Praca oryginalna *Original paper*

Characterization of fertile and infertile dog seminal plasma proteins and their correlation with semen quality: Preliminary study

BURCU ESIN[1], MERVE GIZEM SEZENER KABAY[2], CUMALI KAYA[1], VOLKAN ERGÜDEN[2], MESUT ÇEVIK[1]

[1]Department of Animal Reproduction and Artificial Insemination, University of Ondokuz Mayis, Samsun 55200, Türkiye
[2]Department of Veterinary Microbiology, University of Ondokuz Mayis, Samsun 55200, Türkiye

Received 10.06.2024 *Accepted 21.08.2024*

Esin B., Kabay M. G. S., Kaya C., Ergüden V., Çevik M.
Characterization of fertile and infertile dog seminal plasma proteins and their correlation with semen quality: Preliminary study

Summary

Significant deterioration in semen quality is associated with increased male infertility. Alterations in the sperm proteome resulting from infertility are not well understood. The aim of this study was to examine the connection between the proteome and the quality of spermatozoa in fertile and infertile dogs. The research was conducted on 11 male dogs, including 3 German shepherds, 3 Turkish Kangal, 2 Cane Corso, and 3 Golden Retrievers, aged 4 and 6 years (4.81 ± 0.75). Four of the 11 dogs constituted the infertile study group because of conception failure observed in at least 3 (3-5) matings with different fertile females within 1 year, and seven dogs from different owners constituted the fertile control group. Semen was manually manipulated and collected from all dogs in the presence of a female dog in heat. Ejaculates were used for spermatological examination and protein analysis. Computer-assisted semen analysis (CASA) was used to evaluate semen samples. In spermatological parameters, motility, kinematic parameters, morphology and plasma membrane integrity were evaluated. Gel electrophoresis (SDS-PAGE) was used to analyze proteins in sperm. The semen quality of infertile dogs was significantly lower. Motility, progressive motility, viability and plasma membrane integrity were statistically significantly lower in the infertile group than in the fertile group ($P < 0.05$). In morphological parameters, acrosome, head, middle part, tail and total defects were found to be statistically significantly higher in sterile dogs than in fertile dogs ($P < 0.05$). In the study, a total of 8 protein bands with molecular weights (MW) ranging from 11.0 to 245.0 kDa were identified in the protein analysis of semen. Variations in the sperm proteome composition were shown to be dependent on fertility. These proteins were associated with important metabolite pathways. Additionally, correlations of these bands with various spermatological parameters were revealed. The result of the study suggests that seminal plasma proteins play a role in semen quality and may be potential fertility biomarkers.

Keywords: canine, proteomics, SDS-PAGE, sperm quality

Infertility in dogs is a prevalent phenomenon (11, 12). Male dog infertility is becoming increasingly significant in clinical practice. The causes of infertility in males are not well understood (11). A comprehensive assessment of semen is required to evaluate fertility and to select suitable techniques and extenders for semen preservation (2). Semen quality plays an important role in the fertilization capacity of spermatozoa. Various spermatological parameters, such as motility, morphology, mitochondrial potential, the integrity of sperm plasma and acrosome membranes and the presence of proteins in the ejaculate, are closely linked with the fertilizing potential of spermatozoa. Semen motility is considered to be one of the most important factors to consider when evaluating the fertilization capacity of sperm (6). Numerous factors, including post-testicular molecular processes, influence semen motility. Evaluation of male dogs' reproductive status based only on semen analysis is insufficient (4). Therefore, applying proteomics and metabolomics in the field of andrology will aid in overcoming the limitations of the standard semen analysis.

The complex process of spermatogenesis includes the development of undifferentiated germ cells into

highly specialized sperm that are capable of fertilizing an oocyte (19). When spermatozoa mature in the epididymis after spermatogenesis, they acquire motility among other gains (8). In spermatogenesis, protein components derived from the epididymal duct play a crucial role (16).

Seminal plasma consists of secretions from the testicles, epididymis and male accessory glands. By providing a suitable environment for spermatozoa, it acts as a vehicle on the travels to meet the oocyte (29, 31). The very complex biological fluid known as seminal plasma is made up of proteins, enzymes, lipids, amino acids, carbohydrates, trace elements and minerals. Special proteins found in seminal plasma are essential to the survival and function of sperm (10, 31).

