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Motility of boar spermatozoa supplemented with homologous seminal plasma of high or low protein content after storage for three days

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ABSTRACT: The effect of adding high or low protein content homologous seminal plasma to boar spermatozoa on the progressive motility of the spermatozoa after storage for three days, and at a 1 : 4 dilution ratio was investigated. A total of 32 ejaculates collected from four boars (eight ejaculates per boar) with high seminal plasma protein content (4%, HH-group) and 32 ejaculates collected from four boars (eight ejaculates per boar) with low seminal plasma protein content (2%, LL-group) were evaluated. The fresh ejaculate samples were centrifuged at 1000 *g* for 10 min at 4 °C to separate the spermatozoa from the seminal plasma. After centrifugation, one of the centrifuged spermatozoa samples was used to form an autologous fresh ejaculate sample, and another to form a homologous sample by adding low or high protein content seminal plasma from other boars. It was found that semen samples formed with spermatozoa from HH-group boars, supplemented with seminal plasma from LL-group, boars have significantly ($P < 0.01$) lower progressive motility (55%) after storage for 72 h than samples containing the boar's own (autologous) seminal plasma (65%). Conversely, when homologous seminal plasma with high protein content was added to the spermatozoa isolated from the boar ejaculate with low protein content in its seminal plasma, progressive motility significantly ($P < 0.01$) increased from 52% in samples with autologous seminal plasma, to 65% in samples with homologous seminal plasma. It was concluded that addition of homologous high protein content seminal plasma to the spermatozoa of boars with low protein content in their seminal plasma increases their progressive motility after storage for 72 h at a 1 : 4 dilution ratio. This could be a useful tool for increasing reproductive performance in lower fertility high genetic quality boars.

Keywords: seminal plasma; homologous; autologous; protein; dilution; spermatozoa; motility; boar

The reproductive efficiency of expensive genetically superior boars has great economic importance in conventional intracervical artificial insemination (AI) by diluted liquid semen, which is performed in about 99% of worldwide intensive pig production (Singleton 2001;Kommisrud et al. 2002; Stancic and Dragin 2011; Stancic et al. 2013; Khalifa et al. 2014). In the contemporary technology of conventional pig AI, diluted liquid semen doses of 80 ml to 100 ml are used, which contain $3–5 \times 10^9$ progressively motile spermatozoa, and which are stored at 17 °C for zero to five days (Johnson et al. 2000; Haugan et al. 2005; Alm et al. 2006; Stancic et al. 2009; Khalifa et al. 2014). However, the fertilising potential of extended boar semen declines within the first 72 h of *in vitro* storage (Dziekonska and Strzezek 2011; Waberski et al. 2011).

In modern intensive pig production, the 1200 average of annual insemination doses produced per boar is inefficient due to the high cost of genetically

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high quality boars (Singleton 2001; Stancic et al. 2009). Increasing the number of AI doses per ejaculate is possible to achieve by significantly reducing the spermatozoa count per AI dose, but this can often result in ejaculate overextension (Kommisrud et al. 2002; Roca et al. 2006; Stancic et al. 2009). Unfortunately, ejaculate overextension leads to a reduction in the progressive motility of spermatozoa, damaged acrosomes or disintegrated acrosomal membranes (Kommisrud et al. 2002; Haugan et al. 2005; Maxwell et al. 2007; Caballero et al. 2008; Stancic et al. 2012a). Using overextended AI doses is frequently implicated as a cause of reduced fertility in artificially inseminated, compared to naturally mated sows (Tummaruk et al. 2000; Zvekic 2003; Gadea 2005; Alm et al. 2006). Furthermore, significant variation between individual boars has been found in the motility tolerance of spermatozoa to the degree of extension and storage time (Waberski et al. 1994; Stancic et al. 2002; Stancic et al. 2003; Katanic 2004). On the other hand, it has been shown that addition of seminal plasma to highly diluted semen increases the motility and fertilisation capacity of boar spermatozoa during storage (Centurion et al. 2003; Muino-Blanco et al. 2008). Seminal plasma proteins appear to play an active role in these processes (Strzezek et al. 2005; Garcia et al. 2009). Moreover, it has been found that ejaculates with the highest protein levels in their seminal plasma exhibited the highest farrowing rates and greatest number of live born piglets, compared with lower protein level ejaculates (Flowers 2001). Finally, a significant variation in the protein content of the seminal plasma has also been found between individual boars (Flowers 2001; Novak et al. 2010).

