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THE EFFECT OF TREHALOSE, CAFFEINE AND GLUTATHIONE ON BOVINE SPERMATOZOA: 1. MOTILITY *IN VITRO**

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SUMMARY: Results of this study show that trehalose, caffeine and glutathione supplementations have stimulating effects on spermatozoa motility parameters. Stimulating effects of trehalose were lasting over a longer time period, with recommendation of trehalose supplementation for a long-time spermatozoa cultivation. Caffeine and glutathione have the most markedly stimulating effects especially over the first 24 hours of cultivation, and they could be used as additives for a short-term in vitro spermatozoa preservation.

Key words: *trehalose, caffeine, glutathione, spermatozoa, bulls, motility.*

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 motility 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 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 in-

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semination 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).

The aim of this study was to examine the effects of glutathione, trehalose and caffeine on selected bovine spermatozoa motility parameters 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.

The 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.

Spermatozoa motility analysis was carried out using the Computer Assisted Sperm Analysis (CASA) system – SpermVision (MiniTüb, Tiefenbach, Germany) with Olympus BX 51 phase contrast microscope (Olympus, Japan). Each sample was placed into Makler Counting Chamber (depth 10 μm, 37±1°C; Sefi–Medical Instruments, Haifa, Israel) and the following parameters were evaluated: percentage of motile spermatozoa (motility>5 μm/s), percentage of progressive motile spermatozoa (motility > 20 μm/s), VCL (velocity curved line, μm/s) ALH (amplitude of lateral head displacement, μm) and BCF (beat cross frequency, Hz) with minimum 1000 cells examined in each sample (Massanyi et al., 2008).

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

The overall motility of spermatozoa cultured at 37°C and 5 °C is shown in Table 1. In all of the groups the highest motility was detected at time 0 and decreased over the course of time.

The overall spermatozoa motility was recorded as 78.56 – 46.08% in the C1 A/B groups and 86.34 – 43.94% in the C2 A/B groups.

The motility varied from 84.02% to 43.52% in the T1 groups and from 89.00% to 56.04% in the T2 groups. When comparing the motility between T1 and T2, no significant differences ($p>0.05$) were observed. Compared to the control groups, higher motility parameters were found in groups T2A and T2B during all of the time periods, as well as in the T1A group from 0h to 4 h.

The overall motility in the experimental caffeine groups decreased with time, however no significance was proved ($p>0.05$). An interesting observation was made in the motility development in both of the KA groups as it decreased after 1h and 2 h of culture, then surprisingly increased at 3h and 4h, with subsequent decrease.

Controversial results were detected in the experimental groups with glutathione addition. Insignificantly ($p>0.05$) higher motility parameters were detected in the G2A group at a time period from 0h to 4h when compared with the control. However, a significant decrease was recorded in the G2B group at 48h and 72h ($p<0.01$). No significant differences ($p>0.05$) were observed in the G1 groups compared to the controls, however relatively higher, even though insignificant values ($p>0.05$) were observed at timeframes from 0h to 4h at 37°C as well as at 24h/5°C.

Table1. Spermatozoa motility (in %) in groups and time periods (mean±SD)

Group	Time 0h, 37°C	Time 1h, 37°C	Time 2h, 37°C	Time 3h, 37°C	Time 4h, 37°C
			Time 0h, 37°C		
C1A	78.56±16.07	76.81±9.23	76.07±8.38	78.20±6.46	77.14±12.08
T1A	84.02±10.87	79.77±11.61	81.86±9.66	76.16±8.67	76.25±7.18
K1A	79.49±14.81	75.36±10.86	71.97±11.10	77.96±9.33	78.58±10.45
G1A	80.66±14.64	82.94±6.97	81.18±7.13	79.26±9.92	77.75±7.90
C2A	86.34±9.03	79.58±7.64	77.38±7.79	79.74±11.32	79.27±10.47
T2A	89.00±4.98	84.43±8.07	80.01±8.79	81.21±8.17	79.01±13.08
K2A	75.41±17.05	78.54±6.95	78.94±7.86	78.48±7.87	75.04±13.31
G2A	85.46±10.24	83.53±7.00	80.48±7.65	80.91±9.47	83.76±6.42

