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Original article

Use of biochemical and protein profiles of seminal plasma to prediction of semen quality and fertility in stallions

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Abstract

The identification of various substances in seminal plasma has opened the way to study their functionality. It was aimed to identify the electrophoretic protein profile (EPP) and biochemical parameters (BP) of seminal plasma (SP) as predictors of semen quality and fertility in stallion. Forty-six ejaculates from 7 fertile stallions, aged between 6-26 years, were collected from May to July and 117 mares were used to obtain fertility data. For each ejaculate, volume, sperm motility, concentration were determined and seminal plasma samples were collected to perform one-dimensional electrophoresis and biochemical profiling. Following the estrus detection, mares were inseminated with fresh sperm. Pregnancy rates and foal rates were recorded. The concentration of 15-18 kDa molecular weight (MW) proteins has shown a positive correlation with sperm concentration and foal rate. Besides, a strong positive correlation was found between sperm concentration and 23-28 kDa MW proteins (r=0.77). The volume of 19-22 kDa MW proteins was negatively correlated with pregnancy and foal rate. Similarly, the volume of high MW proteins (173-385 kDa) correlated negatively with sperm motility and foal rate. Apart from the protein profile, while Magnesium and Glucose levels were negatively correlated with sperm quality and foal rate, Cholesterol level was a positive indicator of the quality of semen as well as the foaling rate. Moreover, the total protein level was correlated negatively with the sperm concentration whereas triglyceride was correlated positively. In conclusion, EPP and BP of seminal plasma are valuable clinical tools as predictors of fertility and semen quality in the stallion.

Key words: fertility, seminal plasma biochemistry, seminal plasma proteins, stallion

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Introduction

The seminal plasma is a combined secretion of the male genital accessory glands added to epididymal and testicular fluids. In horses, the vesicular glands, the prostate and the vas deferens contribute to the composition of the liquid fraction of the ejaculate (Mann 1974). The content of SP includes various proteins, hormones, sugars, enzymes, organic ions, lipids amino and fatty acids (Mann 1964, Tischner et al. 1974, Kosiniak 1975, Mann 1975, Pickett et al. 1975, Gebauer et al. 1976, Kosiniak 1980, Kosiniak and Bittmar 1981, Druart and De Graaf 2018) in the stallion. In each species, the above mentioned substances are used to accomplish one final aim, which is to keep the spermatozoa alive until they reach the oocyte in order to achieve a successful fertilization. However, the equine species presents some peculiarities such as a high concentration of glucose (82 mg/dl) and low levels of fructose (2 mg/dl), unlike what was found in ruminants, in which the latter is the main substrate usable for glycolysis (Mann 1974). The glucose metabolism of stallion sperm is mainly aerobic. However, at particular times, such as immediately after the deposition of semen into the uterus, sperm cells can exploit the anaerobic glycolysis due to the limited presence of oxygen. For the ascension of the sperm along the female reproductive tract, the sperm concentration decreases and the partial pressure of the oxygen increases; thus, creating the conditions for the aerobic metabolism. In anaerobic conditions, the sperms can use fructose less efficient than the aerobic glycolysis (Mann 1975).

The SP also contains molecules such as glyceryl phosphoryl-choline (GPC), which is an important indicator of epididymal function, and mainly produced in the epididymis and accessory organs (Mann 1964, 1975). Among the organic acids, besides lactate and ascorbate, a characteristic constituent of the seminal plasma is citric acid (citrate), present with a very wide range of concentrations (8-53 mg/dl, on average, 26.1 mg/dl) (Mann 1964). Citrate is a product of the vesicular gland secretion not only in the stallion but also in the bull, ram, and boar (Mann 1964), while in humans, it is predominantly prostate-derived (Costello and Franklin 1991). The function of citric acid in semen has not yet been fully elucidated, although, its role in forming complexes with the calcium ion has been suggested in acting as a buffer, maintaining the osmotic balance and particularly in man, intervening in semen coagulation-liquefaction process (Costello and Franklin 1991, Melotti et al. 1996). In stallion, ejaculation starts with a pre-spermatic and aqueous fraction without sperms, which contains GPC, ergothioneine, citrate and derives from the prostate and bulbourethral glands. Subsequently, a second fraction that is rich in sperm, GPC, and ergothioneine, ejected from the epididymal tail, ductus deferens, the ampulla and prostate. Finally, the third, the poorest fraction in terms of sperms and ergothioneine but rich in citrate, is originated from the vesicular glands (Mann 1964). Given the subtle differences in its composition, the biochemical investigation of seminal fluid may provide useful information about the physio-pathological conditions of the male or even the possible location of a malfunction. In fact, the pathological secretory activity of one or more male reproductive compartments could lead to a specific change in the biochemical composition of SP.

The SP proteins are relatively low (10 mg/ml) in stallion compared to other species (20-60 mg/ml). Several studies conducted to characterize these proteins. Von Fellenberg et al. (1985) revealed that the SP proteins tend to form multi-protein aggregation of around 800 kDa MW, composed of 11-30 kDa proteins, which constitute, approximately, 70% of all contained proteins in the seminal plasma. The 13-122 kDa molecular weight proteins have been studied by Amann et al. (1985) and Brandon et al. (1999) and 14 of them were correlated with stallions fertility. Several smaller seminal plasma components, such as lactoferrin (Inagaki et al. 2002), known as dog testis originated protein (Kikuchi et al. 2003) and spermatozoon life span/ /viability promoters like leptin and growth factors (Champion et al. 2002, Lackey et al. 2002), lipase (Carver and Ball 2002), the α1,4-glucosidase (Dias et al. 2004) and angiotensin-converting enzyme (Ball et al. 2003), have been described.

