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Original Research Article

THE INFLUENCE OF DIFFERENT MOLECULAR WEIGHT SEMINAL PLASMA PROTEIN CONTENT ON SOME FERTILITY PARAMETERS IN BOAR'S EJACULATES

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Abstract: The aim of this study was to investigate the effect of different percentage of seminal plasma proteins with different molecular weight on sperm motility and fertility parameters (farrowing rate (FR), number of live-born pigs (PBA) per litter and percentage of unsuccessful insemination). A total of 50 sperm-rich ejaculate fractions were collected (one per boar) using the gloved hand method. The quality parameters of the semen samples were first evaluated at the farm. Further assessment of sperm quality was performed on a CASA - computer assisted semen analysis by two competent operators. Seminal plasma protein fractions were obtained by AOAC - Association of Official Analytical Chemists as a chemical method. The assessment of reproductive performance was carried out based on collected data of three parameters in selected 9696 sows: FR, PBA per litter and percentage of unsuccessful insemination. Protein fractions were divided in to three groups (10 – 20kDa, 21 – 30kDa and 31 – 40kDa) Proteins with 10 – 20kDa did not have significant effect and correlation with analyzed parameters. Significant differences were recorded in farrowing rate between samples with up to 80 % compared to samples with 10% of proteins with 21 – 30kDa. Significant differences were recorded in unsuccessful insemination between samples with different percentage of proteins with 31 – 40kDa. Results of this study have shown the effect of different percentage of certain fraction of seminal plasma proteins on boar ejaculates fertility potential.

Key words: boars; semen quality; seminal plasma proteins; reproductive results

Introduction

The main reason for using the technique of artificial insemination (AI) is to increase reproductive efficiency and rates of genetic improvement in pig industry. Obtaining good reproductive material for AI includes continuous improvements that follow new technologies in the assessment of good quality semen which have proved that impact on farrowing rate. Farrowing rate in sows and gilts is an important data and commonly used for measure of reproductive performance. The primary determinants of boar fertility that represents the measured progress in pig production are the quantity and quality of semen. Since there is no established gold standard in assessment of good quality semen, prediction of the fertilizing potential is based on objective analysis of semen quality parameters that includes good boar selection and relations with fertility rate data that reflects the reproductive performance. Knowledge of the physiological characteristics of semen, seminal plasma, spermatozoa and the influence of breed, lines and individual boar characteristics are prerequisites for the successful

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improvement of reproductive efficiency and for inheritance of the most desirable semen genotype traits (22).

Boar semen is ejaculated in sequential fractions with a large volume of seminal plasma and the relatively diluted sperm concentration. Seminal plasma is a complex mixture of secretions from testis, epididymis and male accessory glands that may affect the semen quality. An important physiological function of seminal plasma is to carry out spermatozoa through the female reproductive tract, nourishing the sperm, and what is the most important, seminal plasma is directly involved in sperm capacitation and fertilization. This was also supported by the recent observations where the interactions between sperm viability and semen plasma presence or absence were detected during semen storage (2). Therefore, conventional semen evaluation generally includes seminal volume, sperm cell concentration and percentage of motile, viable and morphologically normal spermatozoa, are not sufficient indicators that accurately predict the fertility rate variations among the breeding males (1). Subsequently, the evaluation of semen quality is also directed toward other semen components and seminal plasma as useful predictors of fertility. Given the fact that seminal plasma represents composite secretion, many authors emphasize the importance of seminal plasma protein content since the protein composition could affect the spermatozoa function and this could be considered as valuable biomarker of boar fertility (3, 1, 4, 5) . The SP proteins cover and protect the spermatozoa during ejaculation and the higher protein concentration results in the better protection and preservation of spermatozoa cell membrane stability (21) .

