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Influence of the protein content of boar seminal plasma on spermatozoa viability, motility and acrosome integrity in diluted semen stored for 3 days

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Abstract

The aim of the present study was to investigate the influence of the protein content of seminal plasma on the motility, viability and acrosome integrity of spermatozoa in extended semen stored for 3 days. A total of 32 semen samples (from four boars) with high (4 mg/ml) and 32 semen samples (from four boars) with low (2 mg/ml) protein content were investigated. The semen samples were diluted by BTS at a ratio of 1:4, and stored for 72 h at 17°C. The percentages of live sperm (LS), live sperm with damaged acrosome (LDA) and total sperm with damaged acrosome (TDA) were detected by flow cytometry. Sperm progressive motility (PM) was detected using CASA. After 72 h of storage, the percentage of LS and PM was significantly ($P < 0.01$) higher, and the LDA and TDA were significantly ($P < 0.01$) lower in samples with high protein content than in the samples with low protein content (LS = 66 *vs*. 44%, PM = 64 *vs*. 48%, LDA = 15 *vs*. 21% and TDA = 29 *vs*. 45%, respectively). When comparing the difference between 0 and 72 h of storage, the percentage decrease in LS and PM, while increase in LDA and TDA were significantly higher in the samples with low (LS: 75 to 44%; PM: 68 to 48%; LDA: 11 to 21% and TDA: 23 to 45%) than in the samples with high protein content (LS: 78 to 66%; PM: 70 to 64%; LDA: 9 to 15% and TDA: 17 to 29%). We concluded that protein content in seminal plasma has a significant influence on progressive motility, viability and acrosome integrity in diluted semen stored for 3 days.

Keywords: boar, protein, semen, sperm, storage.

Introduction

Artificial insemination (AI) is the most important reproductive biotechnology for successful intensive pig production (Erikson *et al.,* 2000; Gerrits *et al.,* 2005). In this technology, the reproductive efficiency of expensive genetically superior boars has great economic importance (Gadea *et al.,* 2005). This is often the reason for the extreme extension of ejaculate to produce more AI doses and/or prolonging the preservation time of extended liquid semen. Unfortunately, extreme semen extension (Maxwell and Johnson, 1999; Bößenrodt *et al.,* 2008; Lipenský *et al.,* 2013) and/or the *in vitro* preservation of extended liquid boar semen for a longer time, i.e. 3 to 5 days (Alexopoulos *et al.,* 1996; Boe-Hansen *et al.,* 2005; Barrabés *et al.,* 2008; Waberski *et al.,* 2011; Stančić *et al.,* 2012) is the most common reason for a gradual decline in sperm quality and fertility. Consequently, it is often the reason for a reduced fertility rate in artificially inseminated sows. In fact, it has been shown that artificial insemination by AI doses, obtained from extremely extended ejaculates, often results in lower fertility rates than those obtained by naturally mated sows (Tummaruk *et al.,* 2000; Tanavots *et al.,* 2002; Gadea*,* 2005; Alm *et al.,* 2006, Stančić *et al.,* 2009).

Procedures involving the *in vitro* handling and preservation of liquid boar semen promote a decrease in sperm progressive motility, as well as damage to the acrosome and sperm plasma membranes (Kommisurd *et al.,* 2002; Maxwell *et al.,* 2007; Bößenrodt *et al.,* 2008; Gączarzewicz *et al.,* 2010; Stančić *et al.,* 2012; González-Cadavid *et al.,* 2014). Namely, it has been shown that a reduction in the protein concentration, as a result of seminal plasma dilution, plays a key role in decreasing the fertility of stored liquid semen (Maxwell and Johnson, 1999; Mogielnicka-Brzozowska and Kordan, 2011; Nasrin and Calogero, 2012; González-Cadavid *et al.,* 2014). The average total protein content in boar seminal plasma is approximately 4%. Namely, Moore and Hibbit (1976) found an average 3.9% of total protein in boar seminal plasma, and Frunză *et al*. (2008) refer that this value range from 3.26 to 4.0%. In our recent investigation (Apić *et al.* 2015), that included 106 boars, it was been demonstrated that average protein content in seminal plasma was 2.9%, and ranged from 1.25 to 6.50%. However, it has been found that ejaculates with the highest protein levels in seminal plasma exhibit the highest farrowing rates and greatest number of live born piglets, compared with ejaculates that have lower levels of protein (Flowers, 2001; Novak *et al.,* 2010).