Male infertility may be influenced by molecular changes in spermatozoa and seminal plasma (31). Thus, seminal plasma proteins can function as significant biomarkers of male infertility (28). Protein characterization is made possible by contemporary research techniques, such as one-dimensional gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis (2D-PAGE), mass spectrometry, chromatography and flow cytometry (7). However, even with the advancements in proteomics, there is still a lack of comprehensive information regarding the comparative analysis of seminal plasma proteins linked to male infertility (31). The purpose of this study was to compare the semen quality and proteomic analysis of seminal plasma proteins from fertile and infertile dogs.

Material and methods

Ethics statement. This study was approved by the Ondokuz Mayis University, Animal Experiments Local Ethics Committee (Approval no: E-68489742-604.01.03-2300061883).

Animals and semen collection. The research was conducted using 11 different breeds of dogs (3 German shepherds, 3 Turkish Kangal, 2 Cane Corso and 3 Golden Retrievers). The dogs were between 4 and 6 years (4.81 ± 0.75) of age and weighed between 28 and 55 kg (40.54 ± 10.99). Four male dogs were referred to our Department of Animal Reproduction and Artificial Insemination with the Clinic because of conception failure in at least 3 (3-5) matings of fertile bitches during the preceding year. These dogs had previously experienced normal reproduction. Seven fertile dogs from different owners served as the control group. The general condition of all the males was good. In the presence of a teaser bitch in heat, semen was manually manipulated and collected (23). The three fractions of the ejaculate were collected into a prewarmed (37°C) three separate glass tubes, and the volume of each fraction was recorded.

Semen analysis. Semen motility, kinematic parameters and concentration were evaluated using a Computer-Aided Sperm Analyzer (CASA) (SCA®, Microptic, Barcelona, Spain) in at least 5 microscope fields or by reading at least 500 cells. A negative phase-contrast microscope (Nikon, Eclipse, Tokyo, Japan) with a 10 × objective and a heating plate kept at 37°C was used. Using CASA, total motility %, progressive motility %, kinematic parameters and total concentration parameters were measured and recorded.

The morphology of the spermatozoa was evaluated using a SpermBlue® kit (Microptic, Spain). For this, 10 µl of 50 × 10^6/mL semen was placed on a glass slide, and a coverslip was held at an angle of about 45° and pushed over the slide to make the smear. The smear was stained according to the kit manufacturer's instructions. After the staining process, the morphology module was selected in CASA, and evaluations were performed on at least 200 spermatozoa. Head, acrosome, midpiece, tail abnormalities and total abnormal spermatozoa were evaluated and recorded.

The percentage of viability and sperm plasma membrane integrity were analyzed with the hypoosmotic eosin staining test (HE-test) (25). Samples of semen were diluted at 1 : 10 (v/v) in 100 mOsm/kg fructose solution with 1% (w/v) eosin-Y and incubated in a water bath at 37°C for 30 min. Following incubation, a 5-µL drop was placed on a pre-warmed clean glass slide mounted with a cover glass, and 400 sperm per sample were analyzed using a phase-contrast microscope (400 ×). Spermatozoa were evaluated as follows: tail swollen and head white (HOS+/E–), tail non-swollen and head white (HOS–/E–), tail swollen and head red (HOS+/E+), tail non-swollen and head red (HOS–/E+).

Seminal plasma preparation for proteomics. The second fraction and part of the third fraction of the ejaculate from 11 dogs were collected separately by digital manipulation of the penis. After semen was collected, seminal plasma and sperm cells were separated by centrifugation (800 × g / 15 min). To remove the remaining cells, the seminal plasma was recentrifuged at 10,000 × g / 30 min at 4°C. It was then refrigerated at –20°C until protein extraction.

SDS-PAGE analysis. Seminal plasma samples were prepared for SDS-PAGE, according to the 9 with minor modifications. One-dimensional electrophoresis was employed to display the protein band's configuration and to indicate molecular weights by comparing them with the molecular marker (Fig. 1).