Group	Time 24h, 5°C	Time 48h, 5°C	Time 72h, 5°C	Time 168h, 5°C
C1B	76.60±11.66	75.03±13.64	75.75±14.61	46.08±14.93
T1B	71.72±9.96	72.74±16.75	75.84±13.68	43.52±15.92
K1B	69.80±12.26	71.82±16.57	68.81±17.05	28.33±16.48
G1B	77.48±10.34	70.61±18.76	65.76±18.04	42.35±14.85
C2B	76.72±9.77	79.11±12.65	77.30±11.07	43.94±27.06
T2B	78.30±10.12	81.03±8.84	74.78±15.73	56.04±25.05
K2B	74.66±10.98	69.47±17.60	68.35±14.57	33.49±12.54
G2B	72.50±14.00	58.13^{B}±22.92	57.34^{B}±17.10	40.47±10.96

C – control group; T – trehalose; K – caffeine; G – glutathione; 1 – 1 mg/mL of the supplement; 2 – 2 mg/mL of the supplement; $^{A}p<0.05$; $^{B}p<0.01$; ^{C} – $p<0.001$; X – mean; SD – standard deviation.

The overall progressive spermatozoa motility in the C2A/B groups (79.09 – 34.29%) was generally slightly higher than in the C1A/B groups (68.76 – 32.00%; Table 2).

No significant differences ($p>0.05$) were observed in medias with trehalose supplementation. In all cases, the highest progressive motility was recorded at time 0. The lowest progressive motility in the group T2 was detected after 168h. Nevertheless, comparing the data with the control, insignificantly ($p>0.05$) higher values were observed in all of the time periods, except for 4h and 72h. A similar decrease of progressive motility was observed in the T1 group. The progressive motility was relatively ($p>0.05$) higher at times 0h – 4h and at 72h.

Considering caffeine as a culture medium supplement, the K2 progressive motility decreased during the first two hours, subsequently it increased at time 3h, however it decreased afterwards, and the lowest progressive motility was detected at time 168h. No significant differences ($p>0.05$) were observed in this group. Equally, the lowest progressive motility rates were detected in the K1 groups at 168h, with a significant decrease ($p<0.05$) when compared with the control.

Similarly as in previous groups, the progressive motility rates in the glutathione groups were the highest at time 0h. A relatively higher progressive motility was detected in the T2 groups at times 1h, 2h, 3h and 4h. However afterwards, the progressive motility started to decrease radically, with a significant reduction ($p<0.001$) at time 48h. Later on, the decrease was non-significant ($p>0.05$). No significant increase or decrease of progressive motility was recorded in the T1 groups, even though the rates were relatively increased in the timeframe from 0h to 4h. As in the T2 groups, the progressive motility visibly decreased from time 24h and the lowest rates were observed at 168h.

Table 2. Progressive spermatozoa motility (in %) in groups and time periods (mean±SD)

Group	Time 0h, 37°C	Time 1h, 37°C	Time 2h, 37°C	Time 3h, 37°C	Time 4h, 37°C
C1A	68.76±17.70	68.17±9.96	69.10±7.59	67.09±8.09	66.88±14.29
T1A	75.83±11.06	73.61±12.95	71.77±10.88	67.88±10.86	67.13±9.29
K1A	71.70±13.72	67.71±11.55	64.40±10.53	68.93±8.66	67.98±12.91
G1A	73.96±13.45	74.45±8.32	71.94±7.00	69.76±10.43	67.47±9.51
C2A	79.07±11.12	71.68±7.51	66.73±9.88	71.48±12.66	71.52±8.92
T2A	80.80±5.00	76.50±7.85	70.84±10.83	71.85±8.87	69.91±15.28
K2A	68.48±16.96	69.44±7.76	69.58±9.02	70.67±7.61	65.62±14.02
G2A	76.98±10.65	74.85±8.72	70.61±9.64	72.43±10.10	72.00±8.76