Taking all these considerations into account, the aim of the present study was to assess the correlations among electrophoretic protein profile (EPP) and biochemical parameters (BP) of seminal plasma with the semen quality and fertility indexes in stallion.

Materials and Methods

Animals and semen treatment

This study was conducted with 46 ejaculated of seven Italian trotter stallions (clinically healthy and fertile) aged 9 to 26 years. The samples were collected from April to July at 3-7 days of intervals, using an artificial vagina (Mod. Hannover, Colorado, and Missouri) and a dummy. The semen evaluation was performed immediately after the collection. Motility was evaluated using light microscopy. The volume was directly read on the graduated tube used for the collection.

For each ejaculate, the standard analysis was performed according to Varner et al. (1991). For the assessment of sperm concentration, a photometer (Spermacue,

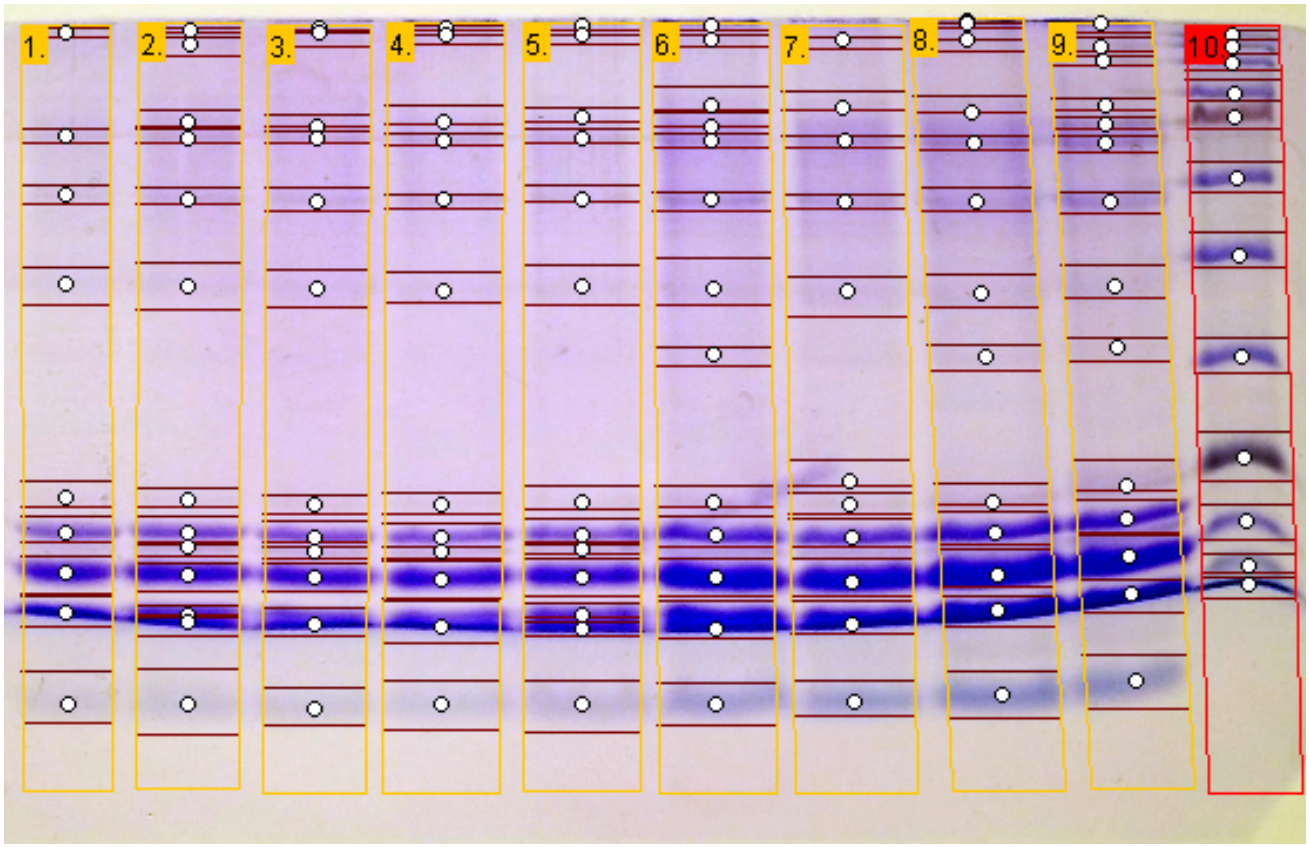

Fig. 1. Example of electrophoresis runs of different seminal plasma samples from the same stallion.

Minitüb, Germany) was used. A sample of 2 ml was taken with a sterile pipette and placed in a centrifuge at 3000 revolutions/minute for 30 minutes. The supernatant was extracted and frozen at a temperature of -24°C until the use of the sample. All the procedures were performed following the EU Directive 2010/63/ /EU for animal experiments.

Selected classes of parameters

Obtained parameters of motility, pregnancy, biochemical profile compared to motility and pregnancy classes, seminal plasma protein profile compared to pregnancy and motility classes were divided into a different class of parameters for correlation indexes as reported below:

Class of motility 1 < 70%; Class of motility 2> 70%,
Class of Pregnancy 1 <50%; Class of Pregnancy 2 >50%,
SP biochemical profile compared to the class of motility 1 and 2,
SP biochemical profile compared to the class pregnancy 1 and 2,
SP protein profile compared to the class of pregnancy 1 and 2,
SP protein profile compared to the class of motility 1 and 2,

Variation of the seminal plasma composition, semen parameters and fertility throughout the subsequent breeding times (I-VII).

