 2009). Nevertheless, antioxidants present in the seminal plasma, are usually attenuated by dilution of the semen during the preparation of insemination doses (Pivko et al., 2009).

It was confirmed that the addition of glutathione, as an antioxidant and trehalose, a nonpermeant cryoprotectant has elicited beneficial effects in many facets of AI and *in vitro* fertilization: increase of semen quality, spermatozoa motility and viability. Furthermore, it was documented that caffeine as a cyclic nucleotide phosphodiesterase inhibitor markedly increased and maintained the respiration and motility of ejaculated spermatozoa, which resulted in a higher fertilization rate of oocytes (Tatham et al, 2003; Spalekova et al, 2011).

As a follow-up of our previous study (Massanyi et al., 2011), we examined the effects of glutathione, trehalose and caffeine on selected bovine spermatozoa morphology parameters, as well as lipid peroxidation following an *in vitro* cultivation at different temperatures and time periods.

## MATERIAL AND METHODS

Bovine semen samples (n=48) were obtained from 6 randomly selected adult breeding bulls (Slovak Biological Services, Nitra, Slovakia) on a regular collection schedule using an artificial vagina. The semen was cooled down to 4°C and transported to the laboratory, where the samples were divided into four main groups, according to the concentration of the used experimental supplement and the cultivation temperature together with the time intervals of analysis.

Basic spermatozoa diluent medium consisted of Triladyl® (250 mL; Minitüb, Tiefenbach Germany), distilled water (750 mL) and egg yolk (62.5 mL). The treatment was based on the addition of trehalose (Sigma-Aldrich, St. Louis, USA) caffeine (Sigma-Aldrich, St. Louis, USA) and glutathione (Sigma-Aldrich, St. Louis, USA) into the semen diluent medium at dosages of 0 (Control), 1 (Group 1) and 2 (Group 2) mg/mL. Fresh semen was added to each medium with a final dilution rate of 1:50. Group A was cultured at 37 °C and analyzed at time intervals of 0h, 1h, 2h, 3h and 4h after the experiment had started. Group B was cultured at 5°C and analyzed at 24h, 48h, 72h and 168h after the experiment had begun.

For the analysis of morphologically altered spermatozoa the semen samples were fixed in Hancock's solution and stained according to the Giemsa–Romanowski staining method. The frequency of abnormal spermatozoa was quantified microscopically at 500x magnification, and the following abnormal morphological changes were evaluated: knob-twisted flagellum, separated flagellum, flagellum torso, broken flagellum, retention of cytoplasmatic drop, acrosomal changes, large head, small head, flagellum ball, and other abnormal spermatozoa Morphologically changed spermatozoa were sorted to the classification table of morphological malformed forms of spermatozoa (Lukac et al., 2009).

Decomposition of unstable peroxides derived from lipid peroxidation results in the formation of malondialdehyde (MDA), which can be quantified colorimetrically following its controlled reaction with thiobarbituric acid (TBA). The measurement of these is a well-established method for screening and monitoring lipid peroxidation. Cayman's Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit (Cayman chemical company, Ann Arbor, Michigan, USA) was used for the MDA evaluation. The MDA-TBA

product formed by the reaction of MDA and TBA at high temperatures (90-100°C) and acidic conditions was measured colorimetrically at 530-540 nm.

Statistical analysis of the obtained data was carried out using the SAS statistical program (SAS Institute Inc., Cary, USA). Basic statistical parameters (mean, standard deviation, coefficient of variation) were calculated at first. Subsequently, a paired t–test and Scheffe's test were used to compare the results between the control and experimental groups. The level of significance was set at *** ($p < 0.001$); ** ($p < 0.01$); * ($p < 0.05$).

## RESULTS AND DISCUSSION

### Group 1 (1 mg/mL)

The morphological analysis showed that more than 85% of the examined spermatozoa were intact (Table 1). The highest percentage of normal spermatozoa was found after trehalose (88.88%) and glutathione treatment (88.75%). The percentage of abnormal (morphologically changed) spermatozoa was almost equal in the control (14.62%) and caffeine (14.87%) group. The dominant abnormalities included flagellum torso and broken flagellum, as well as a small head in case of caffeine and glutathione treatment.