Therefore, recent interest of investigation is pointed at extensive proteomic analysis of boar seminal plasma in order to provide the major characterization of the boar seminal plasma proteins as a first step for further understanding the role of proteins in reproductive outcomes as well as the identification of biomarkers for sperm quality and fertility. Namely, *Perez–Patino et al.,* 2016 (6) found decrypting of the boar seminal plasma proteins challenging since most total protein mass is taken up by a few number of low molecular weight proteins. In order to overcome the limitations, these authors suggested improving the accuracy of later analysis in fractionation of protein according their molecular weight. Using SDS-PAGE electrophoresis, *Karunakaran et al 2016* (7) reported a total of 11 proteins bands in porcine seminal plasma ranging with the molecular weight from 14 – 200kDa, of which, proteins with higher molecular weight were more abundant compared to proteins with lower molecular weight. *Duart and coworkers 2013* (8) reported that the proportion of proteins with molecular weight below 25kDa prevail in boar seminal plasma.

Many studies have shown that proteins with different molecular weight have different individual contribution on sperm quality and fertility potential. In boar seminal plasma protein with molecular weight of 55kDa and 65kDa showed positive correlation with fertilization capacity manifested by a higher percentage of fertilization and number of pigs born alive in the litter. The protein composed of four polypeptides of 43, 55, 65 and 100kDa defined as Platelet-activating factor – acetyl-hydrolase (PAF-AH), that was isolated and purified form boar sperm has been suggested that enhanced sperm motility, capacitation and acrosome reaction (9, 5, 3). The role of protecting sperm against oxidative stress caused by lipid oxidation plays the albumin presented in boar seminal plasma as a series of proteins with the molecular weight in range 76 – 78kDa (5). It has been suggested that protein fraction with greater molecular weight promote binding between sperm and polymorph-nuclear neutrophils (10, 3). Therefore, most of these studies support thesis that proteins with lower molecular weight may be considered as a natural protectors and play crucial role in spermatozoa viability (11). But there is some evidence that proteins with very low molecular weight (5.7kDa) can be deleterious to sperm function. The removal of very low-molecular weight proteins from the seminal plasma enhances sperm motility, plasma membrane integrity, mitochondrial function and sperm exhibit higher ATP content (5).

More than 75% of all proteins in boar's seminal plasma belong to the family of spermadhesins (10). These are multifunctional proteins important in sperm–cell development, sperm capacitation and formation of the oviductal sperm reservoir that preserves the fertilizing capacity of spermatozoa, interaction of gamets and protection of sperm against the female immune system by uterine immunomodulation. Considering the functions of these proteins some authors suggested that

spermadhesins could be considered as potential candidate for markers of boar fertility (5).

Hence, most studies dealing with seminal plasma proteins in boars are aimed at identifying and evaluating its function, but the effect of different abundance of certain seminal plasma proteins on fertility parameters has not been fully understood. Therefore, the aim of this study was to contribute in evaluation of the effects of seminal plasma proteins with different molecular weight in a different proportion in boar's seminal plasma on the spermatozoa viability and fertility.

Materials and methods

Animals and materials

The experiment was conducted at nine large commercial pig farms in Serbia (Autonomous Province of Vojvodina) with the capacity of 1000 sows in the breeding herd. The farms are in radius of approximately 50 km away from the laboratory where the semen quality control was performed. Fifty boars (Large White x Yorkshire n = 22, Swedish Landrace n = 16, Duroc n = 10 and other n = 2) aged between 18 to 36 months when the study period began, were selected. Fifty sperm-rich ejaculate fractions were collected one per boar using the gloved hand method while the boar mounts a dummy sow. Semen samples were filtered through Minitube® filter immediately after collection. The quality parameters of the ejaculate were evaluated, as is the usual practice at the farm.

Semen quality assessment

The fresh native semen samples, with volume range between 60 to 70ml taken from sperm rich fractions prepared for regular use in AI, and placed in sterile plastic flasks with caps and stored in a thermo-box at +17°C. After that, semen are transported to the laboratory at the Scientific Veterinary Institute in Novi Sad, Serbia (within 2-4h after semen collection at the farm), for semen quality assessment on CASA - computer assisted semen analysis. In the laboratory sperm was prepared and diluted for assessment of sperm progressive motility, concentration and morphology evaluation. The average of all measurements per sample was used for data analysis according to *Kommisrud et al. 2002* (13). Only fresh ejaculates with a progressive motility ≥ 65% were used.