One dimensional electrophoresis

The seminal plasma total protein concentration was determined for each ejaculate. The samples were divided into 2 aliquots: >50 kDa and <50 kDa, using firstly 50 000 MW, MW cut off (MWCO) Amicon ® Ultra-2, and then 3000 MWCO Amicon ® Ultra-0.5 filters (Millipore), Milan, Italy). The one-dimensional (1D) electrophoresis (PAGE) was done according to Laemmli (1970). In all studies, 4% stacking gel was used; concentrated samples (35 µg), 10% (>50 kDa fractions) and 20% SDS-polyacrylamide gels (<50 kDa fractions) were carefully installed. Molecular mass markers (pre-painted protein marker VI; AppliChem, Darmstadt, Germany) that adequately covered the possible range of 10-245 kDa were applied to each gel. Gels were run at 150 V using a Bio-Rad power supply unit (PowerPac™ Universal Power Supply; Bio-Rad Laboratories Ltd., UK). Subsequently, gels were stained with Colloidal Coomassie Blue G-250 (Bio-Rad Laboratories Ltd., UK) and de-stained with a solution of 10% methanol and 10% glacial acetic acid. The gel images were obtained using a used Pentax Optio M90 camera

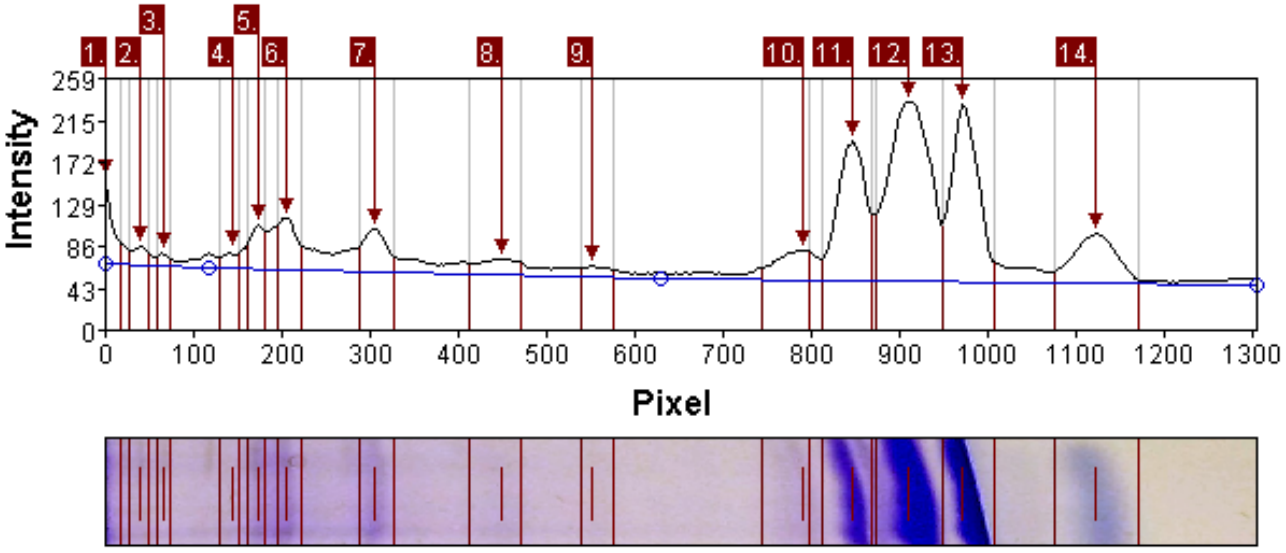

Fig. 2. Example of quantification of volume and concentration of single electrophoresis band.

Table 1. The semen parameters compared regarding the classes of sperm motility and pregnancy rates.

	Motility < 70%	Motility > 70%	p Value	Pregnancy < 50%	Pregnancy > 50%	p Value
Semen volume (ml)	52.94 ± 4.73	49.82 ± 3.56	0.60	51.20 ± 3.93	50.71 ± 4.15	0.93
Sperm concentration (x10^6/ml)	152.06 ± 16.82	214.79 ± 13.95	0.007	178.68 ± 17.05	207.00 ± 14.94	0.23
Sperm motility (%)	62.94 ± 1.21	70.17 ± 0.17	<0.001	67.00 ± 1.19	68.10 ± 0.54	0.44
Pregnancy rate (%)	62.23 ± 9.27	52.72 ± 5.99	0.37	30.76 ± 4.57	86.57 ± 3.59	0.001
Foal rate (%)	49.06 ± 4.50	59.21 ± 1.23	0.01	52.28 ± 3.28	59.24 ± 1.42	0.008

(Pentax, Milan, Italy). The visible molecular mass of the bands on gel electrophoresis (SDS-PAGE) was estimated by GelAnalyzer version 2010a freeware software (Copyright 2010 by Istvan Lazar and Dr. Istvan Lazar, Hungary) (Arganaraz et al. 2015). The same software was run to determine the density of the bands detected in the digitized gel images (Figs. 1 and 2).

Biochemical analysis

Biochemical analysis was performed using an automated analysis system (BM Hitachi 911; Roche, Basel, Switzerland). In each sample, the values of glucose, cholesterol, triglycerides, total protein (TP), urea, creatinine, Ca, Cl, K, Na, Mg, ALP, LDH and CK were determined.

Artificial insemination and fertility results

The collected ejaculates were extended up to 400 million progressive motile sperm/ml, chilled and used for artificial inseminations within 24 hours. The pregnancy rate for each ejaculate and total foal rate of the 117 mares inseminated following the estrous monitoring, were collected on pregnancy detection (28th day) and at the parturition time, respectively.