The columns on the left side of the figure represent the seminal plasma obtained from fertile and infertile dogs. The figure illustrates the seminal plasma samples from fertile (F) and infertile (I) dogs, along with its computer-drawn representation. Briefly, a volume of sample containing 21 mg/mL of protein was mixed with sample buffer (0.25 M TrisHCl, pH 6.8, 5% SDS, 25% (v/v) glycerol, 0.1% 2-mercaptoethanol, 0.05% bromophenol blue, 0.05% ultrapure H_2O), boiled for 5 min and loaded into the wells of a stacking gel (4% acrylamide) laid on top of a 12.5% gradient polyacrylamide resolving gel. In one well of stacking gel, 1 µL of 10-250 kDa molecular weight marker (Thermo Scientific™) was loaded. The remaining wells of the stacking gel were loaded with 15 µL seminal plasma samples. Initially, the 40 mA current was applied for 10 min, which was resulted with the samples pass to the separation gel. Then the current was reduced to 25 mA and applied for 40 min. The gel was fixated with fixation solution containing methanol (40%) and acetic acid (10%) in distilled water for

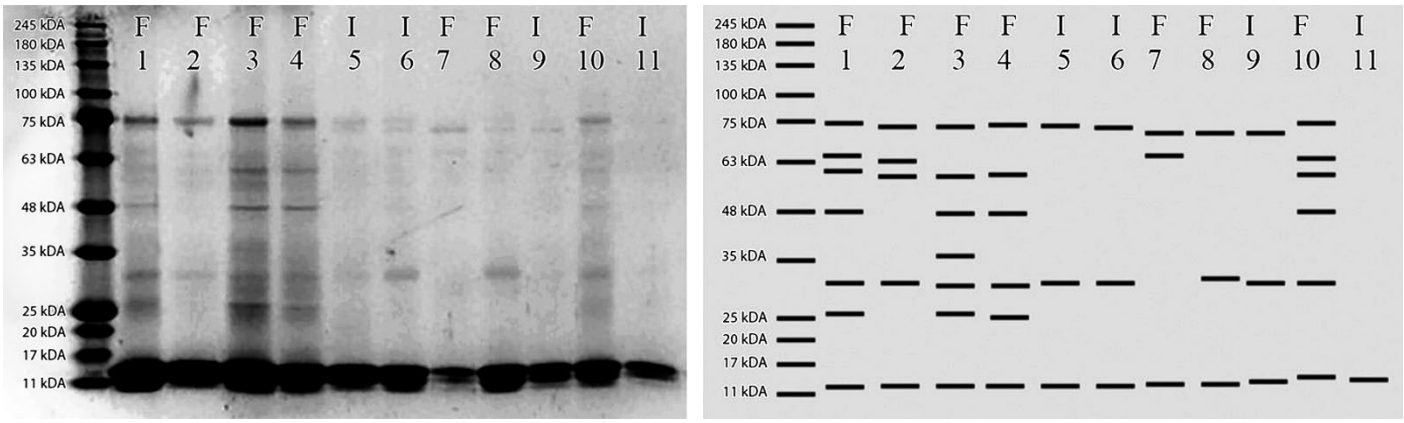

Fig. 1. Left: SDS-PAGE protein profiles of seminal plasma in fertile and infertile dogs (F – Fertile, I – Infertile), Right: Representation of SDS-PAGE protein profiles

4 hr and then stained with Coomassie Blue R-250 (Sigma-Aldrich) for 24 hr. After staining, the gel was destained with distilled water. The gel was examined and photographed by Istvan Lazar Jr., PhD and Istvan Lazar Sr., PhD, CSc. using GelAnalyzer 19.1 (www.gelanalyzer.com).

Statistical analysis. To ensure the appropriate sample size, an analysis was conducted with the G*Power software. Data analysis was performed in RStudio version 2022.07.2+576 (R Core Team, 2022). The α level was set at 0.05. In the statistical analysis of the parameters, Student's T-test, Welch's T-test and Mann-Whitney U test were employed based on the normality distributions and variances. Spearman's rank correlation coefficient was used to calculate correlations.