Table1. Spermatozoa morphology (in %) in groups with 1 mg/mL of supplement addition (mean±SD)

| Morphology | C1 | T1 | K1 | G1 |
|---|---|---|---|---|
| TN | 13.73±5.62 | 11.62±3.74 | 17.75±3.96 | 13.62±4.00 |
| AC | 0.82±0.75 | 1.25±1.16 | 1.12±1.13 | 1.62±0.92 |
| SF | 2.73±1.10 | 2.00±1.07 | 3.50±1.31 | 2.62±1.41 |
| KTF | 2.64±2.46 | 1.50±1.31 | 2.00±1.37 | 2.37±1.30 |
| FT | 2.18±1.47 | 2.37±1.06 | 2.62±1.19 | 2.12±1.81 |
| FB | 0.64±0.92 | 0.75±0.89 | 1.75±1.04 | 1.50±1.31 |
| BF | 1.45±1.29 | 0.88±0.83 | 1.62±0.52 | 0.88±1.36 |
| RCD | 0.55±0.93 | 0.38±0.52 | 0.38±0.52 | 0.50±0.53 |
| SH | 2.45±1.21 | 1.62±0.92 | 3.25±1.04 | 1.50±1.07 |
| LH | 0.27±0.65 | 0.50±0.76 | 1.12±1.36 | 0.50±0.76 |
| OPS | 0.00±0.00 | 0.38±0.52 | 0.38±0.74 | 0.00±0.00 |

TN -Total number of pathological spermatozoa, SF - Separated flagellum, KTF - Knob-twisted flagellum,

FB - Flagellum ball, BF - Broken flagellum, RCD - Retention of cytoplasmic drop, FT - Flagellum torso,

AC - Acrosomal changes, SH - Small head, LH - Large head, OPS - Other pathological spermatozoa

C – control group; T – trehalose; K – caffeine; G – glutathione

### Group 2 (2 mg/mL)

The examination demonstrated that over 80% of the observed spermatozoa showed normal morphology (Table 2). A higher number of normal spermatozoa was found in the group with trehalose addition (88.38%), the lowest number was detected after caffeine supplementation (82.25%). The dominant morphological abnormalities in all of the groups included a separated flagellum, knob-twisted flagellum, as well as flagellum torso in case of trehalose and a small head in the case of caffeine. Overall, no significant

differences were observed comparing the experimental groups with the control group.

Table 2. Spermatozoa morphology (in %) in groups with 2 mg/mL of supplement addition (mean±SD)

| Morphology | C2 | T2 | K2 | G2 |
|---|---|---|---|---|
| TN | 13.73±5.62 | 11.62±3.74 | 17.75±3.96 | 13.62±4.00 |
| AC | 0.82±0.75 | 1.25±1.16 | 1.12±1.13 | 1.62±0.92 |
| SF | 2.73±1.10 | 2.00±1.07 | 3.50±1.31 | 2.62±1.41 |
| KTF | 2.64±2.46 | 1.50±1.31 | 2.00±1.07 | 2.37±1.30 |
| FT | 2.18±1.47 | 2.37±1.06 | 2.62±1.19 | 2.12±1.81 |
| FB | 0.64±0.92 | 0.75±0.89 | 1.75±1.04 | 1.50±1.31 |
| BF | 1.45±1.29 | 0.88±0.83 | 1.62±0.52 | 0.88±1.36 |
| RCD | 0.55±0.93 | 0.38±0.52 | 0.38±0.52 | 0.50±0.53 |
| SH | 2.45±1.21 | 1.62±0.92 | 3.25±1.04 | 1.50±1.07 |
| LH | 0.27±0.65 | 0.50±0.76 | 1.12±1.36 | 0.50±0.76 |
| OPS | 0.00±0.00 | 0.38±0.52 | 0.38±0.74 | 0.00±0.00 |

TN -Total number of pathological spermatozoa, SF - Separated flagellum, KTF - Knob-twisted flagellum,

FB - Flagellum ball, BF - Broken flagellum, RCD - Retention of cytoplasmic drop,

FT - Flagellum torso, AC - Acrosomal changes, SH - Small head, LH - Large head,

OPS - Other pathological spermatozoa, C – control group; T – trehalose; K – caffeine; G – glutathione

Generally, no significant differences were observed ($p>0.05$), even though a tendency of a higher occurrence of morphological abnormalities with a higher dosage of the supplements (especially caffeine) was detected.