Based on the above-mentioned facts, we assumed that replacing a boar's own (autologous) low protein content seminal plasma, before diluting the ejaculate, with high protein content seminal plasma from other boars (homologous seminal plasma) can increase the fertilising ability of those spermatozoa during storage. If this is true, then it could serve as a tool for increasing the reproductive performance of lower fertility boars, i.e. boars with low protein content in their seminal plasma. The final result could be an increased reproductive efficiency of genetically superior AI boars used in commercial pig production.

Therefore, the aim of the present study was to determine the influence of replacing low protein content autologous seminal plasma with high protein content homologous seminal plasma, and vice versa, on spermatozoa progressive motility in diluted semen, stored for three days.

MATERIAL AND METHODS

The boars and ejaculate collection. Experimental boars were used for the artificial insemination (AI) of sows at a commercial farm unit with a capacity of 1000 breeding sows, located in the northern part of Serbia (AP Vojvodina). Four (two Swedish Landrace and two Large White) boars whose ejaculates contained high levels of protein (average 4.03%) in the seminal plasma, and four (two Swedish Landrace and two Large White) boars whose ejaculates contained low levels of protein (average 2.03%) in the seminal plasma were selected. The age of the boars ranged from 18 to 36 months when the study period began. The boars were selected based on the previously determined concentration of protein in the seminal plasma of eight ejaculates from each boar. Sperm-rich ejaculate fractions from each boar were collected, with the gloved-hand technique once a week for two months (every Thursday or Friday, depending on the AI boars scheduled for semen production). The semen was filtered through gauze immediately after collection. The protein content in the seminal plasma of the boars used in the trial is shown in Table 1.

Preparation and evaluation of the ejaculates for the trial were performed at the farm's AI laboratory.

Table 1. Protein content in seminal plasma of the experimental boars

Item	Total protein content in seminal plasma (%)	
	high (HP)	low (LP)
Boars (*n*)	4	4
Examined ejaculate(*n*)	32 + 32 = 64[1]	32 + 32 = 64[1]
Av. total protein content in seminal plasma (%)	4.03 (3.44–4.43)	2.02 (1.40–2.60)
Av. total protein content in seminal plasma, HP + LP (%)	3.03 (1.4–4.43)	

[1]Eight ejaculates per boar made up the total volumes of high and low protein seminal plasma + eight ejaculates per boar were used in the trial with diluted semen. Minimal and maximal values are in parentheses

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Prior to processing, the fresh ejaculates were heated at 35 °C in a water bath for 30 min. The following parameters of the ejaculates were determined in the laboratory: volume (ml), spermatozoa concentration ($\times 10^6$/ml), total number of spermatozoa per ejaculate ($\times 10^9$) and progressive motility (%). The concentration of spermatozoa was measured with a photometer (SDM5, Minitub, Germany), and the progressive motility was determined by a visual microscopic method (using a phase contrast microscope at × 100 magnification and a heating stage at 35 °C). Sperm motility was always tested simultaneously but independently by two competent operators. Three different fields in two semen droplets from each sample were examined (six per operator). The average of all the measurements per sample was used for data analysis (according to Kommisrud et al. 2002). These methods used for ejaculate quality evaluation are suitable for commercial farm practice. Only fresh ejaculates with a progressive motility ≥ 65%, volume ≤ 120 ml and spermatozoa concentration ≥ 200 × 10^6/ml were used.

Analysis of protein content in seminal plasma. After the on-farm quality evaluation, the ejaculates was placed in closed plastic flasks and transported to the Faculty of Agriculture in a thermo-box at 17 °C, within 2–3 h. Chemical analysis was performed in the chemical laboratory at the Faculty of Agriculture, Department of Animal Sciences, University of Novi Sad, Serbia. The total protein content in the seminal plasma was determined by the AOAC chemical method (Official Method 2001.11). The samples for analysis were prepared as described below, and stored in a refrigerator at 4 °C. The analysis was performed within 24 h after the ejaculates were collected at the farm.