Therefore, the aim of the present study was to determine the influence of protein content in the native seminal plasma on the sperm motility and acrosome integrity in diluted semen after storage for 3 days.

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Materials and Methods

Farm and animals

The experiment was conducted at a commercial pig farm in Serbia with 1,000 sows in the breeding herd. The farm is approximately 10 km from the laboratory where the semen quality control was performed.

Four AI boars (two Landrace and two Large White) with high (4.1%) and four boars (two Landrace and two Large White) with low (2.2%) protein content in their seminal plasma, aged between 18 and 36 months, were allocated to the trial. The boars were selected based on the previously determined concentration of protein in the seminal plasma of eight ejaculates from each boar.

Semen preparation at the farm

Eight ejaculates were collected per boar (one ejaculate per week). Thus, 32 ejaculates were obtained from the four boars with high protein content, and 32 ejaculates from the four boars with low protein content in their seminal plasma. A gel-free spermatic and post-spermatic semen fraction was collected from each boar, using the gloved hand method. Gel-fraction was separated by a sterile disposable filter. Immediately after collection the semen was filtered through gauze, and the quality parameters of the ejaculate were evaluated, as is the usual practice at the farm. In the experiment only ejaculates with a volume of 190 to 310 ml, a spermatozoa concentration from 200×10^6/ml to 300×10^6/ml and progressive motility ≥65% were included. The ejaculate quality parameters (volume, concentration, total number and progressive motility of spermatozoa) were not significantly different ($P > 0.05$) between the boars with high and those with low protein content in their seminal plasma. Thus, the proportion of spermatozoa concentration and protein content per unit volume of seminal plasma was similar in each sample tested. However, the protein content of the seminal plasma was significantly different ($P < 0.01$) between the two groups of boars (4.1 *vs*. 2.2%; Table 1).

Table 1. Sperm quality parameters assessed in native sperm samples (mean ± SD)

	High protein content (n = 32)	Low protein content (n = 32)
Protein content in seminal plasma (%)	4.1 ± 0.25^{A} (3.5-4.7)	2.2 ± 0.30^{B} (1.5-2.6)
Ejaculate volume (ml)	249 ± 36^{a}	240 ± 23^{a}
Spermatozoa concentration ($\times 10^6$/ml)	277 ± 33^{a}	269 ± 25^{a}
Total number of spermatozoa ($\times 10^9$)	68 ± 12.5^{a}	63 ± 8.7^{a}
Progressive motility (%)	81 ± 4.1^{a}	78 ± 4.5^{a}

Values with a different superscript, within a row, differ ($^{AB}P < 0.01$; $^{ab}P < 0.05$).

After evaluating the quality of the ejaculate, one 10 ml of fresh gel-free semen sample, and one sample of the same volume, but extended with BTS - Beltsville Thawing Solution (Minitüb, Germany) at exactly 1:4 rate, were prepared from each ejaculate. The semen samples were placed in sterile plastic tubes with caps and stored in a thermo-box at 17°C. They were then transported to the laboratory at the Scientific Veterinary Institute in Novi Sad, Serbia, within 2-3 h of semen collection at the farm, for semen quality assessment.

Semen quality assessment in the laboratory

Immediately after arrival in the laboratory, the semen samples were reactivated in a water bath at 35°C for 30 min before the testing began. The native semen samples were tested for spermatozoa concentration ($\times 10^6$/ml), total number of spermatozoa in the ejaculate ($\times 10^9$) and progressive motility (%) using CASA - Computer Assisted Sperm Analysis (ISAS Proiser, Paterna, Spain) with an integrated software system for the sperm analysis (ISAS) and a USB 200i light microscope (Proiser, Paterna, Spain) at 100X magnification.