Group	Time 24h, 5°C	Time 48h, 5°C	Time 72h, 5°C	Time 168h, 5°C
C1B	65.79±13.29	62.72±15.16	63.83±15.91	32.00±14.00
T1B	60.40±11.31	60.87±16.39	64.43±15.75	28.78±14.03
K1B	58.87±12.91	61.07±17.05	58.77±17.58	13.45^{A}±9.67
G1B	63.91±11.42	54.09±17.46	53.72±18.30	22.70±7.11
C2B	66.51±11.55	67.37±12.85	64.37±13.10	34.29±25.68

T2B	67.19±9.96	68.07±13.17	63.48±17.57	41.26±23.51
K2B	65.29±11.39	55.27±16.61	54.17±14.74	19.15±11.96
G2B	59.80±15.56	44.05^{C}±22.28	40.68±18.59	19.96±10.96

C – control group; T – trehalose; K – caffeine; G – glutathione; 1 – 1 mg/mL of the supplement; 2 – 2 mg/mL of the supplement; $^{A}p<0.05$; $^{B}p<0.01$; $^{C}p<0.001$; X – mean; SD – standard deviation.

The VCL was elevated in all of the T groups with a significant increase ($p<0.001$) in the T2A group at time 4h, as compared to the control. A relative VCL increase was observed in the C2 and G2 media at times 2h, 3h and 4h, however a significant ($p<0.05$) decrease was recorded in both experimental media at time 72h. Regarding groups C1 and G1, a significant difference was not detected, even though a relative increase was observed in all of the experimental groups, especially at times 0h, 1h, 3h, 4h and 72h (Table 3).

Table3. Spermatozoa velocity curved line (VCL; in μm/s) in groups and time periods (mean±SD)

Group	Time 0h, 37°C	Time 1h, 37°C	Time 2h, 37°C	Time 3h, 37°C	Time 4h, 37°C
C1A	98.00±13.75	119.72±16.66	121.54±18.41	109.07±9.90	109.41±15.28
T1A	95.38±10.97	130.41±24.27	118.43±20.49	117.20±16.02	119.21±22.51
K1A	99.52±17.86	124.10±22.45	121.20±17.29	113.22±15.74	114.29±16.61
G1A	106.16±23.31	131.24±15.42	128.43±18.48	124.83±16.29	118.89±16.96
C2A	117.44±15.99	131.04±19.90	121.44±19.19	121.32±27.93	119.93±24.49
T2A	117.69±28.69	128.73±10.55	130.92±17.62	130.17±21.16	122.35^{C}±24.96
K2A	104.74±18.73	119.74±11.82	127.44±14.30	130.65±21.20	123.44±24.27
G2A	103.75±13.73	128.01±14.28	130.07±13.62	132.23±12.29	126.91±17.81

Group	Time 24h, 5°C	Time 48h, 5°C	Time 72h, 5°C	Time 168h, 5°C
C1B	118.66±17.21	27.70±24.02	18.11±2.64	15.94±17.56
T1B	116.70±17.19	25.86±21.38	19.58±2.83	16.36±18.11
K1B	116.53±15.29	16.98±16.33	13.22±1.91	28.14±37.21
G1B	111.25±13.67	20.28±19.01	16.34±2.36	14.51±17.67
C2B	127.37±18.85	14.20±11.47	14.71±2.10	23.22±24.81
T2B	124.24±26.15	18.15±15.44	22.04±3.18	12.09±12.96
K2B	117.37±21.92	15.87±15.72	15.54±2.24	17.61±24.00
G2B	124.64±27.09	20.86±20.31	24.02±3.47	12.59±16.76

C – control group; T – trehalose; K – caffeine; G – glutathione; 1 – 1 mg/mL of the supplement; 2 – 2 mg/mL of the supplement; $^{A}p<0.05$; $^{B}p<0.01$; $^{C}p<0.001$; X – mean; SD – standard deviation.

Analyzing the ALH, significant ($p<0.05$ and $p<0.01$ respectively) increases in all of the experimental groups A at time 2h was detected. Other important, but not significant differences ($p>0.05$) were detected at times 0h, 1h, 3h, 4h and 24h. Focusing on the B group we observed no significant differences ($p>0.05$), however a relative increase was observed in all of the media over the first 24 hours (Table 4).

