CONTEMPORARY AGRICULTURE / *SAVREMENA POLJOPRIVREDA* 62(3-4)139-148, 2013.
UDC: 63(497.1)-“540-2” ISSN 0350-1205

UDC: 581.35:599.735.5

THE EFFECT OF BOVINE SERUM ALBUMIN IN CULTURE MEDIA ON BOVINE SPERMATOZOA MOTILITY PARAMETERS *IN VITRO**

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SUMMARY: The aim of this study was to analyze the influence of bovine serum albumin (BSA) used in different culture media on motility and viability of bovine spermatozoa during a 24-hour in vitro cultivation. We compared native samples (N) and samples cultivated in a commercial egg yolk medium (R) with samples cultivated in several experimental media containing 10% or 20% BSA. The analysis was carried out during three time periods (time 0, time 1 hour and time 24 hours) using Sperm VisionTM CASA system. Our study shows an obvious time-dependent decrease of the spermatozoa viability parameters in all experimental groups cultured for 24 hours. The lowest spermatozoa motility parameters were detected in native sample. Culture media with 10% BSA content showed only average quality and unsatisfactory results. Overall best motility parameters were detected when applying 20% BSA, especially in the medium D containig triladyl, 20% BSA and 5% glucose. Comparing results detected in this group with the results from the commercial egg yolk medium significant (P<0.01) differences for progressive motility, as well as for distance average path (P<0.001), velocity average path (P<0.001) and amplitude of lateral head displacement (P<0.001) were detected. This study demonstrates that BSA could be a good protein suplement for long-term bovine spermatozoa cultivation and further processing.

Key words: *BSA, spermatozoa cultivation, protein supplementation, bulls, CASA.*

Original scientific paper / *Originalni naučni rad*
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*This work was supported by the Scientific Agency of the Slovak Republic VEGA no. 1/0532/11 and by the SF EU ITMS 26220220180.

INTRODUCTION

Artificial insemination (AI) has become one of the most important pillars in animal biotechnology. Especially in the cattle AI industry, 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; Lukáč et al., 2007). Before preservation and distribution for usage in AI, several laboratory tests are routinely conducted in an attempt to predict the fertilization potential of the processed semen. Such tests include but are not limited to sperm motility or viability, total sperm output, and morphology (Ibrahim et al., 2000). Working with semen samples in laboratory environment requires a provision of optimal conditions for their *in vitro* cultivation.

In vitro cultivation of spermatozoa is a relatively complicated process, since the sperm cells are extremely sensible to *ex vivo* conditions. Therefore, preparing a culture medium, which ensures high spermatozoa motility and viability during extended periods of cultivation, is complicated (Balaban et al., 1999). Semen cultivation media ususally contain glucose or fructose as an energetic substrate, egg yolk as a protein supply and glycerol (McPhie et al., 2000; Matsuoka et al., 2006). However, preparation of a uniform semen cultivation medium may vary because of the quality of the egg yolk. Also, extended cultivation changes the structure of egg yolk and decreases its quality, what complicates the obtention of relevant results. For this reason, investigations have been conducted to find an alternative protein substrate for spermatozoa cultivation (Muller–Schlosser et al., 1995).

Bovine serum albumin (BSA) has been recently used as a protein alternative to egg yolk. It is a large globular protein (66 000 Da) with a good essential amino acid profile (Peters et al., 1977), which has been added to culture media of various mammalian cells to mimic some growth enhancing effects of serum. Previous studies have reported that the role of albumin appears to be protective as a result of its general capacity and ability to trap toxic substances in the culture media (Yamane et al., 1976) and lipid binding properties (Fox and Kelly, 2003). It may play a role in mediating lipid oxidation, since BSA has been shown to protect lipids against phenolic induced oxidation *in vitro* (Smith et al., 1992). Investigations using semen from various mammalian species have indicated that BSA stimulates sperm motility by an unknown mechanism (Harrison et al., 1982; Klem et al., 1986).

Most of the experiments have been designed to look at the function of BSA on sperm capacitation, fertilization and cryoconservation hence there is a lack of information about the effect of BSA as a culture medium component on the general spermatozoa *in vitro* viability.

Extended spermatozoa cultivation is essential for studies related with questions of male infertility origin. The aim of such analysis is to see how spermatozoa viability or fertilization capacity is changed by administration of a certain substance (i.e. heavy metals, antioxidants or growth factors; Lovásová et al., 2002) or under the influence of a specific factor (i.e. UV radiation, nutrition; Formicki et al., 2010). Therefore, results of such experiments could be helpful in further prevention and possible treatment of infertility.