Seminal plasma preparation. Each fresh ejaculate was divided into 45 ml samples, placed in 50 ml plastic tubes with caps, and centrifuged at 1000 × *g* for 10 min at 4 °C to remove the spermatozoa. The supernatant was re-centrifuged (at 3000 × *g* for 15 min at 4 °C) to purify the seminal plasma from any residual sperm and other organic particles. The total obtained daily volume of seminal plasma with high protein content was mixed together. The total volume of seminal plasma was divided into aliquots of 15 ml, placed in plastic tubes with caps, and stored in a freezer at –20 °C. The same was done with the volume of low protein content seminal plasma. The total volume of seminal plasma collected was approximately 400 ml from the boars with a high, and 400 ml from the boars with a low protein level in their seminal plasma. This seminal plasma served as high or low protein content homologous seminal plasma to replace the autologous seminal plasma in the ejaculate samples, and was used in the trial with diluted semen. Homologous seminal plasma = seminal plasma from other boars; Autologous seminal plasma = a boar's own seminal plasma.

Preparation of diluted semen samples. For the trial we used four boars with high (HP-boar group) and four boars with low protein content in their seminal plasma (LP-boar group), which were used for obtaining the required amount of seminal plasma. Eight new ejaculates were collected per boar (one ejaculate per week). Thus, we obtained 32 ejaculates from the boars with high protein content, and 32 ejaculates from the boars with low protein content in their seminal plasma.

Two samples with a volume 100 times smaller than that of the total fresh ejaculate volume were prepared from each ejaculate. For example, if the volume of the fresh ejaculate was 300 ml, the volume of the ejaculate sample was 3 ml. The thus formed fresh ejaculate samples were centrifuged at 1000 × *g* for 10 min at 4 °C, to separate the spermatozoa from the seminal plasma. After centrifugation, one of the centrifuged spermatozoa samples was used to form an autologous, and the other to form a homologous fresh ejaculate sample. Frozen doses of seminal plasma were thawed in a refrigerator at 4 °C, from about 24 h before starting, to prepare the ejaculate for dilution. Immediately before centrifuging the ejaculate, the thawed seminal plasma doses were warmed at 35 °C in a water bath for 30 min.

The autologous fresh ejaculate sample. After centrifugation, the seminal plasma was left in the sample, and the spermatozoa were re-dispersed in the same seminal plasma by hand mixing the tube.

The homologous fresh ejaculate sample. After centrifugation, autologous seminal plasma was separated from the centrifuged spermatozoa. After that, the same amounts of homologous seminal plasma were added to the spermatozoa. Homologous seminal plasma with high protein content was added to the spermatozoa samples with low protein content in their own seminal plasma, and vice versa. The spermatozoa were re-dispersed in the homologous seminal plasma by hand mixing the tube with the new ejaculate sample.

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By this procedure, the following combinations of samples were obtained: (1) autologous samples with own spermatozoa + own high protein content seminal plasma, (2) autologous samples with own spermatozoa + own low protein content seminal plasma, (3) homologous samples with own spermatozoa + high protein content seminal plasma from another boar, and (4) homologous samples with own spermatozoa + low protein content seminal plasma from other boars.

Evaluation of the autologous and homologous semen samples. Each autologous and homologous fresh ejaculate semen sample was diluted in a 1 : 4 ratio with BTS1 extender, and stored for three days at 17 °C in a thermo-box. The tubes with diluted semen samples were hand mixed every 24 h to redisperse the spermatozoa in the seminal plasma. An examination of progressive motility was performed 72 h after dilution, using the same method as described above. Prior to determining the progressive motility, the diluted semen samples were reactivated in a water bath at 35 °C for 30 min. The average progressive motility of three diluted samples per ejaculate was used for the data analysis. The progressive motility of the fresh ejaculates immediately before dilution determined the initial point of the trial. Semen samples with ≥ 65% progressive motility after 72 h storage at a 1 : 4 dilution ratio were considered as good semen samples (usable for AI).

Statistical analysis. The differences between the results were tested using Student's *t*-test. The data were analysed in the Statistics 12 software package. The mean ± standard deviation and minimum and maximum values of the experimental data are presented in Table 2.