Data analysis

All these data were entered in an Excel table from which the mean values for each parameter for each range of MWs were obtained. The collected data were analyzed by ANOVA using the GLM procedure of SigmaStat 2.3 software considering as independent variables the classes of sperm motility (< or > 70%) and pregnancy rate (< or > 50%). Pearson correlation scores between the evaluated parameters (the biochemical composition of SP, results of the electrophoretic analysis, sperm quality and, fertility) were evaluated.

Results

The semen parameters were compared regarding the different classes of sperm motility and pregnancy rate (Table 1). According to the obtained results, both the sperm concentration and foal rate were significantly higher in the class of motility > 70 ($p<0.05$). However, the semen parameters have not differed between the classes of pregnancy.

Table 2. The biochemical profile of seminal plasma compared regarding the classes of motility and pregnancy rates.

	Motility < 70%	Motility > 70%	p Value	Pregnancy < 50%	Pregnancy > 50%	p Value
Glucose (mg/dL)	5.08 ± 0.98	5.19 ± 1.19	0.95	5.32 ± 1.29	4.97 ± 1.05	0.84
Cholesterol (mg/dL)	3.92 ± 0.47	5.75 ± 0.62	0.05	4.50 ± 0.48	5.72 ± 0.78	0.18
Triglycerides (mg/dL)	41.92 ± 5.79	58.92 ± 6.51	0.09	46.33 ± 5.94	60.39 ± 7.55	0.15
Total Protein (g/L)	1.12 ± 0.13	1.33 ± 0.15	0.36	1.27 ± 0.14	1.47 ± 0.75	0.86
Urea (mg/dL)	42.48 ± 1.66	45.09 ± 1.58	0.29	43.88 ± 2.00	44.48 ± 1.13	0.81
Creatinine (mg/dL)	0.51 ± 0.05	0.68 ± 0.06	0.05	0.59 ± 0.07	0.65 ± 0.05	0.50
Calcium (mg/dL)	11.5 ± 1.65	9.97 ± 1.74	0.56	10.73 ± 1.48	10.63 ± 1.82	0.95
Chloride (mEq/L)	103.32 ± 3.07	107.2 ± 0.85	0.14	105.16 ± 2.29	106.40 ± 0.99	0.63
Potassium (mEq/L)	20.63 ± 1.80	21.78 ± 1.34	0.61	21.06 ± 1.65	21.67 ± 1.37	0.78
Sodium (mEq/L)	104.93 ± 4.02	113.96 ± 3.22	0.09	109.00 ± 3.10	112.32 ± 4.43	0.54
Magnesium (mEq/L)	7.46 ± 1.02	4.26 ± 0.65	0.009	5.58 ± 0.79	5.22 ± 0.95	0.77
Alkaline phosphatase (IU/L)	5320.33 ± 1362.76	7510.72 ± 783.93	0.15	6779.64 ± 921.82	6660.67 ± 1270.37	0.94
Lactate dehydrogenase (IU/L)	40.60 ± 13.94	204.87 ± 72.18	0.08	71.55 ± 25.59	215.53 ± 89.11	0.12
Creatine kinase (IU/L)	59.26 ± 21.96	349.84 ± 150.39	0.15	76.91 ± 32.37	422.11 ± 193.75	0.07

Table 3. The protein profile of seminal plasma compared regarding the classes of motility and pregnancy rate.

Range of molecular weight (kDa)	Value	Motility < 70%	Motility > 70%	p Value	Pregnancy < 50%	Pregnancy > 50%	p Value
3-14	Volume (%)	14.77 ± 1.6	14.64 ± 0.95	0.94	15.23 ± 0.05	14.04 ± 0.04	0.48
	Concentration (mg/dl)	0.15 ± 0.02	0.21±0.03	0.18	0.19 ± 0.03	0.19 ± 0.03	0.89
15-18	Volume (%)	16.56 ± 1.69	16.9 ± 1.59	0.88	15.76 ± 1.71	17.94 ± 1.56	0.36
	Concentration (mg/dl)	0.18 ± 0.03	0.22 ± 0.03	0.33	0.17 ± 0.03	0.25 ± 0.04	0.13
19-22	Volume (%)	11.93 ± 2.37	9.45 ± 2.85	0.51	13.08 ± 2.94	8.31 ± 2.13	0.20
	Concentration (mg/dl)	0.12 ± 0.03	0.08 ± 0.02	0.33	0.12 ± 0.04	0.08 ± 0.02	0.41
23-28	Volume (%)	5.99 ± 2.05	6.01 ± 1.81	0.99	5.67 ± 2.10	6.28 ± 0.47	0.83
	Concentration (mg/dl)	0.04 ± 0.34	0.045 ± 0.02	0.75	0.06 ± 0.03	0.04 ± 0.01	0.61
34-42	Volume (%)	4.71 ± 1.02	4.69 ± 0.59	0.98	5.09 ± 0.87	4.19 ± 0.24	0.42
	Concentration (mg/dl)	0.06 ± 0.01	0.05 ± 0.001	0.68	0.05 ± 0.01	0.06 ± 0.01	0.85
44-56	Volume (%)	3.47 ± 0.24	4.22 ± 0.31	0.11	4.15 ± 0.35	3.71 ± 0.99	0.33
	Concentration (mg/dl)	0.04 ± 0.007	0.06 ± 0.007	0.026	0.054 ± 0.01	0.05 ± 0.02	0.96
57-70	Volume (%)	5.17 ± 1.29	5.38 ± 0.90	0.89	4.41 ± 1.07	6.06 ± 0.76	0.27
	Concentration (mg/dl)	0.06 ± 0.02	0.07 ± 0.01	0.72	0.06 ± 0.02	0.08 ± 0.01	0.48
72-88	Volume (%)	3.81 ± 0.62	4.16 ± 0.73	0.71	3.45 ± 0.58	4.81 ± 0.22	0.17
	Concentration (mg/dl)	0.04 ± 0.008	0.05 ± 0.02	0.37	0.043 ± 0.01	0.05 ± 0.01	0.58
122-160	Volume (%)	0.78 ± 0.27	1.47 ± 0.18	0.18	1.51 ± 0.28	1.27 ± 0.43	0.52
	Concentration (mg/dl)	0.01 ± 0.01	0.02 ± 0.003	0.14	0.02 ± 0.01	0.02 ± 0.01	0.96
173-385	Volume (%)	3.10 ± 0.51	2.45 ± 0.31	0.26	2.74 ± 0.37	2.64 ± 0.43	0.86
	Concentration (mg/dl)	0.03 ± 0.006	0.3 ± 0.006	0.74	0.04 ± 0.01	0.31 ± 0.01	0.73