Results and discussion

The mean values of semen concentration and velocity parameters of the fertile and infertile dogs included in the study are shown in Table 1. When a comparison was made between the motility and progressive motility values of dogs in the fertile (80.34 ± 9.37, 44.27 ± 5.85) and infertile groups (32.83 ± 11.08, 14.80 ± 9.67), significantly decreased in dogs in the infertile group ($P < 0.05$). On the other hand, while there was a significant difference between the two groups ($P < 0.05$) in the kinematic velocity parameters, VCL, VAP, STR and BCF values, there was no statistically significant difference in VSL, LIN, WOB and ALH values.

The mean values of semen characteristics (viability, plasma membrane integrity, acrosome status and morphological parameters) of infertile and fertile dogs are presented in Table 2. The results showed differences ($P < 0.05$) in viability and plasma membrane integrity between the two groups. In morphological parameters, acrosome, head, middle piece, tail and total abnormal sperm morphology were found to be statistically significantly higher in sterile dogs than in fertile dogs ($P < 0.05$).

SDS-PAGE protein profiles of seminal plasma from fertile and infertile dogs were analyzed. The profile of 13% gel images and average bands in the seminal plasma from all 11 dogs is shown in Figure 1.

Protein profiles for the entire fertile group were similar and characterized by protein fractions with molecular weights (MW) ranging from 11 to 245.0 kDa.

Tab. 1. Semen concentration and kinematic velocity parameters (mean ± SD) in fertile and infertile dogs

Parameter	Unit	Fertile dogs (n = 7)	Infertile dogs (n = 4)	Probability (P)	CI 95%	
					LL	UL
Concentration	× 10^6/ml	345.28 ± 226.67	141.13 ± 46.67	0.060	–0.03	2.18
Second fraction	ml	2.57 ± 0.53	1.75 ± 0.50	0.060	0.09	0.92
Motility	%	80.34 ± 9.37	44.27 ± 5.85	0.001***	1.99	6.39
Progressive motility	%	32.83 ± 11.08	14.80 ± 9.67	0.020*	0.18	2.84
VAP	µm/s	46.56 ± 6.81	33.52 ± 11.82	0.040*	–0.23	2.32
VSL	µm/s	39.92 ± 5.60	36.35 ± 25.79	0.720	–0.98	1.15
VCL	µm/s	59.96 ± 7.43	41.28 ± 11.05	0.008**	0.12	3.08
ALH	µm	1.68 ± 0.32	2.18 ± 0.41	0.060	–0.94	–0.23
BCF	Hz	7.39 ± 0.66	5.25 ± 2.06	0.030*	–0.27	2.30
STR	%	72.98 ± 5.31	62.38 ± 7.43	0.020*	–0.03	2.69
LIN	%	57.05 ± 3.88	55.61 ± 14.53	0.800	–0.93	1.12

Explanations: Data are expressed as means ± SD; significance: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Tab. 2. Semen quality parameters (mean ± SD) in fertile and infertile dogs

Parameter (%)	Fertile dogs (n = 7)	Infertile dogs (n = 4)	Probability (P)	CI 95%	
				LL	UL
Viability	79.86 ± 6.31	47.75 ± 6.24	0.010**	1	1
Plasma membrane integrity	84.43 ± 3.74	55.25 ± 6.18	0.001***	1.48	7.8
Acrosomal defect	1.43 ± 0.98	4.5 ± 2.38	0.007**	–1	–1
Head defect	2.43 ± 0.98	5.25 ± 0.50	0.009**	–1	–1
Mid-piece defect	3.29 ± 1.11	5.75 ± 2.22	0.030*	–2.36	0.22
Tail defect	3.29 ± 0.95	6.50 ± 1.0	0.010**	–0.99	–0.85
Total abnormal sperm morphology	10.43 ± 1.90	22.0 ± 5.42	0.001***	–4	–0.28

Explanations: Data are expressed as means ± SD; significance: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Tab. 3. Correlations between the eight protein bands and some spermatological parameters