Diluted semen samples (1:4 dilution ratio) were prepared from the ejaculates with a high (average 4.1 mg/ml) and a low (average 2.2 mg/ml) content of protein in their seminal plasma. From each sample, spermatozoa were evaluated for acrosome damage and spermatozoa progressive motility immediately after arrival at the laboratory (0 h) and after storage for 72 h at 17°C. The acrosome status was evaluated using flow cytometry (Guava Milipore-IMV, USA). A combination of fluorometric colors Lectin PNA, Alexa Flour® 488 conjugate (Life technologies, Carlsbad, CA, USA) and propidium iodide (Life technologies, Carlsbad, CA,USA) was used for assessing the acrosome status.

To prepare the samples for acrosome status testing: (1) fluorometric colors (2 µl propidium iodide and 1 µl Lectin PNA) were placed in a closed plastic Eppendorf tube; (2) 97 µl EasyBuffer A – Swine (IMV-technologies) was added to the Eppendorf tube; (3) followed by 8 µl fresh semen; and (4) another 292 µl EasyBuffer was added to the Eppendorf tube. This procedure lasted for about 1 min. The samples were incubated for 10 min at 37°C in a thermo-mixer for better homogenisation. After incubation, a flow cytometry device was used to read the results. Progressive motility (%) in the diluted semen was evaluated using CASA - Computer Assisted Sperm

Analysis (ISAS Proiser, Spain), as described above. The sperm cells were assessed as having normal or altered acrosomes. The acrosomal alterations were expressed as a percentage of sperm cells with normal status. Progressive motility was expressed as a percentage of the total number of sperm cells. Taking the value of each parameter (Live spermatozoa-LS, Progressive motility-PM, Live spermatozoa with damaged acrosomes-LDA and Total spermatozoa with damaged acrosomes-TDA) as 100% at the beginning of testing (0 h), the percentage differences between the initial values and those after 72 h of storage were calculated for the samples with high and low protein content.

Analysis of protein content in seminal plasma

After arrival in the laboratory, the fresh gel-free semen samples (20 ml) were centrifuged at 1000 × g for 10 min at 4°C, to separate the spermatozoa from the seminal plasma. The supernatant was re-centrifuged at 3000 × g for 15 min at 4°C to purify the seminal plasma from the residual sperm and other organic particles. The seminal plasma samples obtained were placed in plastic tubes with caps, and stored in a refrigerator at 4°C. The total protein content of the seminal plasma was determined by the AOAC chemical method (Official Method 2001.11, Büchi Device). The analysis was performed within 4 to 6 h after collecting the ejaculate at the farm.

Statistical analysis

Data on the percentage value of spermatozoa motility and acrosome integrity assessments, expressed as a number of good spermatozoa number/total spermatozoa number ×100, were analyzed by the Fischer's egzact test. The data were analyzed by the Statistics 12 software package (StatSoft Inc**.,** Tulsa, OK, USA). The mean ± standard deviation values of the experimental data are presented in Tables 1 and 2.

Results

The average total live number of spermatozoa (LS), progressive motility (PM), live spermatozoa with damaged acrosomes (LDA) and total spermatozoa with damaged acrosomes (TDA) in the diluted semen samples with high or low protein content, after 0 and 72 h of storage, are shown in Table 2. The percentages of LS and PM were significantly ($P < 0.01$) lower, while LDA and TDA were significantly ($P < 0.01$) higher after storage for 72 h, than those at the start of the test (0 h), both for the samples with a high (LS = 78: 66%, PM = 70: 64%, LDA = 9: 15% and TDA = 17: 29%, respectively) and low protein content (LS = 75: 44%, PM = 68: 48%, LDA = 11: 21% and TDA = 23: 45%).