This study was designed to test the effects of two BSA concentrations (10% and 20%) on the bovine spermatozoa motility parameters during a 24 hour *in vitro* cultivation.

MATERIAL AND METHODS

Bovine semen samples were obtained from 15 adult breeding bulls (Slovak Biological Services, Nitra, Slovak republic). The samples had to accomplish the basic criteria given for the corresponding breed. Semen was obtained on a regular collection schedule using an artificial vagina.

After collecting the samples were stored in the laboratory at room temperature (22-25°C) and basic measurements were performed – volume (ml), pH and concentration (x 10^9/ml). Basic characteristics of the samples are shown in Table 1.

Table 1. Basic characteristics of bull semen samples used in the experiment

No	Breed	Volume [ml]	pH	Concentration [x 10^9 ml^{-1}]
1	Limousine	7.0	6.48	5.20
2	Pinzgau	8.0	6.44	5.70
3	Holstein	4.5	6.35	2.80
4	Fleckvieh	6.5	6.59	2.10
5	Fleckvieh	5.5	6.40	1.90
6	Fleckvieh	5.0	6.14	1.02
7	Holstein	8.0	6.30	2.50
8	Holstein	5.0	6.44	2.50
9	Holstein	8.0	6.50	4.90
10	Holstein	6.0	6.32	2.20
11	Holstein	6.0	6.52	2.20
12	Holstein	7.0	6.18	2.30
13	Holstein	6.0	6.21	1.30
14	Holstein	7.0	6.24	5.30
15	Holstein	10.0	6.15	2.60

Six cultivation media with a different composition (Table 2) were prepared, five of them containing BSA (Fluka, Sigma-Aldrich, USA). Fresh semen was added to each medium in a dilution ratio 1:40 and subsequently cultivated in an incubator (37°C) for 24 hours. Semen diluted in physiological saline solution (sodium chloride 0.9% w/v, Bieffe Medital, Italia) was used as native sample.

Table 2. Culture media used in the experiment

Group	Composition
N - Native group	Native sample diluted in physiological saline solution.
R - Commercial medium	Triladyl (MiniTüb, Tiefenbach, Germany), egg yolk and redistilled water.
A	Triladyl, 10% BSA (Sigma Alrdrich, St. Louis, USA), 5% glucose (D-glukosa monohydrat p.a; Penta, Chrudim, Czech republic) and distilled water.
B	Triladyl, 10% BSA, 5% glucose, 1% trehalose (D(+)-trehalose; Sigma-Aldrich, USA) and distilled water.
C	Triladyl, 20% BSA and distilled water.
D	Triladyl, 20% BSA, 5% glucose and distilled water.
E	Triladyl, 20% BSA, 5% glucose, 1% trehalose) and distilled water.

Motility analysis was carried out using the CASA – SpermVision (MiniTüb, Tiefenbach, Germany) system with Olympus BX 51 phase microscope (Olympus, Japan) at cultivation times 0 hour, 1 hour and 24 hours. Each sample was placed into Makler Counting Chamber (deph 10 µm, Sefi-Medical Instruments, Izrael) and the following parameters were evaluated: percentage of motile spermatozoa (motility > 5µm/s), percentage of progressive motile spermatozoa (motility > 20µm/s), distance average path (DAP, µm), velocity average path (VAP, µm/s) and amplitude of of lateral head displacement (ALH, µm).
Statistical analysis was carried out using the statistical program GraphPad Prism 3.02 (Graphpad Software incorporated, San Diego, California, USA). Results are quoted as arithmetic mean±standard deviation (SD). Comparison of semen parameters in groups was evaluated with the Dunnett's Post Test. The level of significance was set at A ($P<0.001$); B ($P<0.01$); C ($P<0.05$).

RESULTS AND DISCUSSION

In vitro effects of different BSA concentrations on bovine spermatozoa motility parameters were analyzed in three time periods (Time 0 hours, 1 hour, 24 hours).
Evaluation of the percentage of motile spermatozoa showed decreased values in all experimental groups cultured for 24 hours (Table 3). Initial (time 0) spermatozoa motility was significantly ($P<0.001$) high in the group D containing 20% BSA, and 5% glucose, compared to group R containig the egg yolk commercial medium. This observation could be explained by an initaially high concentration of energetic and protein substrate in the medium. The highest inhibitory effect of spermatozoa motility after 24 hours was detected in the group N (28.86%; $P<0.001$), followed by group B (42.89%; $P<0.001$) containing 10% BSA, 5% glucose and 1% trehalose. The lowest motility inhibition was found in group D (66.46%) and group R (62.81%).