Parameter (%)	Band 1	Band 2	Band 3	Band 4	Band 5	Band 6	Band 7	Band 8
Motility	0.500	0.359	0.635*	0.717*	0.100	0.373	0.645*	0.747**
Density	0.500	–0.359	0.289	0.418	0.500	0.671*	0.645*	0.541
Viability	0.502	0.360	0.754**	0.840**	0.251	0.374	0.746**	0.857**
Plasma membrane integrity	0.201	0.840**	0.638*	0.360	–0.100	–0.037	0.130	0.491
Acrosomal defect	–0.527	–0.472	–0.730*	–0.693*	–0.105	–0.314	–0.680*	–0.793**
Head defect	–0.508	–0.425	–0.733*	–0.850**	–0.305	–0.341	–0.722*	–0.872**
Mid-piece defect	–0.512	–0.428	–0.384	–0.122	–0.256	–0.381	0.033	–0.417
Tail defect	–0.514	–0.215	–0.505	–0.615*	–0.257	–0.383	–0.498	–0.646*
Total abnormal sperm morphology	–0.506	–0.363	–0.613*	–0.665*	–0.202	–0.415	–0.588	–0.746**

Explanations: * Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed)

The protein profiles of the infertile group were also similar and characterized by protein fractions with molecular weights (MW) ranging from 11 to 94.0 kDa. This study identified a total of 8 protein bands. However, no dog had all 8 bands. In the 13% gel, Band 1 (75 kDa) and Band 8 (11 kDa) were present in all fertile dogs. Additionally, Band 8 was observed in all infertile dogs. Correlations between the eight protein bands and some spermatological parameters in the data obtained are presented in Table 3.

Seminal plasma is the supernatant that accounts for > 90% of the semen secreted from different appendage glands of male animals and is recovered after centrifugation of the semen. It serves as a potential non-invasive clinical sample for discovering fertility-related biomarkers. Seminal plasma, which is generally considered an indicator of fertility and has different properties from species to species, ensures the survival and transport of spermatozoa by participating in the inflammatory and immune response against sperm during passage through the female reproductive system (13). The identification of seminal plasma proteins can potentially be used to predict or increase the fertility rate (22). There is an increasing interest in proteomic analysis in different animal species. Dog semen is also a biological fluid that can be evaluated in this context (27). Research on dogs is crucial for advancements in medical science and can serve as a model for technologies that aid in reproduction for threatened or endangered animals (30). However, it should also be noted that the use of proteomic technologies in dogs may be a limiting factor because their genomes are not fully sequenced. As in proteomic analysis of blood plasma, identifying abundant proteins in seminal plasma is difficult due to the wide dynamic range of protein concentrations, with a high abundance of proteins expressed in seminal vesicles and the prostate (13). This study evaluated seminal plasma proteins with regard to semen quality in fertile and infertile dogs. The polyacrylamide denaturation test (gel electrophoresis) used in the study is a method used to detect protein metabolites and it helps identify specific proteins that may serve as fertility biomarkers. The fertilization abilities of the dogs used in this study were known, and significant correlations were found between seminal plasma proteins and spermatological parameters in the data obtained.

In the first study characterizing canine seminal plasma proteins, three protein subsets were found (15). It has been reported that there is no significant difference between protein concentrations detected in the seminal plasma of fertile and non-fertile dogs. Bruschi et al. (5) reported three protein fractions separated from dog seminal plasma, but relationships between these proteins and fertility or spermatological parameters could not be determined.

In a study investigating the effect of lactoferrin on gonadal functions in dogs, it was reported that lactoferrin purified from seminal plasma had a molecular mass of 75.2 kDa (21). The molecular group of lactoferrin determined in the study was equivalent to that of purified lactoferrin from humans (76 kDa) (32) and horse seminal plasma (80 kDa) (20), and its concentration range was found in porcine or human (26) seminal plasma. In that study, it was reported that lactoferrin is related to semen quantity and semen concentration. In the presented study, this protein can be considered lactoferrin, since protein bands detected at an average concentration of 63-75 kDa were found in the seminal plasma samples from both groups, and no statistical difference was observed in the amount and concentration between the two groups.

In a different study investigating the proteomic characterization of dog semen, 268 proteins were identified in seminal plasma by tandem mass spectrometry. These proteins and their properties were estimated. In the study, the 67.74 kDa protein was predicted to be a basal cell adhesion molecule. The glycoprotein on the cell surface known as the basal cell adhesion molecule is a member of the immunoglobulin superfamily membrane (1, 24). In the presented study, the second band, which was predicted to be this protein, was not detected in the seminal plasma of infertile dogs. This band was formed in four of the fertile dogs. A positive correlation was observed between this band and plasma membrane integrity. The basal cell adhesion molecule serves as a membrane surface protein and is thought to protect sperm membrane integrity.