RESULTS

The average percentage of progressive motility in the diluted semen samples with a high autologous level of seminal plasma protein (HH group) was significantly ($P < 0.01$) lower after 72 h of storage at a 1 : 4 dilution ratio than the fresh ejaculate immediately before dilution. A similar significant decrease ($P < 0.01$) in progressive motility was obtained in the diluted samples with low autologous seminal plasma protein level (LL group), compared to the fresh ejaculate. However, when high protein level seminal plasma was added to the centrifuged spermatozoa from the LL ejaculate (LH sample group), the average progressive motility increased significantly ($P < 0.01$). In contrast, progressive motility was significantly diminished ($P < 0.01$) when the seminal plasma with low protein content was added to the HH ejaculate spermatozoa (HL sample group) (Table 2).

After storage for 72 h at a 1 : 4 dilution ratio, a higher percentage of semen samples in the HH group kept ≥ 65% progressive motility, and were considered as good samples, compared to a significantly ($P < 0.01$) lower percentage of good diluted samples in the LL group. However, when high protein level seminal plasma was added to the centrifuged spermatozoa from the LL ejaculate (LH sample group), the percentage of good diluted

Table 2. Progressive motility (%) of all sperm samples diluted with autologous and homologous seminal plasma ($\bar{x}$ ± SD)

The protein level in seminal plasma of the ejaculate from which was taken the spermatozoa or seminal plasma		Group (simple number)	Progressive motility (%)	
Spermatozoa	seminal plasma		fresh ejaculate	diluted semen[1]
High[2]	high[2]	HH (32)	82 ± 4.16^{AX} 75–90)	65 ± 5.87^{BX} 50–76)
Low[2]	low[2]	LL (32)	76 ± 6.01^{AY} (65–85)	52 ± 12.03^{BZ} (40–75)
High[3]	low[3]	HL (32)	–	55 ± 11.56^{Y} (30–70)
Low[3]	high[3]	LH (32)	–	65 ± 6.64^{W} (50–80)

Values with different superscripts differ ($^{AB,XY,ZW}P < 0.01$; $^{ab,xy,zw}P < 0.05$)

$^{ABab\ ABab}$within the same row; $^{XYxy,\ ZWzw}$ within the same column (^{Xyxy} for HH/HL; ^{ZWzw} for LL/LH)

[1]after 72 h preservation at +17 °C, in a 1 : 4 dilution, [2]autologous (HH and LL), [3]homologous diluted semen (HL and LH). Min. and max in parentheses

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Table 3. The number and progressive motility of good* semen samples diluted with autologous and homologous seminal plasma ($\bar{x}$ ± SD)

The protein level in seminal plasma of the ejaculate from which was taken the spermatozoa or seminal plasma		Group (simple number)	Good samples		Progressive motility (%)
Spermatozoa	seminal plasma		*n*/*n*	(%)	
High[1]	high[1]	HH (32)	24/32	75 ± 0.45^{A}	67 ± 2.94^{a}
Low[1]	low[1]	LL (32)	8/32	25 ± 0.43^{Y}	71 ± 3.20^{x}
High[2]	low[2]	HL (32)	16/32	50 ± 0.51^{B}	66 ± 2.01^{a}
Low[2]	high[2]	LH (32)	24/32	75 ± 0.45^{Y}	68 ± 4.34^{x}

Values with different superscripts differ ($^{AB, XY}P < 0.01$; $^{ab, xy}P < 0.05$)

^{AB,ab} for high-high and high-low group; ^{XY,xy}for low-low and low-high group

n/*n* = number of good samples/total number of examined samples

*≥ 65% progressive motility, after 72 h preservation at +17 °C, in a 1 : 4 dilution

[1]autologous and [2]homologous diluted semen samples. Max. and min. in parentheses

samples significantly ($P < 0.01$) increased. On the other hand, the percentage of good diluted samples was significantly ($P < 0.01$) lower when the seminal plasma with low protein content was added to the HH ejaculate spermatozoa (HL sample group) (Table 2).

The average progressive motility after storage for 72 h at a 1 : 4 dilution ratio was not significantly different ($P > 0.05$) between the groups of diluted semen (Table 3).

DISCUSSION

The results obtained in the present study clearly show that the protein content in boar seminal plasma has a significant effect on sperm progressive motility in 1 : 4 diluted semen samples, stored for 72 h at 17 °C. Namely, the addition of homologous seminal plasma with high protein content to spermatozoa isolated from the ejaculates of boars with low protein content in the seminal plasma significantly ($P < 0.01$) increased progressive motility. Conversely, when homologous seminal plasma with low protein content was added to the spermatozoa separated from ejaculates with high protein content, their progressive motility significantly ($P < 0.01$) decreased (Table 2).