The only significant difference was related to the magnesium concentration of the SP which was found lower in the higher class of motility ($p<0.05$), however, the biochemical composition of SP did not differ statistically between the two classes of pregnancy (Table 2).

Other than the significant difference regarding

Table 4. The correlation indices among the semen parameters, related fertility and seminal plasma composition ($p<0.05$).

	Semen volume (ml)	Sperm concentration ($x10^6$/ml)	Sperm motility (%)	Pregnancy rate (%)	Foaling rate (%)
3-14 kDa vol (%)	0.32				
3-14 kDa conc (mg/dl)					
15-18 kDa vol (%)	-0.52	0.35			
15-18 kDa conc (mg/dl)		0.39			0.32
19-22 kDa vol (%)	-0.57			-0.48	-0.43
19-22 kDa conc (mg/dl)	-0.34			-0.45	
23-28 kDa vol (%)	-0.69	0.77			
23-28 kDa conc (mg/dl)					
34-42 kDa vol (%)		0.39			
34-42 kDa conc (mg/dl)					
44-56 kDa vol (%)					
44-56 kDa conc (mg/dl)					
57-70 kDa vol (%)					
57-70 kDa conc (mg/dl)					
72-88 kDa vol (%)		0.39			
72-88 kDa conc (mg/dl)		0.47			
122-160 kDa vol (%)	0.70				
122-160 kDa conc (mg/dl)					
173-385 kDa vol (%)			-0.27		-0.28
173-385 kDa conc (mg/dl)		0.28			
Urea					
Glucose	0.34	-0.28			-0.31
Cholesterol		0.37	0.28		0.31
Triglyceride	0.28	0.40			
Creatinine	0.31				
Calcium	0.29				
Chloride					
Potassium					
Sodium					
Magnesium		-0.33	-0.42		-0.39
Alkaline phosphatase					
Lactate dehydrogenase					
Creatine kinase					
Total Protein		-0.30			

the concentration of 44-56 kDa MW proteins between the classes of motility ($p<0.05$), the volumes and the concentrations of the SP proteins grouped according to the ranges of molecular weight showed no significant difference neither between the classes of pregnancy rate nor regarding the motility classes (Table 3).

Furthermore, various correlations among the composition of seminal plasma, sperm quality and fertility were determined (Table 4). The concentration of 122-160 kDa MW proteins was positively correlated with the volume ($r=0.70$) whereas the volumes of 23-28 kDa ($r=0.69$), 19-22 kDa ($r=0.57$) and 15-18 kDa ($r=0.52$) MW proteins were correlated negatively. The quantity of 15-18 kDa MW proteins revealed a positive correlation ($r=0.32$) with sperm concentration and foal rate. Besides, a strong positive correlation was found between sperm concentration and 23-28 kDa MW proteins ($r=0.77$). The volume of 19-22 kDa MW proteins was negatively correlated with pregnancy and foal rate. Similarly, the volume of high MW proteins (173-385 kDa) correlated negatively with sperm motility and foal rate.

Apart from the protein content of SP, various biochemical parameters were related to semen quality and fertility as well. While magnesium and glucose levels were negatively correlated with sperm quality and foal rate, cholesterol level was a positive indicator of the quality of semen as well as the foaling rate. Moreover, the total protein level was correlated negatively with the sperm concentration whereas triglyceride was positively correlated.

Discussion

The fertilization process depends on the different interactions between male and female related factors such as the mission of SP composition in sperm capacitation, sperm reservoir formation and sperm-oocyte interactions. Several researchers have documented the link between poor semen quality and the low MW proteins (Calvete et al. 1995), while other have reported the connection between high MW SP proteins and semen quality (Schambony et al. 1998, Töpfer-Peterson et al. 2005). In parallel with above-mentioned studies, we aimed to determine the correlation indexes among seminal plasma components, essential semen quality parameters (volume, concentration, motility) and fertility as well, in order to introduce indicators of fertility and semen quality in stallions by a low cost and less labor-intensive simple clinical approach for AI centers.