Table 3. Spermatozoa motility (%) in groups and time periods (mean±SD)

24 h	0 h	1 h	
N/MOT	87.31±5.16	85.66±2.35	28.86±9.09^{A}
R/MOT	83.86±6.57	87.23±5.28	62.81±8.04
A/MOT	89.99±2.96	91.07±4.12	52.28±16.12^{B}
B/MOT	92.62±3.37	83.25±5.81	42.89±13.17^{A}
C/MOT	96.15±1.49	84.43±13.15	55.41±13.96
D/MOT	97.39±2.03^{A}	91.29±3.77	66.46±9.99
E/MOT	87.46±3.67	89.17±4.39	57.75±12.87

x – mean, S.D. – standard deviation
^{A} P<0.001, ^{B} P<0.01, ^{C} P<0.05

Different observarions were made in the percentage of progressive spermatozoa motility (Table 4). The initial progressive motility was significantly (P<0.001) high in group D compared to group R. The parameter descreased slowly in all groups after 1 hour but a significant (P<0.01) difference between groups A (10% BSA and 5% glucose) and D compared to group R was detected. A radical spermatozoa progressive motility inhibition was observed after 24 hours in all of the groups. Comparing experimental medias with the commercial medium we recorded a significant (P<0.01) difference between group D and group R. Group A, which appeared to have good progressive motility parameters after 1 hour (87.18%), showed just an average progressive motility (37.79%) after 24 hours. The lowest progressive spermatozoa motility was detected in the group N (14.67%). The highest parameters were found in the group D (55.67%; P<0.001) - it was indeed the only group presenting a progressive motility over 50 %.

Table 4. Spermatozoa progressive motility (%) in groups and time periods (mean±SD)

Group	0 h	1 h	24 h
N/PROG	86.65±5.41	77.97±4.15	14.67±5.968^{A}
R/ PROG	80.99±5.54	78.55±6.47	35.33±11.44
A/ PROG	87.65±1.59	87.18±4.42^{B}	37.79±12.31
B/ PROG	89.71±2.95	79.52±8.12	26.43±10.33
C/ PROG	93.20±2.13	79.18±13.59	37.33±16.12
D/ PROG	93.85±2.05^{A}	89.08±3.99^{B}	55.76±12.06^{A}
E/ PROG	86.79±3.54	83.41±4.96	45.27±11.58^{B}

x – mean, S.D. – standard deviation
^{A} P<0.001, ^{B} P<0.01, ^{C} P<0.05

The DAP analysis revealed significant differences (P<0.001) between groups C (20% BSA) and D as compared to the R group at time 1 as well as at time 24 (39.66 and 34.24 μm versus 27.43 μm, 20.66 and 20.31 μm versus 14.24 μm, respectively). Distance average path after 24 hours was the shortest in the group R and the longest in the group C (Table 5).

Table 5. Spermatozoa distance average path (µm) in groups and time periods (mean±SD)

Group	0 h	1 h	24 h
N/DAP	45.59±3.91	27.43±0.61	14.24±1.36
R/DAP	38.36±11.86	28.70±1.56	14.88±1.92
A/DAP	43.37±1.25	37.06±3.39	14.63±3.49
B/DAP	42.50±2.25	35.98±4.29	14.86±1.88
C/DAP	37.12±2.11	39.66±3.48^{A}	20.66±2.86^{A}
D/DAP	31.22±0.63	34.24±1.51^{A}	20.31±2.62^{A}
E/DAP	40.82±6.71	32.62±2.41	16.85±2.13

x – mean, S.D. – standard deviation

^{A} P<0.001, ^{B} P<0.01, ^{C} P<0.05

Measurement of spermatozoa VAP (Table 6) after 1 hour demonstrated significantly (P<0.001) higher values in groups A, B and especially in group C when compared to group R (83.80, 81.28 and 90.70 µm/s respectively versus 62.88 µm/s). After 24 hours of cultivation, VAP decreased in all of the groups but significant differences were found in groups C (45.04 µm/s) and D (43.45 µm/s) with the highest values of VAP compared to the commercial medium group with the lowest value (31.25 µm/s).