Analysis of protein content in seminal plasma

Semen samples were divided in to 20ml samples, placed in 50ml plastic tubes with caps and centrifuged at 1000×g for 10min at 4°C to remove the spermatozoa. The supernatant was re-centrifuged (at 3000×g for 15min at 4°C) to purify the seminal plasma from any residual sperm and other organic particles. The prepared seminal plasma samples were stored in a refrigerator at +4°C. The analysis was performed within 24h after the ejaculates were collected at the farm. Protein analysis was performed in cooperation of the Laboratory or Reproduction at the Faculty of Agriculture -Department of Animal Sciences, and FINS institute from University of Novi Sad. The total protein content in the seminal plasma was determined by the AOAC - Association of Official Analytical Chemists as a chemical method (Official Method 2001.11). The chip-based separations were performed on the Agilent 2100 bio-analyzer (Agilent Technologies, Santa Clara, CA) in combination with the Protein 230 Plus LabChip kit and the dedicated Protein 230 software assay on 2100 expert software. All chips were prepared according to the protocol provided with the Protein 230 LabChip kit. Before loading onto the chip, the samples were diluted with deionized water (1:2 v/v sample: water). After homogenization, samples (4µl) were denatured using 2µL of Agilent denaturing solution (3.5% ß-mercaptoethanol) and heated for 5min at 100°C. After dilution with deionized water, 6µL were applied to the Protein 230+ LabChip. For each investigated sample, analysis was conducted in two independent replications.

All semen samples were divided in to four groups according protein molecular weight including the percentage of each group in total protein content (Table 1).

Assessment of reproductive performance

The assessment of reproductive performance was carried out based on collected data of three parameters in selected 9696 sows: farrowing rate

(FR), number of pigs born-alive (PBA) per litter and percentage of unsuccessful insemination. Insemination of sows was performed with doses which containing in practice on farms in Serbia, 3 bilion Sptz./ 90ml per dose and AI was performed with classic intracervical insemination method. The evaluation was performed on litters immediately after the delivery was completed. The age structure of the sows involved in the experiment was similar.

Statistical analysis

The data was evaluated using the software package "Statistics 12". Mean, standard deviation, minimum and maximum values of the studied traits were determined. Analysis of variance (ANOVA) test with subsequent Kruskal Wallis test, Fisher exact two tailed test was used for comparison of mean values. Pearson's correlation coefficient was examined to evaluate the correlation of the semen parameters and parameters of reproductive performance between the groups. Differences were considered significant at P-values less than 0.05.

Since this research was performed only with biological material, Ethics Committee has not been taken into consideration because the animals used in the research was in the regular intensive production process, from which are used only written results on the end of the reproductive cycle.

Table 1: Average values (Median ± SD) of seminal plasma proteins with different molecular weight (kDa) in ejaculate from different boar breeds

	Molecular weight (kDa) % of Total		
Breed	10-20	21-30	31-40
Large White x Yorkshire	25.12±4.7	51.03±16.3	18.67±16.3
Swedish Landrace Duroc	28.92±9.1	58.12±10.9	9.83±14.1
Other	28.1±7.5	45±19.8	18.63±21.4
	28.4± 7.2	48.45 ± 3.0	21.15 ± 5.3

Table 2: The effect of different percentage of 10-20kD molecular weight proteins on some fertility parameters (means ± SD)

N	Molecular weight (kDa) % of Total			
	10-20% 8 Mean ± SD	21-30% 28 Mean ± SD	31-40% 12 Mean ± SD	p value
Progresive motility	69.37±9.42[a]	72.17±21.14[a]	71.66±15.85[a]	p > 0.05
Unssecsesful inseminations	49.00±19.69[a]	55.32±43.61[a]	64.91±56.96[a]	p > 0.05
FR	68.35±13.16[a]	70.55±21.77[a]	71.66±20.18[a]	p > 0.05
PBA per litter	11.71±1.40[a]	11.69±1.75[a]	11.62±1.35[a]	p > 0.05

Values with a superscript describe statistical significance p value < 0.05

Table 3: Pearson's correlation coefficient between percentage of proteins with 10 – 20kDa molecular weight and progressive motility, unsuccessful inseminations and piglets born per litter