Data on the concentration of certain biochemical elements have shown possible relationships with both sperm motility and fertility. The concentration of magnesium in SP was lower in ejaculates with higher motility, besides, both Mg and glucose levels correlated negatively with sperm quality and foaling rate. In general, Mg is also known as a marker for seminal secretions (Wong et al. 2001) and the motility of spermatozoa (Jobim et al. 2004). In stallions, both pre-secretions and the sperm-rich fraction have low levels of Mg (Kareskoski et al. 2011). The presence of a high amount of Mg is correlated with infertility in human medicine (Pandy et al. 1983, Stanwell-Smith et al. 1983, Huang et al. 2007). Although there was no significant difference in terms of CASA parameters such as VAP, VCL, VSL and STR between the high and low concentrations of magnesium in human SP, the linearity percentage was found lower in ejaculates with higher Mg concentrations ($p<0.001$) (Sørensen et al. 1999). Usuaga et al. (2017) reported that a medium level of Mg gave the best results for post-thaw total motility, progressive motility, straight-line velocity, average path velocity, sperm vitality, abnormal morphology, and plasma membrane integrity. On the contrary, in another research, low levels of Mg in stallion SP showed the lowest motility results (Halo Jr et al. 2018). Apart from these studies, Barrier-Battut et al. (2002) have stated that variation in the concentrations of magnesium, calcium, zinc and copper in stallion SP did not affect freezability. Although there are contradictive results regarding the effect of Mg in SP, it should be kept in mind that excessive exposure of Mg and calcium in SP correlate with infertility and could have an adverse effect on the male reproductive system (Pesch et al. 2006). In a study conducted on Marwari stallions and Poitou jacks, a significant positive correlation was found between glucose levels and motility of sperm. However, in the present study, the glucose levels in SP were almost 4 times lower than their mean levels of 24.31 mg/dl in Marwari stallions (Talluri et al. 2017). In the present study, the total protein level was correlated negatively with sperm concentration as well. Similar results were obtained by Usaga et al. (2017) regarding the effect of total protein levels on sperm quality. Possibly due to the increasement in the high protein level causes increasement in protein oxidation thus lower the semen quality. Apart from that, previous studies (Strzezek et al. 2005, Akcay et al. 2006) have argued that the concentration of the total protein in SP intensified during puberty and decreases with the age of the stallions. As another biochemical parameter of SP, cholesterol is the main sterol in sperm membranes of the boar, bull, stallion and rooster (Parks and Lynch 1992) thus, it is important to maintain the integrity and functional sperm membrane (Sieber 1987, Gadella and Harrison 2002, Cross 2003). In the present study, sperm concentration was found to have a positive correlation with cholesterol and triglycerides. Besides, cholesterol levels were positively correlated with sperm motility and foaling rate as well.

Although these results are quite intuitive, it is more interesting to observe that various bands of proteins with a higher MW have a negative effect on sperm quality and/or fertility such as 173-385 kDa MW, while protein bands with a lower MW such as 15-18 kDa, 23-28 kDa and 44-56 kDa have resulted with positive correlations. The exception for this implication is the 19-22 kDa MW proteins, which had a moderate negative correlation with pregnancy and foaling rates.

Prostaglandin D2 synthase appeared as a polymorphic and monomeric 22- to 30-kDa molecule with liquid origin and a variable pH range depending on the species. In fact, the wide distribution of this enzyme in the genital tract has a sustained effect on the gamete or its environment, more likely than a local effect on sperm, as in epididymal sperm maturation processes (Fouchecourt et al. 1999). In the present study a strong positive correlation was observed between sperm concentration and 23-28 kDa proteins. Similarly, osteopon-

tin (72 kDa) has been associated with fertility in stallions (Brandon et al. 1999), bulls (Killian et al. 1993) and pigs (Hao et al. 2006, Hao et al. 2008). Although a positive correlation between the 72-88 kDa proteins and sperm concentration was obtained in the present study, no significant correlation was found with pregnancy or foaling rate.

Jobim et al. (2005) observed a protein found only in ejaculates of high fertile stallions (20-25kDa, pI 8.5-8.7) and another protein with a higher relative protein content (25-30kDa, pI 7.5-7.7) in the ejaculations of low fertile stallions. The high frequency of cysteine-rich secretory protein 3 (CRISP3) was positively correlated with the first cycle conception rate while the kallikrein-1E2 (KLK2), clusterin, seminal plasma proteins 1 (SP1) and 2 (SP2) were found negatively associated with fertility (Novak et al. 2010).

On contrary, other researchers reported the HSP-1 and HSP-2 are associated with heparin binding ability (Töpfer-Petersen et al. 2005) and capacitation (Garcia et al. 2015) which accounts for 70-80 % of the total proteins of stallion SP (Calvete et al. 1995). More interestingly, HSP-1 and HSP-2 have protective activity on spermatozoa from chemical, oxidative and thermal stress by saving a client protein either against aggregation or the loss of activity evoked by stress *in vivo* (chaperon activity) (Kumar and Swamy 2016).

Another conspicuous protein, which has a sperm binding activity is equine homologue of equine AWN, also known as sperm adhesin HSP-7. This carbohydrate-binding protein secreted and existing in spermatogonia, rete testis, ductus epididymis, and seminal vesicles have a key role to bind to the intact horse zona pellucidae, thus fertilization (Töpfer-Peterson et al. 2005). Recently, the CRISP protein family has gained interest in molecular research due to the large concentration found in the stallion ejaculate. Multiple members of the CRISP family have been identified, including CRISP-1, CRISP-2, CRISP-3 and CRISP-4. CRISP proteins have been found to be sperm bound in various locations, including the equator and post-acrosome region, and the midpiece. Among these, human and mouse CRISP-1 also taking a part by binding to a complementary site during gamete fusion is reported (Da Ros et al. 2008). The addition of equine CRISP-3 to semen resulted in decreased binding activity among live spermatozoa and PMNs in vitro (Doty et al. 2011). Recently, it has also been shown that abundant CRISP-3 in ejaculate positively correlates with first cycle pregnancy rates and high semen freezability (Hamann et al. 2007). Equine seminal protein-4 (HSP-4) has been found to be associated with the calcitonin-like protein family. In humans and mice, calcitonin levels are associated with sperm motility, suggesting that HSP-4 may play a role in equine sperm motility (Kareoski 2011). Equine seminal protein-6 (HSP-6) and equine seminal protein-8 (HSP-8) are thought to be isoforms of a single protein similar in structure to human prostate specific antigen (PSA) which participate in the division of the seminal coagulum (Calvete et al. 1994).