Table 6. Spermatozoa velocity average path (µm/s) in groups and time periods (mean±SD)

Group	0 h	1 h	24 h
N/VAP	104.40±9.38^{A}	62.88±2.17	31.25±3.55
R/VAP	86.45±27.00	66.97±4.12	32.30±4.65
A/VAP	101.90±2.23^{A}	83.80±8.21^{A}	32.51±9.05
B/VAP	100.00±4.46^{A}	81.28±11.11^{B}	31.86±3.79
C/VAP	88.12±4.68	90.70±6.77^{A}	45.04±6.57^{A}
D/VAP	72.92±1.83^{B}	77.66±4.18	43.45±5.73^{A}
E/VAP	91.98±15.45	77.51±5.64	36.93±3.99

x – mean, S.D. – standard deviation

^{A} P<0.001, ^{B} P<0.01, ^{C} P<0.05

Important statistical differences were observed in the amplitude of lateral displacement (Table 7). ALH was significantly (P<0.001) higher in all experinmental groups compared to the commercial medium. Similar results were observed after 1 hour with the exception of group N. Significant (P<0.001) differences were observed in groups B, C, D (with the highest value of 3.616 µm) and E compared to R group with the lowest vaue of 2.264 µm. The decrease of ALH in the group N slowed down, revealing significantly (P<0.05) higher values compared to the R group after 24 hours.

Table 7. Spermatozoa amplitude of lateral displacement (μm) in groups and time periods (mean±SD)

Group	0 h	1 h	24 h
N/ALH	3.05±0.41	4.23±0.16	2.88±0.21^{C}
R/ALH	3.13±0.76	4.04±0.15	2.26±0.21
A/ALH	5.39±0.11^{A}	5.14±0.51^{A}	2.39±0.43
B/ALH	5.03±0.20^{A}	5.20±0.58^{A}	3.20±0.29^{A}
C/ALH	5.68±0.34^{A}	5.85±0.54^{A}	3.53±0.67^{A}
D/ALH	5.76±0.18^{A}	5.10±0.38^{A}	3.62±0.56^{A}
E/ALH	3.97±0.56^{A}	5.92±0.37^{A}	3.56±0.28^{A}

x – mean, S.D. – standard deviation
^{A} $P<0.001$, ^{B} $P<0.01$, ^{C} $P<0.05$

The results demonstrate that spermatozoa samples cultivated in media containig BSA had better viability parameters when compared to the commercial medium based on egg yolk. Overall best viability parameters were observed in the medium consisting of triladyl, 20% BSA, 5% glucose and distilled water.

Proteins have been extensively employed as a supplement for culture media used to handle and cultivate spermatozoa, gametes and embryos. Protein molecules provide nutrients and protection to the cell, behave as colloids in solution and contribute to the osmotic pressure of fluids (Gebauer et al., 1970; Correa–Pérez et al., 2003).

The discovery that egg yolk has a beneficial effect on fertility led to its widespread use in bull semen extenders (Pace and Graham, 1974). Additionally it has been widely regarded as an essential ingredient for freezing diluents of bovine semen, apparently providing protection to spermatozoa membranes against cold shock and damage during cooling, freezing and thawing (Foulkes et al., 1980). Still, it is difficult to produce egg yolk semen diluents of uniform quality, because of individual quality differences of the yolk. Egg yolk is also relatively unstable for extended periods of time because of high content of fatty acids sensitive to degradation. Sperm cells usually form clusters making it almost impossible to perform an adequate analysis. Therefore, it seems that removal of chicken egg yolk from semen diluents offers several advantages, such as consistency improvement and elimination of various patogens (Muller–Schlosser et al., 1995; Matsuoka et al., 2006).

BSA could be a good protein alternative because of its stability, nutritional quality and protective functions (Bakst and Cecil, 1992). There are several studies focusing on the possible effects of BSA on spermatozoa viability in different animal species.

Harrison et al. (1982) washed and diluted rabbit and ram spermatozoa in media containing various protein substances. BSA was found to be better than polyvinylpyrrolidone, ovalbumin, or alpha-lactalbumin at stimulating and maintaining motility levels as well as reducing the tendency of washed spermatozoa to stick to glass. According to Matsuoka et al. (2006) the rates of progressive motility in post-thawn ram semen were significantly higher in media containing BSA than in the Tris-fructose-egg yolk control group. Also, the spermatozoa viability was significantly improved and the rates of intact acrosomes were higher. Yamashiro, et al. (2006) found that collection of goat semen into tubes containing BSA resulted in improvement of the sperm freezability, higher sperm motility and intact acrosomes. Moreover, a field trial by Fukui, et al. (2008) revealed that a semi defined semen extender containing BSA provides a fertility potential after intrauterine insemination higher to that achieved with semen extender containing

egg yolk in sheep. On the contrary, Serniene et al. (2001) state that the addition of BSA was not significant for boar spermatozoa motility, vigor rate and number of viable and non-damaged spermatozoa per ejaculation.