Table 4. Spermatozoa amplitude of lateral head displacement (ALH; in μm) in groups and time periods (mean±SD)

Group	Time 0h, 37°C	Time 1h, 37°C	Time 2h, 37°C	Time 3h, 37°C	Time 4h, 37°C
C1A	4.57±1.15	4.78±0.94	4.76±0.97	4.51±0.84	4.57±0.84
T1A	4.90±0.73	5.35±0.95	5.01±1.00	4.67±0.88	4.67±0.87
K1A	4.90±1.00	5.17±0.99	4.73±0.86	4.69±0.98	4.59±0.81
G1A	4.63±0.87	5.51±0.70	5.26±0.78	5.18±1.13	5.08±0.96
C2A	5.37±0.70	5.15±1.16	4.78±1.07	4.91±1.31	4.84±0.93
T2A	5.39±0.43	5.47±1.01	5.66^{A}±0.41	5.31±0.61	5.01±0.70
K2A	5.14±0.82	5.46±0.88	5.70^{A}±0.92	5.40±0.93	5.20±0.94
G2A	4.93±0.78	5.30±0.84	5.81^{B}±0.62	5.64±0.74	5.66±0.65

Group	Time 24h, 5°C	Time 48h, 5°C	Time 72h, 5°C	Time 168h, 5°C
C1B	5.24±0.78	5.51±0.95	5.65±0.83	4.88±0.47
T1B	5.37±0.72	5.74±1.04	5.83±0.80	4.75±0.54
K1B	5.31±1.18	5.13±0.94	5.27±0.69	3.84±1.29
G1B	5.67±0.52	5.45±0.81	5.63±0.72	4.28±0.62
C2B	5.72±0.85	5.97±0.74	5.96±0.75	4.91±1.10
T2B	5.90±0.71	5.95±0.80	6.09±0.86	5.17±0.50
K2B	5.73±0.94	5.06±0.72	5.11±0.75	4.11±0.65
G2B	6.13±0.94	5.18±0.97	5.11±0.93	4.25±0.57

C – control group; T – trehalose; K – caffeine; G – glutathione; 1 – 1 mg/mL of the supplement; 2 – 2 mg/mL of the supplement; $^{A}p<0.05$; $^{B}p<0.01$; $^{C}p<0.001$; X – mean; SD – standard deviation.

Relatively controversial results were obtained for the BCF parameter in the A group. No significant differences ($p>0.05$) were observed in the T2 group. Nevertheless, a significant decrease was recorded in the K2 group at 48h ($p<0.05$) and in the G1 group at 2h ($p<0.05$). No significant differences ($p>0.05$) in the BCL parameter were observed in any of the experimental B groups when compared to the control (Table 5).

Table 5. Spermatozoa beat cross frequency (BCF; in Hz) in groups and time periods (mean±SD)

Group	Time 0h, 37°C	Time 1h, 37°C	Time 2h, 37°C	Time 3h, 37°C	Time 4h, 37°C
C1A	26.50±2.74	27.93±4.81	27.53±5.13	28.02±4.55	28.74±5.96
T1A	25.36±1.63	25.99±3.54	26.74±4.86	27.48±5.10	28.28±4.47
K1A	24.56±2.36	26.51±4.11	28.89±4.67	28.22±4.13	29.55±4.80
G1A	27.07±3.65	25.94±4.08	25.91±4.25	26.72±4.99	26.72±5.54
C2A	26.47±2.15	27.63±5.18	27.82±5.21	26.79±4.48	27.07±5.04
T2A	26.41±3.51	26.03±4.57	23.94±1.51	24.79±2.23	25.79±3.36
K2A	24.28±2.43	25.59±3.57	24.84±3.84	26.32±4.86	26.74±4.29
G2A	26.19±2.91	25.84±3.10	23.60^{A}±1.53	24.41±2.91	25.04±3.53

Group	Time 24h, 5°C	Time 48h, 5°C	Time 72h, 5°C	Time 168h, 5°C
C1B	25.62±4.17	23.54±3.34	23.24±3.65	20.20±2.31
T1B	23.05±3.41	23.20±3.85	22.36±3.46	21.41±3.30