The percentage differences between all of the values tested at 0 and 72 h of storage, with high or low protein content in the seminal plasma, were significantly ($P < 0.01$) greater in the samples with low protein content than the samples with high protein content. Namely, the percentage of LS decreased by 14.4% in the high protein samples, and by 40.6% in the low protein samples. Progressive motility decreased by 9.4% in the high and by 31.7% in the low protein samples. The percentage of LDA increased by 64.6% in the high and by 93.8% in the low protein samples, while the percentage of LDA increased by 64.6% in the high and by 93.8% in the low protein samples (Table 2).

Table 2. Sperm motility and acrosome status assessed at 0 and 72 h in diluted semen samples (mean ± SD)

	Protein content in seminal plasma			
	High (4.1**%**)		Low (2.2%)	
	Diluted semen storage time*			
	0 h	72 h	0 h	72 h
Total ejaculate samples tested (n)	32		32	
Live spermatozoa - LS (%)	$78^{AX} \pm 5.4$	$66^{BZ} \pm 6.2$	$75^{AY} \pm 4.1$	$44^{BI} \pm 2.0$
	(-14.4 ± 4.6^{X})		(-40.6 ± 3.7^{Y})	
Progressive motility - PM (%)	70 ± 8.92^{AX}	64 ± 7.56^{BZ}	68 ± 5.28^{AY}	48 ± 10.49^{BI}
	(-9.4 ± 6.4^{X})		(-31.7 ± 7.2^{Y})	
Live spermatozoa with damaged acrosomes - LDA (%)	$9.1^{Ax} \pm 1.9$	$14.8^{BZ} \pm 2.4$	$11.1^{Ay} \pm 3.9$	$20.7^{BI} \pm 6.5$
	$(+64.6 \pm 2.4^{X})$		$(+93.8 \pm 4.7^{Y})$	
Total spermatozoa with damaged acrosomes - TDA (%)	$17.6^{AX} \pm 3.3$	$29.0^{BZ} \pm 5.8$	$22.7^{AY} \pm 3.8$	$44.7^{BI} \pm 9.1$
	$(+63.6 \pm 4.5^{X})$		$(+92.5 \pm 6.7^{Y})$	

Values with a different superscript, within a row and the same protein content, differ ($^{AB}P < 0.01$; $^{ab}P < 0.05$); Values without a common superscript, for different protein content and 0 h of storage, differ ($^{XY}P < 0.01$; $^{xy}P < 0.05$); Values without a common superscript, for different protein content and 72 h of storage, differ ($^{ZI}P < 0.01$; $^{zi}P < 0.05$). In parentheses: Percentage differences between values at 0 and 72 h of storage within high or low protein content in the seminal plasma.

Discussion

The results of this study show that the mean live spermatozoa (LS) and spermatozoa progressive motility (PM) were significantly higher ($P < 0.01$), and live spermatozoa with damaged acrosomes (LDA) and total spermatozoa with damaged acrosomes (TDA) were significantly lower ($P < 0.01$), after storage for 72 h in the diluted semen samples with high protein content than the samples with low protein content. Furthermore, taking the starting (0 h) values as 100%, the percentage differences between the starting values (0 h) and those after storage for 72 h were significantly lower ($P < 0.01$) in the samples with high protein content (LS= - 14.4%, PM= - 9.4%, LDA = + 64.6% and TDA = + 63.6%) than the samples with low protein content (LS = - 40.6%, PM = - 31.7%, LDA= + 93.8% and TDA = + 92.5%). This decrease in the spermatozoa parameters could be due to the diluted semen being stored for a longer time, as previously shown by others (Waberski *et al.,* 1994, 2011; Maxwell and Johnson, 1999; Čerovsky, 2005; Leahy and Gadella, 2011; Lipenský *et al.,* 2013). The preservation of diluted liquid boar semen induces damages over the storage period (Waberski *et al.,* 2011). Our results demonstrate that the decrease in acrosome integrity was significantly higher than spermatozoa motility after 72 h of storage. Similar results have been found by Kommisurd *et al.* (2002). In their study, there was a 94 to 78% decrease in acrosome integrity, while the decrease in spermatozoa motility was significantly lower (80 to 78%) over 5 days of storage. Their results indicate that the acrosome is more susceptible to damage during storage than the organelles responsible for spermatozoa motility.