Discussing our results with other authors, our data showed that the highest percentage of morphologically intact spermatozoa in both groups was detected after trehalose supplementation. These results agree with the study of Yildiz et al. (2000). According to the authors, the proportion of spermatozoa with morphological abnormalities, especially damaged acrosomes was significantly reduced by sugars added to the extender, especially trehalose, galactose, lactose or sucrose. Furthermore, Woelders et al. (1997) showed that sugars have a protective influence against the morphological damage occurring in spermatozoa exposed to *ex vivo* conditions and that sucrose could be even more protective than trehalose for bull spermatozoa. Chen et al. (1993) stated that trehalose improved post-thaw survival of bovine spermatozoa stored at 25°C for 24h. Moreover Storey et al. (1998) reported that trehalose increased the proportion of intact mouse spermatozoa after cryopreservation.

Concerning caffeine, there are controversial data about its effects. Although caffeine may stimulate spermatozoa motility and activity, according to Pivko et al. (2009), it increased the occurrence of spermatozoa with swollen or damaged acrosome or spermatozoa with pseudoacrosomal reaction formed by exocytotic vesicles and accompanied with a loss of acrosomal content, leading to impaired membrane integrity and damages of spermatozoa head membranes. Also Harrison et al. (2003) reported that exposure of spermatozoa to high levels of caffeine may produce surface morphological damage which was more pronounced the longer spermatozoa spent incubated with caffeine. Disruption of the spermatozoa head and swelling of the mid-piece were the most

characteristic features. On the other hand, Barkay et al. (1984) state that no *in vitro* caffeine treatment of fertile donor semen does not damage the spermatozoa, as observed by the electron microscopy.

An efficiency of the addition of antioxidants such as glutathione into ram semen was demonstrated by Sarlos et al. (2002), as following the addition of these substances the frequency of spermatozoa abnormalities was decreased. Pivko et al. (2009) agree and state that glutathione, as an implementor added to insemination doses, did not influence the frequency of spermatozoa with intact head and acrosome but decreased the occurrence of swollen and damaged spermatozoa, ultimately leading to an improvement of spermatozoa membrane stability and maintenance of their functional state. Furthermore Lenzi et al. (1994) reported on a placebo-controlled crossover trial of 600 mg of glutathione, administered intramuscularly on alternate days, for a period of two months, to a group of 20 patients: ten with varicocele, and ten with 'germ-free genital tract inflammation'. The treatment resulted in a significant reduction in the proportion of forms with abnormal morphology.

**MDA Assay**

The MDA concentration as a marker of the lipid oxidative stress was the highest of the G2A group (68.40 μM) and the T1A group (60.36 μM). The lowest MDA concentration was recorded in the T2B group (45.85 μM) and the K1A group (47.40 μM). Significant results were not detected ($p>0.05$), neither in comparison with the control, nor within a concrete condition (Table 3). Based on this results we can assume that that the supplements did not have any effect on spermatozoa lipid peroxidation. On one hand, no enhanced peroxidation was observed, on the other hand no specific antioxidant properties of the substances were revealed.