Our results confirm the stimulating effects of BSA towards selected measurable characteristics describing bovine spermatozoa motility. As media containing 20% BSA presented the overall best viability results, we can assume that this could be the optimal BSA concentration for bovine spermatozoa preservation. Lower BSA concentration (10%) did not ensure enough protection for spermatozoa during cultivation with a very similar effectiveness to the egg yolk control. There is no detailed information on the BSA concentration in semen diluents for any species, which would allow us a direct comparison with our data. Our results agree with the conclusion of Uysal and Bucak suggesting that 20 mg/ml BSA was the best concentration improving post-taw motility and viability of ram spermatozoa. According to the authors, concentrations lower than 20 mg/ml of BSA (e.g. 10 mg/ml and 5 mg/ml) were not sufficient to preserve the quality of frozen-thawed semen. On the other hand, El-Kon (2011) recorded better Egyptian Buffalo spermatozoa motility and viability parameters even with 10 or 15% BSA when compared to the control. 10% and 15% BSA in ram and goat semen extenders proved to be optimal according to the findings of Matsuoka et al. (2006) and Yamashiro et al. (2008). It is however difficult to contrapolate our results with these studies, as the maximum concentration of BSA described, was 15%. Experiments using 20% BSA were not performed in any of the studies. However it is concluded that diluents containing a low concentration of BSA result in sperm characteristics similar to a commercial diluent. On the other hand, the experiments of Bankst and Cecil (1992) studying motility characteristics of turkey spermatozoa before and after storage for 24 h in diluents with and without BSA showed that even 1% BSA significantly increased the percentage of motile spermatozoa and sperm velocity, linearity, lateral head displacement and beat frequency, even the overall fertility potential remained unchanged.

The worst results from our study were obtained from the commercial egg yolk medium therefore we assume that the egg yolk medium may be appropriate for a short-term storage of spermatozoa only. It is not ideal for 24 hour cultivation because of the egg yolk unstableness and cell cluster formation.

CONCLUSION

This study demonstrates that BSA has beneficial effects on bovine spermatozoa motility and viability characteristics, which are essential for further processing of semen and an overall fertility potential. According to our results, BSA could be a good protein supplement for long-term bull spermatozoa cultivation especially when using higher concentrations. We suggest that the optimal concentration of BSA for bovine spermatozoa cultivation is 20%, since experimental media containing 20% BSA presented the overall best viability results. Nevertheless, we have to be aware on the fact that a proper protein supplementation is not the only factor crucial for a satisfactory *in vitro* spermatozoa motility and viability. An appropriate energetic substrate and minerals, as well as optimal laboratory conditions are equally important for a successful *in vitro* spermatozoa cultivation.

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UTICAJ ALBUMINA GOVEĐEG SERUMA U KULTIVACIONOM MEDIUMU NA PARAMETRE POKRETLJIVOSTI SPERMATOZOIDA BIKA *IN VITRO*

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Izvod

Cilj rada je da se ustanovi uticaj albumina goveđeg seruma (BSA) dodatog u različite kultivacione mediume, na pokretljivost i preživljavanje spermatozoida bika, tokom 24h kultivacije *in vitro*. Ovi parametri su upoređivani sa nativnim uzorcima sperme kultivisanim u komercijalnom žumančano-jajčanom mediumu, sa eksperimentalnim mediumima, u koje je dodato 10% ili 20% BSA. Uzorci su analizirani posle 0h, 1h i 24h kultivacije, upotrebom sistema Sperm VisionTM CASA. Rezultati pokazuju progresivno opadanje stepena preživljavanja spermatozoida u svim eksperimentalnim mediumima, sa produžavanjem vremena kultivacije. Naj bolji parametri pokretljivosti spermatozoida su postignuti u mediumu sa dodatkom 20% BSA. Dobijeni rezultati pokazuju da BSA može biti dobar proteinski dodatak u mediume za dugotrajnu kultivaciju spermatozoida bika.

Ključne reči: *BSA, kultivacija spermatozoida, dodavanje proteina, CASA, bik.*

Received / *Primljen*: 21.06.2013.
Accepted / *Prihvaćen*: 02.07.2013.