The other forms of protein like leptin, lipase and angiotensin-converting enzyme have been reported in equine semen, however, their functionality in fertilization process is still uncertain. Future studies are necessary to understand molecular mechanism with the advancing new technology for the purification and identification of those proteins.

In conclusion, high MW proteins are indicative of low fertility, while the presence of low MW proteins are indexes of high fertility. Similarly, increasing concentration in Mg, glucose and total protein are identified as low fertility indexes while the increase of the concentration of cholesterol and triglycerides are indexes of greater fertility. Although it is possible to identify and analyze these molecules deeper owing to the advance of biotechnological applications, as an easy, quicker and cost-effective method, PAGE could give valuable insights about the fertility of an ejaculate and could be more adaptable to the conditions in clinic field such as breeding centers. The use of EPP and BP could be extremely important to monitor the ejaculates and for the adjustment of extenders to improve the fertility of the individual stallion or ejaculate. However, further studies should be conducted on a larger number of animals, with different levels of fertility and breeds.

References

Akcay E, Reilas T, Andersson M, Katila T (**2006**) Effect of seminal plasma fractions on stallion sperm survival after cooled storage. J Vet Med A 53: 481-485.

Amann RP, Cristanelli MJ, Squires EL (**1985**) Proteins in stallion seminal plasma. J Reprod Fertil 35: 113-120.

Argañaraz ME, Apichela SA, Zampini R, Vencato J, Stelletta C (**2015**) Biochemical and Protein Profile of Alpaca (V icugna pacos) Uterine Horn Fluid During Early Pregnancy. Reprod Domest Anim 50: 121-128.

Ball BA, Gravance CG, Wessel MT, Sabeur K (**2003**) Activity of Angiotensin-converting enzyme (ACE) in reproductive tissues of the stallion and effects of angiotensin II on sperm motility. Theriogenology 59: 901-914.

Brandon CI, Heussner GL, Caudle AB, Fayrer-Hosken RA (**1999**) Two-dimensional polyacrylamid electrophoresis of equine seminal plasma proteins and their correlation with fertility. Theriogenology 52: 863-873.

Calvete JJ, Mann K, Schafer W, Sanz L, Reinert M, Nessau S, Raida M, Töpfer-Petersen E. (**1995**) Amino acid sequence of HSP-1, a major protein of stallion seminal plasma: effect of glycosylation on its heparin- and gelatin-binding capabilities. Biochem J 310: 615-622.

Calvete JJ, Nessau S, Mann K, Sanz L, Sieme H, Klug E, Töpfer-Petersen E (**1994**) Isolation and Biochemical characterization of stallion seminal-plasma proteins. Reprod Domest Anim 29: 411-426.

Carver DA, Ball BA (**2002**) Lipase activity in stallion seminal plasma and the effect of lipase on stallion spermatozoa during storage at 5 degrees C. Theriogenology 58: 1587-1595.

Champion ZJ, Vickers MH, Gravance CG, Breier BH, Casey PJ (**2002**) Growth hormone or insulin-like growth factor-I extends longevity of equine spermatozoa in vitro. Theriogenology 57: 1793-1800.

Cross NL (**2003**) Decrease in order of human sperm lipids during capacitation. Biol Reprod 69: 529-534.

Costello LC, Franklin R.B. (**1991**) Concepts of citrate production and secretion by prostate 1. Metabolic relationships. The Prostate 18: 25-46.

Da Ros VG, Maldera JA, Willis WD, Cohen DJ, Goulding EH, Gelman DM, Cuasnicu PS (**2008**) Impaired sperm fertilizing ability in mice lacking Cysteine-Rich Secretory Protein 1 (CRISP1). Dev Biol, 320: 12-18.

Da Ros VG, Muñoz MW, Battistone MA, Brukman NG, Carvajal G, Curci L, Cuasnicu PS (2015) From the epididymis to the egg: participation of CRISP proteins in mammalian fertilization. Asian J Androl 17: 711-715.

Dias AJ, Maia MS, Retamal CA, Lopez ML (**2004**) Identification and partial characterization of alpha-1,4-glucosidase activity in equine epididymal fluid. Theriogenology 61: 1545-1558.

Doty A, Buhi WC, Benson S, Scoggin KE, Pozor M, Macpherson M, Troedsson MH (**2011**) Equine CRISP3 modulates interaction between spermatozoa and polymorphonuclear neutrophils. Biol Reprod 85: 157-164.

Druart X, De Graaf S (**2018**) Seminal plasma proteomes and sperm fertility. Anim Reprod Sci 194: 33-40

Gadella BM, Harrison RA (**2002**) Capacitation induces cyclic adenosine 3,5-mono-phosphate-dependent, but apoptosis--unrelated, exposure of amino-phospholipids at the apical head plasma membrane of boar sperm cells. Biol Reprod 67: 340-50.

Gebauer MR, Pickett BW, Faulkner LC, Remenga EE, Berndtson WE (**1976**) Reproductive physiology of the stallion. Chemical characteristics of seminal plasma and spermatozoa. J Anim Sci 43: 628-632.

Garcia B, González-Fernández L, Loux SC, Rocha AM, Guimarães T, Pena F.J, Hinrichs K (**2015**) Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. Reproduction 149: 87-99.

Huang YL, Tseng WC, Cheng SY, Lin TH (**2007**) Trace elements and lipid peroxidation in human seminal plasma. Biol Trace Elem Res 76: 207–215.

Inagaki M, Kikuchi M, Orino K, Ohnami Y, Watanabe K (**2002**) Purification and quantification of lactoferrin in equine seminal plasma. J Vet Med Sci 64: 75-77.