K1B	24.38±5.79	24.09±3.40	24.18±3.76	19.99±5.93
G1B	22.35±2.65	22.67±2.49	22.05±2.66	21.58±1.93
C2B	23.82±3.84	23.27±2.76	23.24±3.00	19.72±2.41
T2B	22.83±2.88	22.02±2.26	22.10±3.01	21.29±2.28
K2B	23.26±3.89	23.44^{A}±3.06	22.93±2.34	21.50±3.27
G2B	21.77±2.47	20.82±2.79	22.23±2.89	20.84±2.62

C – control group; T – trehalose; K – caffeine; G – glutathione; 1 – 1 mg/mL of the supplement; 2 – 2 mg/mL of the supplement;

$^{A}p<0.05$; $^{B}p<0.01$; $^{C}p<0.001$; X – mean; SD – standard deviation.

Generally, we observed a stimulation of all of the observed motility parameters, even though the stimulation was mostly non-significant ($p>0.05$). The few significant differences as well as occasional decreases in the motility parameters may have been caused by individual characteristics and viability parameters of the samples.

The effect of trehalose in our study was mainly stimulating. All of the motility parameters were increased when compared to the control, some of them significantly, especially during a long-time cultivation. Several investigators have found that the incorporation of trehalose in semen diluents can protect and stimulate the spermatozoa of many species. Woelders et al. (1997) demonstrated that an isotonic sugar medium containing sucrose and trehalose is significantly superior to a standard egg yolk medium in preserving the motility and acrosome integrity of bovine spermatozoa. These results were confirmed by Knazicka et al. (2010) who studied the effects of different energetic substrates used in culture media on bovine spermatozoa motility parameters during a 24-hour *in vitro* cultivation. The authors stated that the maximum viability was recorded while applying glucose (5%), sacharose (5%) and trehalose (1%), pointing out the beneficial effects of trehalose as a potential substance to prevent oxidative stress not only in freezing-thawing medias but also in culture medias used for a short-term cultivation and examination of spermatozoa. Moreover, in a comparison of raffinose and trehalose, Storey et al. (1998) showed that trehalose brings about a significantly better recovery rate in intact mouse spermatozoa. Additionally Aisen et al. (2002) observed that trehalose significantly improved the viability of ram spermatozoa assessed for motility and acrosome integrity.

Despite having evidence in favour of the beneficial aspects of trehalose, not all studies have found the same results. Chen et al. (1993) report that trehalose caused only minor improvement in bull spermatozoa survival, which is in the closest accordance with our data. Furthermore, it is now known that only adequate concentrations of trehalose are beneficial for spermatozoa survival as its high concentrations result to be toxic. Aisen et al. (2002) observed a favorable effect of reduced trehalose concentrations on spermatozoa motility and a deleterious effect of greater trehalose concentrations, which was consistent with observations by Hu et al. (2009) carried out in boar semen. In a later study by Hu et al. (2010), the greatest protective effects of trehalose were detected at the concentration of 100 mM, and a more reduced extent at 200 mM. The latter concentration resulted in increased osmolarity of the extender, which was in itself deleterious to the spermatozoa (Hu et al., 2009; 2010).

Regarding caffeine as a supplement for *in vitro* spermatozoa cultivation, we observed a stimulation of the motility parameters, especially over the first time periods,

from which we can conclude that caffeine could be an effective supplement for short-term preservation of spermatozoa. Moreover, various stimulating effects of caffeine on animal spermatozoa have been reported before. Positive effects of caffeine on the bovine semen at a concentration of 2.5 mmol/L were confirmed by Bird et al. (1989). Caffeine may also promote capacitation and/or acrosome reaction of boar spermatozoa and, when added to the fertilization medium, it accelerates spermatozoa penetration *in vitro* in pigs (Nagai et al., 1993) and mice (Fraser, 1979). At 10 mmol/L, caffeine increased rabbit spermatozoa motility after 24 h of semen refrigeration, whilst lower concentrations (2.5 or 5 mmol/L) did not affect spermatozoa motility (Lopez and Alvarino, 2000). According to Spalekova et al. (2011) the total and progressive motility of ram spermatozoa increased after 24h of incubation in presence of 1 or 4 mmol/L of caffeine.