In another study, 37 bands with molecular weights ranging from 3.5 to 136 kDa were identified in seminal plasma samples from five dogs with unknown fertility. Electrophoretic profiles of canine seminal plasma were performed by SDS-PAGE and correlated with semen quality parameters (33). In the same study, a range of 20-37 protein bands were detected, with two bands of 67 and 58.6 kDa showing a positive correlation with semen motility, but their identities were unknown. A 3rd band of 58.6 kDa obtained in our study was seen in all six fertile dogs; it showed a positive correlation with semen viability, plasma membrane integrity and semen motility. It also showed a negative correlation between total morphological sperm abnormality and acrosome damage. In previous studies, this 58.6 kDa band was reported as a heparin binding protein and fertility marker (33, 34). As seen in the data presented in this study, the 58.6 kDa band, which is thought to be a fertility marker, was not detected in infertile dogs.

It is noteworthy that this study was conducted by correlating protein band densitometry with semen characteristics in infertile and fertile dogs. Semen motility in fertile dogs and other essential semen characteristics, the rate of dead organisms in semen, total morphological disorder and acrosome integrity were associated with two bands (Band 5 and Band 7). Band 5 was observed in four fertile dogs and Band 7 was observed in six fertile dogs. Similarly, in other studies, proteins such as arginine esterase, glutathione peroxidase and protein lifeguard 3, which are among predicted proteins in bands with a similar concentration range (24-31 kDa), were identified and reported to be among proteins that play essential roles in reproductive physiology (2, 14). However, the function of these proteins in seminal plasma has not been entirely determined. Arginine esterase, found in high concentrations in canine prostate secretions, has been reported to be responsible for > 90% of canine prostate-secreted protein and 30% of canine seminal plasma proteins. It is an important sign of human prostate cancer and may also be considered as a particular immunological marker to evaluate the prostate gland's normality. It is very similar to prostatic specific antigen (PSA). In this study, protein bands identified in the seminal plasma of fertile dogs were associated with motility and other spermatological characteristics, both *in vitro* and *in vivo*, while those identified in the seminal plasma of infertile dogs have not been previously described in dogs.

In a study investigating the effect of vasectomy on seminal plasma proteins in dogs, the protein levels of 29.2 kDa and 42.6 kDa were not observed after vasectomy. It has been reported that vasectomy is not observed in dogs due to the inhibition of the formation of sperm that can bind these proteins to the membrane surface (34). These proteins serve as proteins that attach to the sperm surface. In the presented study, the resulting bands with a protein concentration of approximately 35-48 kDa were detected in the seminal plasma of 4 fertile dogs. It was not detected in the seminal plasma of infertile dogs. The data showed that spermatozoon membrane integrity was higher in fertile dogs than in infertile dogs ($p < 0.001$). Therefore, it is thought that these bands seen in fertile dogs are a protein coming from the subunit of sperm surface proteins.

Band 6 (25-35 kDa), observed in the proteomic analysis, was found in both fertile and infertile dogs. Band 6 showed a positive correlation with spermatozoa concentration. No statistical difference was revealed in spermatozoa concentration between the two groups. For this reason, it was predicted that this band could be associated with spermatozoa concentration and evaluated in this context. In previous studies, proteins considered in this protein concentration range were presented as protein Rab-8A and cell division control protein 42 homologs (2). However, their reproductive functions were not fully explained. In line with these results, more comprehensive analysis and advanced tests should be performed to fully determine the functions of these proteins and should be evaluated together with the findings of the current study.

Notably, some previous studies reported a higher number of identified proteins than those in our study (2, 3, 17). The differences in results between the current study and previous studies may be due to the heterogeneity of animals, protein differences between breeds and between individuals of the same breed, and the absence of standardization in proteomics technologies, which use different procedures for identification and sample preparation (such as protein extraction). Additionally, procedures performed before proteomic analysis (e.g., sample collection, processing and storage) may affect the results of a proteomic study (18).