Correlation % Protein molecular weight 10 – 20kDa	r (Pearson's correlation coeficient)	p value
Progresive motility	r = 0.048	p = 0.74
Unssecsesful inseminations	r = 0.073	p = 0.62
PBA per litter	r = - 0.071	p = 0.63

Statistical significance p value < 0.05

Table 4: The effect of different percentage of 21-30kD molecular weight proteins on some fertility parameters (means ± SD): Progressive motility, Unsuccessful inseminations, Farrowing rate (FR), pigs born per litter (PBA)

N	10-20% 4 Mean ± SD	21-40% 5 Mean ± SD	41-50% 8 Mean ± SD	51-60% 17 Mean ± SD	61-70% 12 Mean ± SD	71-80% 4 Mean ± SD	p value
Progressive motility	77.50± 10.408[a]	80.00± 12.747[a]	73.12± 17.100[a]	68.52± 15.588[a]	67.16± 25.992[a]	77.50 ± 6.454[a]	*P*>0.05
Unsuccessful inseminations	43.75± 15.52[a]	39.60± 18.95[a]	29.25± 18.59[a]	36.29± 21.49[a]	32.00± 25.53[a]	18.25± 8.53[b]	*P*<0.05
FR	65.11± 9.28[a]	52.26± 18.81[ab]	72.00± 10.57[a]	57.12± 22.04[ab]	59.03± 22.72[ab]	81.48± 8.20[b]	*P*<0.05
PBA per litter	12.85± 1.26[a]	11.08± 1.33[a]	11.22± 1.40[a]	11.51± 1.75[a]	11.89± 1.71[a]	11.72± 1.75[a]	*P*>0.05

Values with a different superscript, within a row differ (ab p < 0.005)

Table 5: Pearson's correlation coefficient between percentage (%) of proteins with 21 – 30kDa molecular weight and progressive motility, unsuccessful inseminations and piglets born per litter (PBA)

Correlation % Protein molecular weight 21 – 30 kDa	r (Pearson's correlation coeficient)	p value
Progresive motility	r = -0.119	p = 0.42
Unssecsesful inseminations	r = -0.088	p = 0.55
PBA per litter	r = - 0.049	p = 0.74

Statistical significance p value < 0.05

Table 6: The effect of different percentage of 31-40kD molecular weight proteins on some fertility parameters (means ± SD): Progressive motility, Unsuccessful inseminations, Farrowing rate (FR), pigs born per litter (PBA)

N	1-10% 25 Mean ± SD	11-20% 10 Mean ± SD	21-30% 9 Mean ± SD	31-70% 6 Mean ± SD	P value
Progressive motility	68.44± 20.55[a]	75.00± 15.98a	73.33± 16.20a	75.83± 9.70a	*P*>0.05
Unsuccessful inseminations	30.84± 22.68a	47.70± 18.16b	20.00± 10.13bc	42.16± 12.41cd	*P*<0.05
FR	63.07± 20.09a	57.13± 16.83a	63.58± 28.73a	63.78± 7.65a	*P*>0,05
PBA per litter	11.75± 1.45a	10.75± 1.76a	11.90± 1.57a	12.24± 1.37a	*P*>0.05

Statistical significance p value < 0.05

Table 7: Pearson's correlation coefficient between percentage (%) of proteins with 21 – 30kDa molecular weight and progressive motility, unsuccessful inseminations and piglets born per litter (PBA)

Correlation % Protein molecular weight 31 – 40kDa	r (Pearson's correlation coeficient)	p value
Progresive motility	r = 0.194	p = 0.22
Unssecsesful inseminations	r = 0.278	p = 0.078
PBA per litter	r = 0.008	p = 0.96

Statistical significance p value < 0.05

Results

The effect of different percentage of proteins with 10 – 20kDa molecular weight

The effect of proteins with 10 – 20kDa on sperm progressive motility, FR, PBA and %, of unsuccessful insemination shows in Table 2. The effect on progressive sperm motility of proteins with mw of 10 – 20kDa had not been significantly different in samples with different percentage of this fraction of proteins in semen plasma. The similar results were recorded in analysis of parameters for reproductive performance assessment. The analysis on correlation (Table 3) shows that different percentage of the proteins with mw of 10 – 20kDa are not significantly correlated with progressive sperm motility and reproductive performance parameters.