However, along with stimulating effects, a number of negative effects of caffeine have been described as well. In humans caffeine concentrations greater than 2.5 mmol/L may adversely affect the spermatozoa fertilization and cleavage of embryos derived from such spermatozoa (Imoedemhe et al., 1992). Similarly, the results of Aitken et al. (1983) indicate that caffeine at concentrations of 5 mmol/L and above may have potential toxic effect on human spermatozoa. The fertilizing capacity of the normal human spermatozoa exposed to caffeine did not appear to be enhanced at low concentrations, whereas at higher concentrations (5 mmol/L) the capacity was adversely affected despite improving the motility (Imoedemhe et al., 1992). Furthermore, caffeine at higher concentration may cause a reduction of bovine spermatozoa motility (Bird et al., 1989), therefore, could be recommended to use caffeine-free fertilization media for insemination with capacitated spermatozoa in bovine IVF (Momoyawa and Fukuda, 2003). The effects of caffeine on spermatozoa characteristics may be species-specific. Caffeine at concentration of 5 mmol/L may cause decrease in fertilization capacity of human or bovine spermatozoa, however higher concentrations (10 mmol/L) may cause increased motility parameters in rabbits. Nevertheless, this motility increase did not lead to an improvement of the reproductive parameters.

Based on our spermatozoa motility analysis glutathione acts generally as a stimulating supplement as well. However we found controversial results at various time periods, which are in accordance with some previous studies. On one hand, Munsi et al. (2007) who examined the effects of several glutathione concentrations on bovine spermatozoa viability stored at 4-8°C during 5 days. The motility was significantly higher with the addition of 0.5 mM of glutathione. After 5 days, the optimal motility (over 50%) was recorded at concentrations from 0.5 to 2.0 mM, meanwhile at 3.0 mM the motility decreased significantly. These conclusions are proved by our results as well. Results of Triwulanningsih (2003) showed that the addition of 0.5 mM glutathione to the spermatozoa diluent medium was sufficiently effective in the production of liquid semen. Moreover Kim *et al.* (1999) reported that the addition of 1 mM glutathione into a fertilization medium during an *in vitro* fertilization improved its effectiveness.

On the other hand, Donnely et al. (2000) report that supplementation with glutathione to spermatozoa preparation media did not significantly improve spermatozoa motility. Additionally according to Whitaker (2008) a 5.0 mM glutathione supplemented treatment indicates that GSH may reduce the motility of the spermatozoa. Authors concluded that the decrease in motility could be related to the effect of glutathione on the function of the spermatozoa flagellum.

CONCLUSION

Our results show that trehalose, caffeine and glutathione supplementations have stimulating effects on spermatozoa motility parameters. We can conclude that the stimulating effects of trehalose were lasting over a longer time frame, which is why we would recommend trehalose supplementation for a long-time spermatozoa cultivation. Caffeine and glutathione had the most markedly stimulating effects especially over the first 24 hours of cultivation, which is why they could be used as additives for a short-term *in vitro* spermatozoa preservation. Nevertheless, very few of our results were significant, therefore further experiments should be done with a higher variability of trehalose, caffeine and glutathione concentrations and under different conditions to see a more significant positive or negative effect on spermatozoa motility characteristics.

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UTICAJ TREHOLAZE, KOFEINA I GLUTATIONA NA SPERMATOZOIDE BIKA: 1. POKRETLJIVOST *IN VITRO*

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Izvod

Rezultati ovog rada pokazuju da dodavanje treholaze, kofeina i glutationa u spermu bika, ima stimulativni efekt na parametre pokretljivosti spermatozoida. Efekt treholaze traje duže, pa se dodavanje treholaze preporučuje za dugotrajniju kultivaciju spermatozoida. Kofein i glutation ispoljavaju značajno delovanje samo tokom prvih 24h, pa se dodavanje ovih supstanci preporučuje za kratkotrajno čuvanje spermatozoida *in vitro.*

Ključne reči: trehalose, caffeine, glutathione, spermatozoid, bik, pokretljivost.

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