Canine seminal plasma contains various proteins with unique biochemical properties and functions. Some of the proteins identified or predicted in this study can be used as markers of canine fertility. It is also important to be able to determine the individual proteins in the seminal plasma of each species and whether any of these proteins are markers of fertility. Identifying proteins as fertility markers helps understand the proper role or function of seminal plasma and has enormous potential for reproductive technologies. Male seminal plasma can be used to evaluate reproductive potential and cryopreservation suitability. It is possible to isolate and purify these markers for application in female reproductive tracts or extenders that improve spermatozoa survival after thawing. Males with high or low fertility can also be identified before use, which may increase the success of proteomic analysis and the benefits of this analysis in the reproductive field. Furthermore, more advanced research that leads to the isolation of seminal plasma proteins at greater resolutions, comprehensive characterization of those proteins and exploration of their physiological function would contribute to the advancement of knowledge in this field. More studies with comprehensive research protocols are needed to investigate the relationship between proteomic analysis and the quality of semen obtained from larger male samples representative of fertile and infertile populations.

References

1. *Akiyama H., Iwahana Y., Suda M., Yoshimura A., Kogai H., Nagashima A.*: The FBI1/Akirin2 target gene, BCAM, acts as a suppressive oncogene. PLoS One. 2013, 8, 11, e78716
2. *Aquino-Cortez A., Pinheiro B. Q., Lima D. B. C., Silva H. V. R., Mota-Filho A. C., Martins J. A. M.*: Proteomic characterization of canine seminal plasma. Theriogenology 2017, 95, 178-186.
3. *Araujo M. S., de Oliveira Henriques Paulo O. L., Scott C., Paranzini C. S., Codognoto V. M., de Paula Freitas Dell'Aqua C.*: Insights into the influence of canine breed on proteomics of the spermatozoa and seminal plasma. J. Proteomics 2022, 257, 104508.
4. *Barbăroşie C., Agarwal A., Henkel R.*: Diagnostic value of advanced semen analysis in evaluation of male infertility. Andrologia 2021, 53, 2, e13625.
5. *Brushi J., MMC., DAJ.*: Teores de ácido cítrico, frutose, proteína total e seu fraciona-miento eletroforético na semen da cao "Pastor alemao" normal. Arq Esc Vet U Fed Minas Gerais. 1979, 31, 13-17.
6. *Chakraborty S., Saha S.*: Understanding sperm motility mechanisms and the implication of sperm surface molecules in promoting motility. Middle East Fertil Soc J. 2022, 27, 1.
7. *Chambers G., Lawrie L., Cash P., Murray G. I.*: Proteomics: A new approach to the study of disease. Journal of Pathology 2000, 192, 3, 280-288.
8. *Chłopik A., Wysokińska A.*: Canine spermatozoa – What do we know about their morphology and physiology? An overview. Reproduction in Domestic Animals. 2020, 55, 2, 113-126.
9. *Dao T. T. M., Truong D. D., Duong L. N. H., Nguyen N. N. Y., Nguyen H. D.*: Preparation of Bacillus subtilis cell samples and generation of an SDS-PAGE. Biotechniques 2023, 74, 3, 123-129.
10. *Davalieva K., Kiprijanovska S., Noveski P., Plaseski T., Kocevska B., Broussard C.*: Proteomic analysis of seminal plasma in men with different spermatogenic impairment. Andrologia 2012, 44, 4, 256-264.
11. *Domosławska A., Zdunczyk S.*: Clinical and spermatological findings in male dogs with acquired infertility: A retrospective analysis. Andrologia 2020, 52, 11, e13802.
12. *Domosławska A., Zdunczyk S., Franczyk M., Kankofer M., Janowski T.*: Total antioxidant capacity and protein peroxidation intensity in seminal plasma of infertile and fertile dogs. Reproduction in Domestic Animals 2019, 54, 2, 252-257.