The correlation of different percentage of proteins with 21 – 30kDa molecular weight

The effect of different percentage of proteins with 21 – 30kDa mw on progressive sperm motility was not significant. There was significantly ($p<0.05$) lower percentage of unsuccessful insemination in semen with 71 – 80% of proteins with 21 – 30kDa mw compared to semen with the lowest content of this fraction proteins (10 – 20%). Consequently, the group of boars with the greatest content (17 – 80%) of proteins with 21 – 30kDa mw had the significantly higher FR compared to other groups. No significant differences were recorded in PBA between groups (Table 4). The correlation between the percentages of proteins with mw 21 – 30kDa was not strong and significant though the percentage of protein content was negatively correlated with examined semen parameters (Table 5)

The effect of different percentage of proteins with 31 – 40kDa molecular weight

Significant differences were shown in percentage of unsuccessful inseminations between the groups with different percentage of 31 – 40kDa mw protein content in seminal plasma samples. These differences were not significantly influenced by the different concentration of 31 – 40kDa mw protein according the results of linear correlation between proteins and assessed parameters. No significant differences and no significant correlation were shown in different percentage of this fraction semen plasma proteins to sperm motility and reproductive performance parameters (Table 6; Table 7)

Values with a different superscript, within a row differ (^{abcd} $p < 0.005$)

Discussion

The results of this study have demonstrated that different content of fractionated seminal plasma protein may have effects on sperm that reflects on reproductive performance in boars. These results also confirmed previous findings of the effect of seminal plasma proteins on the ejaculate fertility potential. Namely, earlier studies have clearly shown that seminal plasma proteins exert many functions related to sperm development, maturation, transport and survival in the female reproductive tract, as well as capacitation and acrosome reaction, sperm-egg recognition, and protection against microbial and oxidative damages (12, 8, 14, 23). According to *Apić et al 2016* (3) the ejaculates with higher content of semen plasma protein had significantly higher values of progressive motility, live spermatozoa and spermatozoa with intact acrosomes. Electrophoretic profiles of porcine seminal protein are in range up to 200kDa with different percentage of content in seminal plasma. Some author indicates that seminal plasma proteins in native state, forms high-molecular protein aggregates which may associate or dissociate under some conditions in certain molecular weight peptides (15, 14). In this Investigation, analysis was performed on seminal plasma proteins with the range of molecular weight from 10 to 40kDa since proteins in this bands is with most frequent appearance in boar seminal plasma (15). The protein fractions were categorized in to three groups according the molecular weight in order to analyze the effects of different percentage of each protein fraction. According to results, different percentage of proteins with 10 to 20kDa molecular weight range, do not significantly influence on sperm motility and do not affect significantly on fertility performance. Some authors reported that proteins within this range