It has been shown that seminal plasma is important for boar semen fertility *in vitro* (Maxwell et al. 2007; Wolf and Smital 2009; Nasrin and Calogero 2012; Stancic et al. 2012b), as well as *in vivo* owing to its role in promoting transport, survival and fertilisation in the female reproductive tract (Langedijk et al. 2002; Strzezek et al. 2005; Waberski et al. 2006; Madej et al. 2013). Overextended seminal plasma reduces the progressive motility of spermatozoa and increases the number of spermatozoa with damaged acrosomes or disintegrated acrosomal membranes (Kommisrud et al. 2002; Maxwell et al. 2007; Stancic et al. 2012a; Chutia et al. 2014). 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 than in the control samples (51%, 50% and 49%, respectively). It has also been shown that the addition of seminal plasma to the media for thawing frozen boar semen is a key factor in achieving greater fertility in sows inseminated with frozen-thawed AI doses (Okazaki et al. 2009; Garcia et al. 2010; Okazaki et al. 2012).

Flowers (1998) demonstrated that concentrations of seminal plasma proteins were highly correlated with *in vitro* boar semen fertility. Further studies (Flowers 2001) showed a significant correlation between the two proteins (26kDa, pI6.2 and 55kDa, pI4.8) present in boar seminal plasma and the fertilising potential of semen. Namely, this author found that high concentrations of both proteins (relative units ≥ 10) in boar ejaculate corresponded with high farrowing rates (≥ 86%) and number of live born piglets (≥ 11). Moreover, the results of his study show that the inclusion of 10–12% of seminal plasma to an artificial insemination dose may increase the fertility of the boar semen. Significant variation in protein content of the seminal plasma

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between individual boars was also found (Flowers 2001; Novak et al. 2010). Based on these findings, Flowers (2001) concluded that quantification of these two specific proteins in seminal plasma could be used to provide a qualitative rank for boar fertility, prior to using the plasma for the artificial insemination of sows. It has also been shown that adding high-protein content seminal plasma from one boar into highly diluted semen samples from another boar significantly increases the survival rate and progressive motility of the spermatozoa (Caballero et al. 2004). Similar results were obtained by Stancic et al. (2012b). They found that adding high protein homologous seminal plasma to spermatozoa separated from ejaculates with an autologous low protein content increases their progressive motility (from 40% in autologous to 70% in homologous seminal plasma). Chutia 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 the motility, number of live spermatozoa and live spermatozoa with intact acrosomes, respectively. The results of these authors support the findings in the present study, which demonstrate that high protein content in seminal plasma improves the fertilising ability of spermatozoa *in vitro*.

Seminal plasma proteins play a key role in maintaining the fertilising ability of spermatozoa *in vitro* and *in vivo* (Kommisrud et al. 2002; Strzezek et al. 2005; Caballero et al. 2008; Garcia et al. 2009). Namely, it has been demonstrated that specific proteins in seminal plasma (particularly spermadhesins) improve the functional parameters of spermatozoa (progressive motility, survival rate and mitochondrial activity) in highly diluted boar semen (Centurion et al. 2003; Garcia et al. 2006; Garcia et al. 2009). The authors conclude that adding seminal plasma to extremely diluted semen could be a good method for spermatozoa preservation during *in vitro* storage. Besides motility, spermatozoa need intact acrosomes for successful oocyte fertilisation. The results obtained by Kommisrud et al. (2002) indicate that the acrosome is more susceptible to damage during storage than the organelles which are responsible for spermatozoa motility. This is as a result of a reduction in the protein concentration of seminal plasma (Strzezek et al. 2005; Garcia et al. 2009). Assessment of progressive motility and acrosomal and sperm membrane integrity allows detection of any lethal damage to the spermatozoa (Waberski et al. 2011). The influence of seminal plasma proteins on sperm function is not yet fully elucidated. However, it is mostly accepted that the binding of seminal plasma proteins to spermatozoa prevents premature acrosome reaction, masks antigens exposed on the cell surface, and stabilises the components of the plasmalemma (Strzezek et al. 2005).

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