Table 3. Malondialdehyde concentration (in μM) in the control/experimental groups (mean±SD)

| Group | Control | Trehalose | Caffeine | Glutathione |
|---|---|---|---|---|
| 1 mg/mL, 37°C | C1A | T1A | K1A | G1A |
| | 46.82±8.77 | 60.36±1.33 | 47.40±17.19 | 48.35±16.70 |
| 1 mg/mL, 5°C | C1B | T1B | K1B | G1B |
| | 53.94±13.41 | 58.76±11.97 | 49.55±18.10 | 57.04±11.62 |
| 2 mg/mL, 37°C | C2A | T2A | K2A | G2A |
| | 53.83±8.68 | 54.43±0.77 | 52.50±15.01 | 68.40±34.60 |
| 2 mg/mL, 5°C | C2B | T2B | K2B | G2B |
| | 50.26±13.31 | 45.85±3.57 | 54.10±7.23 | 50.12±8.65 |

Our resuts did not confirm the data from Aboagla and Terada (2003) who suggested that trehalose played a major role in increasing membrane fluidity, which led to greater endurance of spermatozoa against oxidative stress. Trehalose had a protective action related to the osmotic effect and to specific interactions with membrane phospholipids, thereby minimizing the degree of spermatozoa oxidative injury. Alternatively, the authors concluded that if trehalose directly protected lipids against peroxidation, there might be a diminished ROS propagation.

Arabi and Shahrokhie (2007) observed the effects of caffeine on lipid peroxidation of bovine spermatozoa without or with the presence of taurine or albumin. All the caffeine treatments showed a significant increase in the MDA level/LPO rate. Also, Arabi et al. (2003) investigated the effects of different concentrations of caffeine (5, 7

and 9 mM) on lipoperoxidation as a marker of spermatozoa integrity of normospermic men. They found out that caffeine is able to produce a mild state of oxidative stress and could be regarded as a potential infertility inducer. On the contrary our results show that tat the caffeine treatment led to a lower MDA concentration, which shows a certain protection of the spermatozoa lipids against oxidative stress development.

Brezezinska-Slebodzinska et al. (1995) studied the protective effects of reduced glutathione against lipid peroxidation in boar semen. The lipid peroxidation was measured by the TBARS assay doubled in the presence of the lipid peroxidation $Fe^{2+}$-sodium ascorbate-inducing system. The ascorbate-induced TBARS was inhibited by about 57% by glutathione. The protective role of glutathione with respect to boar semen against fatty acid peroxidation have been evidenced, even though we detected a higher oxidative state at 2 mg of glutathione supplementation.

## CONCLUSION

Our experiments analysis showed that morphological abnormalities of spermatozoa were elevated especially in case of higher trehalose, caffeine and glutathione concentrations. The TBARS assay revealed that the selected concentrations of the substances did not have a significant effect on lipid peroxidation as a process leading to oxidative stress development in spermatozoa. Based on these results we may conclude that all of the substances are not harmful for the spermatozoa viability and may be suitable for either a short-term (caffeine and glutathione) or a long-term (trehalose) spermatozoa cultivation. Still, only a few of our results were statistically significant, therefore further experiments have to be done with a higher variability in the supplements concentrations and cultivation conditions to see a more significant positive or negative effect on the spermatozoa morpoholgy characteristics and oxidative status.

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# UTICAJ TREHOLAZE, KAFEINA I GLUTATIONA NA SPERMATOZOIDE GOVEDA: 2. MORFOLOGIJA I OKSIDATIVNI STATUS *IN VITRO*

PETER MASSÁNYI, EVA TVRDÁ, MARTINA RAFAJOVÁ, NORBERT LUKÁČ

**Izvod**

Cilj ovog rada je bio da se ispita uticaj glutationa, treholaze i kafeina na morfološke parametre spermatozoida bikova, kao i lipidne peroksidacije, posle in vitro kultivacije tokom različitih perioda i na različitim temperaturama. Nije bilo značajnih razlika ($p>0.05$) u morfologiji spermatozoida, između eksperimentalnih grupa. TBARS isoitivanje nije pokazalo da postoji značajan uticaj ($p>0.05$) ispitivanih koncentracija trehaloze, kafeina i glutationa na lipidnu peroksidaciju, kao procesa koji dovodi do oksidativnog stresa kod spermatozoida.

**Ključne reči:** trehalosa, kafein, glutation, spermatozoidi, bikovi, morpolog, MDA.

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