may have versatile effect on sperm viability and motility (11) suggested that 10 – 20kDa proteins, as the most abundant protein fraction in boar semen plasma, have mostly protective effect on sperm and maintain the stability of sperm plasma membrane. On the other hand, *Caballero et al.,* 2008 (17) reported that supplementation of boar spermatozoa with low molecular weight protein like heparin-binding (AQN-1, AQN-3, AWN) and non-heparin-binding (PSP-I/PSP-II) spermadhesins, exerted opposite effect on sperm viability, motility and mitochondrial activity. At ejaculation the spermadhesins form a protective coat around the sensitive acrosomal region of the sperm head, thus possibly preventing premature acrosome reaction (24). In our research, higher content of this protein fraction had very weak positive correlation with sperm motility, but insignificant negative correlation was recorded to number of pigs born-alive per litter. This indicate that low fertility rate may be related to higher content of proteins with < 20kDa molecular mass range (15) . Significantly increased farrowing rate and lower percentage of unsuccessful inseminations were recorded in ejaculates with over 70% of 20 – 30kDa proteins in seminal plasma. Thus, the results have shown that 20 - 30kDa protein fractions may influence on sperm fertile potential and are consistent with findings of positive correlation with farrowing rate (16). Similar results are reported in bull ejaculates where 28 – 30kDa seminal plasma proteins are considered as a fertility-associated protein. Some fraction of these range seminal proteins are considered as an antioxidant enzyme (glutathione peroxidase-5) that may protect the sperm membranes from oxidative damage and are also positively related with sperm motility and fertility index (18, 14, 21, 22). *Troedsson et al., 2005* (10) reported that proteins with < 35kDa suppress opsonization to polymorph-nuclear neutrophils and play active roles in protection of viable spermatozoa in mare's female reproductive tract. Therefore, the positive effect of 20 - 30kDa seminal plasma protein on fertility index may be the result of multifunctional role of these proteins in maintaining the viability and motility of spermatozoa in native ejaculate. The percentage of 30 – 40kDa seminal plasma proteins in ejaculates were in accordance to findings of other authors (7). The content of this fraction according to *Strzezek et al.,* 2005 (14) is age related. There are insufficient and inconsistent data about physiological significance of these plasma proteins in boar's ejaculates. Protein with 50kDa described as motility factor inhibitor was purified from boar seminal plasma. Protein recognized as platelet-activation factor acetyl-hydrolase (PAF-AH) composed of four polypeptides (43, 55, 65 and 100 kDa) was isolated and purified from boar sperm, enhances sperm motility, capacitation and acrosome reaction. The results in this study did not show significant effect of 30-40kDa seminal plasma proteins content on sperm motility despite very week positive correlation. But results have shown that higher percentage may have positive effect on farrowing rate and PBA per litter although the correlations were not significant. These results could be supported by the findings of *Flowers et al.,* 2001 (20) who reported that increased concentration of 26 and 55kDa proteins had increased the percentage of farrowing rate and number of pigs born alive.

Conclusion

In summary, our results reinforce the findings that content of different fractions of seminal plasma proteins could influence on fertility potential of boar ejaculates. The results have also shown that proteins with molecular weight less than 40kDa could be considered as fertility marker which is in accordance with other investigations. It could be suggested that percentage of proteins with 20 – 30kDa molecular weight may be the most effective since there was significant differences in analyzed parameters.

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VPLIV VSEBNOSTI BELJAKOVIN SEMENSKE PLAZME RAZLIČNIH MOLEKULARNIH TEŽ NA NEKATERE PARAMETRE PLODNOSTI V EJAKULATIH MERJASCEV

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Povzetek: Cilj opisane raziskave je bil proučiti vpliv prisotnosti beljakovin semenske plazme z različno molekulsko maso na gibljivost semenčic in parametre plodnosti kot so: stopnja prasitev, število živorojenih prašičev na leglo in odstotek neuspešnih osemenitev. Po metodi z orokavičeno roko je bilo zbranih 50 frakcij ejakulata, bogatih s semenčicami (1 na merjasca). Na kmetiji smo najprej ocenili parametre kakovosti vzorcev semena. Nadaljnja ocena kakovosti semenčic je bila izvedena z računalniško podprto analizo semena (CASA), ki sta jo izvedla dva usposobljena operaterja. Semenske frakcije beljakovin v plazmi so bile pridobljene s pomočjo AOAC - metode. Ocena reproduktivne učinkovitosti je bila izvedena na podlagi zbranih podatkov treh parametrov pri izbranih 9696 svinjah. Proteinske frakcije so bile razdeljene v tri skupine (10 – 20 kDa, 21 – 30 kDa in 31 – 40 kDa). Beljakovine velikosti 10 - 20 kDa niso imele značilnega učinka in soodvisnosti z analiziranimi parametri. Ugotovili pa smo statistično značilne razlike v stopnji prasitve med vzorci z do 80 % beljakovin velikosti 21 - 30kDa v primerjavi z vzorci s samo 10 % beljakovin velikosti 21 - 30kDa. Statistično značilne razlike so bile ugotovljene tudi pri uspešnosti osemenitev med vzorci z različnim odstotkom beljakovin velikosti 31 - 40kDa. Rezultati te študije kažejo vpliv različnega odstotka določenega deleža beljakovin iz semenske plazme na potencial plodnosti merjascev.

Ključne besede: merjasci; kakovost semena; semenske beljakovine v plazmi; reprodukcijski rezultati