However, it has been shown that seminal plasma is important for maintenance of boar spermatozoa fertility *in vitro* (Maxwell *et al.,* 2007; Wolf and Smital, 2009; Nasrin and Calogero, 2012; Stančić *et al.,* 2012). Namely, it has been found that seminal plasma overextension reduces the sperm progressive motility and increases the number of spermatozoa with damaged acrosomes or disintegrated acrosomal membranes (Kommisurd *et al.,* 2002; Maxwell *et al.,* 2007; Gączarzewicz *et al.,* 2010; Stančić *et al.,* 2012; Chuita *et al.,* 2014).

A study by Caballero *et al.* (2004) demonstrated that the addition of seminal plasma to highly extended spermatozoa significantly ($P < 0.05$) increases their viability. Cremades *et al.* (2004) showed that the addition of seminal plasma to freezing extender has a significant effect on post-thaw sperm parameters compared to the control. Namely, the sperm motility (57%), plasma membrane integrity (57%), and acrosome membrane integrity (57%) were significantly ($P < 0.05$) higher in samples with seminal plasma, compared to the control (51, 50 and 49% respectively). In addition, it has been found that seminal plasma proteins play a key role in this phenomenon (Kommisurd *et al.,* 2002; Strzeżek *et al.,* 2005; Caballero *et al.,* 2008; Garcia *et al.,* 2009; Mogielnicka-Brzozowska and Kordan, 2011; Karsani and Nathan, 2013). A reduction in the concentration of protein in seminal plasma, for example by ejaculate overextension, leads to a gradual reduction in progressive motility, the survival rate, acrosome integrity, and mitochondrial activity, which are important factors for the spermatozoa fertilizing ability (Maxwell and Johnson, 1999; Strzeżek *et al.,* 2005; Garcia *et al.,* 2006, 2009; Ashrafzadeh *et al.,* 2013; González-Cadavid *et al.,* 2014). It has been found that the addition of homologous high protein content seminal plasma to boar spermatozoa from ejaculates with a low protein content, results in a significant ($P < 0.01$) increase in progressive motility (70%) after storage for 72 h, compared with samples that contained autologous seminal plasma (40%; Stančić *et al.,* 2012). Chuita *et al.* (2014) found a significant ($P < 0.01$) effect of washing (6, 66 and 56%) or not washing (66, 76 and 68%) of the seminal plasma on motility, live spermatozoa and live spermatozoa with intact acrosomes, respectively. Furthermore, the results obtained by Flowers (1998) demonstrate that the concentration of seminal plasma proteins is highly correlated with *in vitro* boar semen fertility. A further study (Flowers, 2001) shows a significant correlation between two proteins (26 kDa, pI6.2 and 55 kDa, pI4.8) present in boar seminal plasma and the level of semen fertilizing potential. In this study, ejaculates with the highest protein levels in seminal plasma exhibited the highest farrowing rates ($86.7 \pm 3.4\%$) and greatest number of live born piglets (11.2 ± 0.3), compared with those with lower protein levels (farrowing rate = $78.4 \pm 3.1\%$, number of live born pigs = 10.4 ± 0.3). Based on these findings, Flowers (2001) concludes that quantification of these two proteins in seminal plasma can be used to provide a qualitative rank for boar fertility, prior to their use for the artificial insemination of sows.

In summary, it can be concluded that the values of progressive motility, live spermatozoa, and spermatozoa with intact acrosomes were significantly higher in the diluted samples with high, as opposed to those with low protein content in the seminal plasma, after storage for 3 days. These results support the suggestions of other authors that protein content in seminal plasma can be a useful tool for predicting the fertilizing potential of boar ejaculate. Further research should establish the precise mechanisms by which seminal plasma proteins act on spermatozoa fertility *in vitro*. It is also necessary to compare field fertility data in sows inseminated by AI doses produced from ejaculates with high and low protein content.

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