Jobim MI, Oberst ER, Salbego CG, Souza DO, Wald VB, Tramontina F, Mattos RC (**2004**) Two-dimensional polyacrylamide gel electrophoresis of bovine seminal plasma proteins and their relation with semen freezability. Theriogenology 61: 255-266.

Jobim MI, Oberst ER, Salbego CG, Wald VB, Horn AP, Mattos RC (**2005**) BSP A1/A2-like proteins in ram seminal plasma. Theriogenology 63: 2053-2062.

Kareskoski AM, Sankari S, Johannisson A, Kindahl H, Andersson M, Katila T (**2011**) The association of the presence of seminal plasma and its components with sperm longevity in fractionated stallion ejaculates. Reprod Domest Anim 46: 1073-1081.

Kikuchi M, Mizoroki S, Kubo T, Ohiwa Y, Kubota M, Yamada N, Orino K, Ohnami Y Watanabe K (**2003**) Seminal plasma lactoferrin but not transferrin reflects gonadal function in dogs. J Vet Med Sci 65: 679-684.

Kosiniak K (**1975**) Characteristics of the successive jets of ejaculated semen of stallions." J Reprod Fertil (Suppl) 23: 59-61.

Kosiniak K (**1980**) The role of the accessory gland secretions during the ejaculate production in stallions. Acta Agrar Silvestria Ser Zootech 2: 75-86.

Kosiniak K, Bittmar A (**1981**) Biochemical components of stallion seminal plasma before and after the breeding season. Anim Reprod Sci 4: 39-47.

Lackey BR, Gray SL, Henricks DM (**2002**) Measurement of leptin and insulin-like growth factor-I in seminal plasma from different species. Physiol Res 51: 309-311.

Mann T (**1964**) The Biochemistry of Semen and of the Male Reproductive Tract, 2nd ed., Methuen & Co press, London, pp 334-337

Mann T (**1974**) Secretory function of the prostate, seminal vesicle and other male accessory organs of reproduction. J Reprod Fertil 37: 179-188.

Mann T (**1975**) Biochemistry of stallion semen. J Reprod Fertil (Suppl) 23: 47-52.

Melotti C, Parente R, Di Stasio D, Vitali G, Basunti G, Marchese S, Di Marzio G (**1996**) Citric acid and fructose seminal plasma concentrations and semen characteristics in the stallion. Bioch Clin 20: 90-97.

Novak S, Smith TA, Paradis F, Burwash L, Dyck MK, Foxcroft GR, Dixon WT (**2010**). Biomarkers of in vivo fertility in sperm and seminal plasma of fertile stallions. Theriogenology 74: 956-967.

Pandy VK, Parmeshwaran M, Soman SD, Dacosta JC (**1983**) Concentrations of morphologically normal, motile spermatozoa: Mg, Ca and Zn in the semen of infertile men. Sci Total Environ 27: 49-52.

Pesch S, Bergmann M, Bostedt H (**2006**) Determination of some enzymes and macro-and microelements in stallion seminal plasma and their correlations to semen quality. Theriogenology 66: 307-313.

Pickett BW, Sullivan JJ, Seidel GE Jr (**1975**) Reproductive physiology of the stallion. V. Effect of frequency of ejaculation on seminal characteristics and spermatozoal output. J Anim Sci 40: 917-923.

Restrepo G, Rojano, B, Usuga A (2019). Relationship of cysteine-rich secretory protein-3 gene and protein with semen quality in stallions. Reprod Domest Anim 54: 39-45.

Schambony A, Gentzel M, Wolfes H, Raida M, Neumann U, Töpfer-Petersen E (**1998**) Equine CRISP-3: primary structure and expression in the male genital tract. Biochim Biophys Acta 1387: 206-216.

Sieber F (**1987**) Merocyanine 540. Photochem Photobiol 46: 1035-1042.

Stanwell-Smith R, Thompson SG, Haines AP, Ward RJ, Cashmore G, Stedronska J, Hendry W F (**1983**) A comparative study of zinc, copper, cadmium, and lead levels in fertile and infertile men. Fertil Steril 40: 670-677.

Strzezek J, Wysocki P, Kordan W, Kuklinska M, Mogielnicka M, Soliwoda D, Fraser L (**2005**) Proteomics of boar seminal plasma-current studies and possibility of their application in biotechnology of animal reproduction. Reprod Biol 5: 279-290.

Talluri TR, Mal G, Ravi SK (**2017**) Biochemical components of seminal plasma and their correlation to the fresh seminal characteristics in Marwari stallions and Poitou jacks. Vet World 10: 214-220.

Tischner M, Kosiniak K, Bielanski W (**1974**) Analysis of the pattern of ejaculation in stallions. J Reprod Fertil 41: 329-335.

Töpfer-Petersen E, Ekhlasi-Hundrieser M, Kirchhoff C, Leeb T, Sieme H (**2005**) The role of stallion seminal proteins in fertilisation. Anim Reprod Sci 89: 159-170.

Usuga A, Rojano B, Restrepo G (**2017**) Effect of seminal plasma components on the quality of fresh and cryopreserved stallion semen. J Equine Vet Sci 58: 103-111.

Varner DD, Schumacher J, Blanchard T, Johnson L (**1991**) Breeding soundness examination. In: Diseases and management of breeding stallions. American Veterinary Publications, pp 61-96.

Von Fellenberg R, Zweifel HR, Grunig G, Pellegrini A (**1985**) Proteinase inhibitors of horse seminal plasma. A high molecular mass, acid-soluble proteinase inhibitor. Biol Chem Hoppe Seyler 366: 705-712.

Wong WY, Flik G, Groenen PM, Swinkels DW, Thomas CM, Copius-Peereboom JH, Steegers-Theunissen RP (**2001**) The impact of calcium, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men. Reprod Toxicol 15: 131-136.