13. *Drabovich A. P., Saraon P., Jarvi K., Diamandis E. P.*: Seminal plasma as a diagnostic fluid for male reproductive system disorders. Nat. Rev. Urol. 2014, 11, 5, 278-288.
14. *Druart X., de Graaf S.*: Seminal plasma proteomes and sperm fertility. Anim. Reprod. Sci. 2018, 194, 33-40.
15. *Dubiel A.*: Electrophoretic studies of dog's semen plasma in both fertile and sterile dogs. Pol. Arch. Weter. 1975, 17, 4, 699-706.
16. *Ellerbrock K., Pera I., Hartung S., Ivell R.*: Gene expression in the dog epididymis: a model for human epididymal function. 1994, 17, 6, 314-323.
17. *Gouletsou P. G., Tsangaris G. T., Katsarou E. I., Bourganou M. V., Barbagianni M. S., Venianaki A. P.*: Proteomics evaluation of semen of clinically healthy Beagle-Breed dogs. Vet. Sci. 2022, 9, 12.
18. *Greco V., Piras C., Pieroni L., Urbani A.*: Direct assessment of plasma/serum sample quality for proteomics biomarker investigation. 2017, 3-21.
19. *Hur T.-Y., Lee S.-H., Ock S.-A., Sont H., Park H.-J., Lee R.*: Dose-dependent effects of busulfan on dog testes in preparation for spermatogonial stem cell transplantation. 2017, 3, 264-269.
20. *Inagaki M., Kikuchi M., Orino K., Ohnami Y., Watanabe K.*: Purification and quantification of lactoferrin in equine seminal plasma. 2002.
21. *Kikuchi M., Mizoroki S., Kubo T., Ohiwa Y., Kubota M., Yamada N.*: Seminal plasma lactoferrin but not transferrin reflects gonadal function in dogs. 2003.
22. *Killian G., Chapman D. A., Rogowski L. A.*: Fertility-associated proteins in Holstein Bull seminal plasma. 1993, 49, 6, 1202-1207.
23. *Linde-Forsberg C.*: Achieving canine pregnancy by using frozen or chilled extended semen. Vet. Clin. North. Am. Small Anim. Pract. 1991, 21, 3, 467-485.
24. *Ma Y., Ma Q. W., Sun Y., Chen X. F.*: The emerging role of extracellular vesicles in the testis. Hum. Reprod. 2023, 38, 3, 334-351.
25. *Mansour M. M.*: Modification of hypo-osmotic swelling test to evaluate the integrity of stallion sperm plasma membrane. Glob. Vet. 2009, 3, 4, 302-307.
26. *Masson P. L.*: Studies on lactoferrin, the iron-binding protein of secretions. Prot. Biol. Fluida. 1966, 14, 115-124.
27. *Miller I., Preßlmayer-Hartler A., Wait R., Hummel K., Sensi C., Eberini I.*: In between – Proteomics of dog biological fluids. J. Proteomics. 2014, 106, 30-45.
28. *Nishimune Y., Tanaka H.*: Infertility caused by polymorphisms or mutations in spermatogenesis-specific genes. J. Androl. 2006, 27, 3, 326-334.
29. *Rodriguez-Martinez H., Martinez E. A., Calvete J. J., Peña Vega F. J., Roca J.*: Seminal plasma: Relevant for fertility? Int. J. Mol. Sci. 2021, 22, 9.
30. *Sandhey R., Bhakri Msc G., Gandotra V. K., Kaur C.*: Characterization of mongrel dog seminal plasma proteins and their correlation with semen characteristics. J. Reprod. Stem. Cell Biotech. 2011, 2, 55-63.
31. *Sharma R., Agarwal A., Mohanty G., Jesudasan R., Gopalan B., Willard B.*: Functional proteomic analysis of seminal plasma proteins in men with various semen parameters. Reproductive Biology and Endocrinology 2013, 11, 1.
32. *Sorrentino S., D'alessandro A. M., Maras B., Ciccio L Di., D'andrea G., De Prisco R.*: Purifcation of a 76-kDa iron-binding protein from human seminal plasma by affinity chromatography specific for ribonuclease: structural and functional identity with milk lactoferrin 1. 1999.
33. *Souza F. F. de, Barreto C. S., Lopes M. D.*: Characteristics of seminal plasma proteins and their correlation with canine semen analysis. Theriogenology 2007, 68, 1, 100-106.
34. *Souza F. F. de, Martins M. I. M., Lopes M. D.*: Vasectomy effect on canine seminal plasma biochemical components and their correlation with seminal parameters. Theriogenology 2006, 66, 1621-1625.

Corresponding author: Burcu Esin; e-mail: burcuyalcin@omu.edu.tr