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Original article

Identification of proteoforms of albumin and kallikrein in stallion seminal plasma and their correlations with sperm motility

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Abstract

The aim of this study was to identify the proteoforms of albumin and kallikrein in stallion seminal plasma (SP), and to determine their correlations with sperm motility parameters. The experimental material consisted of ejaculates from 8 stallions, which were collected during the breeding and non-breeding seasons (BS and NBS, respectively). SP proteins were identified by 2-D PAGE and mass spectrometry (MALDI TOT/TOF MS). Sperm motility parameters were analyzed using the CASA system. Protein expression (integrated optical density-IOD) of albumin proteoforms 1 (ALB 1) and 2 (ALB 2) and kallikrein proteoforms 1 (KAL 1) and 2 (KAL 2) was correlated ($p<0.05$) with sperm motility parameters (total motility and progressive motility) during the BS. No significant correlations were found between the expression of albumin or kallikrein and sperm motility parameters during the NBS. The presence of correlations between the expression of ALB 1, ALB 2, KAL 1, KAL 2 and selected sperm motility parameters could suggest that the analyzed components of the SP belong to the group of fertility-associated proteins (FAPs).

Key words: seminal plasma proteins, albumins, kallikreins, stallion

Introduction

The seminal plasma (SP) of stallions is a mixture of fluids secreted by the seminiferous tubules, epididymides, bulbourethral glands, prostate gland, seminal vesicles and ampulla of ductus deferens (Töpfer-Petersen et al. 2005). The SP is involved in sperm epididymal maturation and survival of spermatozoa in the female reproductive tracts (Töpfer-Petersen et al. 2005). Proteins of the SP proteins play an important role in the initiation and maintenance of sperm motility, maintenance of optimal osmotic pressure in semen, prevention of premature acrosome reaction during sperm transport through the female reproductive tract, and stabilization of sperm cell membranes (Mogielnicka-Brzozowska and Kordan 2011). It has been reported that SP proteins

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protect spermatozoa against phagocytosis in the female reproductive system and participate in sperm-oocyte interactions (Doty et al. 2011).

The composition of stallion ejaculates is determined by various factors, including breed, season, age, ejaculate fraction and individual traits (Abou-Ahmed et al. 1993, Kareskoski and Katila 2008, Kareskoski et al. 2011). The quality of stallion semen determines the effectiveness of many biotechnological procedures, such as insemination and *in vitro* fertilization. Since these methods of reproduction are very expensive, SP components have been investigated to identify potential markers for ejaculate quality and male fertility (Caballero et al. 2012). The male fertility is dependent on complex orchestrated biological reactions, which are controlled by the action of various proteins (Ashrafzadeh et al. 2013). However, the role of the exact SP proteins in male reproductive processes is still unclear. Furthermore, comparisons between good quality and bad quality semen samples revealed differences in protein expression (Jobim et al. 2011). Nevertheless, current information about fertility-associated proteins (FAPs) is still not sufficient to propose diagnostic protocols.

In cattle, pigs, horses, sheep and dogs, FAPs are positively or negatively correlated with the sperm fertilizing capacity (Mogielnicka-Brzozowska and Kordan 2011). These proteins have been categorized into energy related, structural and other functional proteins, which play a major role in sperm motility, capacitation and sperm-oocyte binding (Ashrafzadeh et al. 2013). Little is known about the influence of seasonal changes in the SP proteome and the reproductive capacity of stallions. We suggest that the analysis of the expression of sperm and SP proteins is required to increase our knowledge of the biochemical processes, which affect the ejaculate quality and reproductive performance of the stallion. The aim of this study was to identify the proteoforms of albumin and kallikrein in stallion SP, and to determine their correlations with sperm motility parameters.

Materials and Methods

Chemicals and media

All chemicals were of the highest purity grade and were purchased from the Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Animals and semen collection

Semen was collected from eight mature light-breed stallions of normal reproductive status with an average age of 15.5 years (range 10-21 years), using an artificial vagina (Missouri Model AV, Nasco, Ft. Atkinson, WI, USA). Stallions used in our experiment were of estimated fertility and were kept in a horse stud as regular sires. The first cycle pregnancies of analysed stallions ranged from 45 to 87% (average 66%), and the final mare pregnancies ranged from 66 to 100% (average 91%). Four stallions were housed in Stack Stallions in Łąck (Region of Mazowsze, Poland) and the remaining four animals were kept in the stable of Marek Romanowski in Wozławki (Region of Warmia and Mazury, Poland). Three ejaculates were collected from each stallion during the breeding season (BS) (April - May) (n = 24) and another three ejaculates were collected from each animal during the non-breeding season (NBS) (October - December) (n = 24). All experimental procedures were carried out in accordance with the guidelines set out by the Local Ethics Committee for Animal Experimentation.

Experimental design

This study was divided into two experiments: (i) Experiment 1 (Exp. 1), which involved the analysis of the motility of fresh spermatozoa, and (ii) Experiment 2 (Exp. 2), which was based on the purification of SP proteins, 2D electrophoresis and mass spectrometry.

Semen processing procedure

A nylon in-line filter was used to remove the gel fraction from each whole ejaculate. Each ejaculate was routinely assessed by the same trained individual for semen quality parameters, such as ejaculate volume and percentage of motile spermatozoa. Sperm concentration was determined using a Bürker counting chamber (Equimed-Medical Instruments, Cracow, Poland). A portion of the ejaculate was diluted with the EquiPro™ extender (Minitübe, Tiefenbach, Germany) to a final concentration of 30×10^6 sperm cells/ml, and cooled to 5°C before being transported to the Cryopreservation Laboratory at the Department of Animal Biochemistry and Biotechnology of the University of Warmia and Mazury in Olsztyn, Poland. Sperm motility parameters were analyzed within 3 hours of sample delivery (Exp. 1).

The remaining portion of the ejaculate was subjected to centrifugation at room temperature at 10 000 × g for 10 min. The supernatant was separated from the sperm pellets and centrifuged once more at room temperature at 10 000 × *g* for 10 min, then frozen at -80°C for further analysis (Exp. 2).

Experiment 1

Sperm motility and motion patterns

Sperm motility characteristics were analyzed using a computer-assisted sperm analysis (CASA) system (Hamilton-Thorne Research, HTR, IVOS version 12.3; Beverley, MA, USA). Aliquots of the sperm samples (3 µl) were placed in a prewarmed Makler counting chamber (Sefi-Medical Instruments Ltd., Israel) and examined at 38ºC. The IVOS analyzer had the following settings: frame acquired – 30, frame rate – 60 Hz, minimum cell contrast – 75, minimum cell size – 6 pixels, straightness threshold – 75%, low VAP cut-off – 9.9 µm/s^{-1}, low VSL cut-off – 20 µm/s^{-1}, static size gates – 0.80-4.93, static intensity gates – 0.49-1.68, static elongation gates – 22-84. Six fields were randomly selected and were analyzed for each sperm sample (Mogielnicka-Brzozowska et al. 2017). In the experiment we used sperm samples with total motility (TMOT) averaging 66.7% (range, 36 to 94%) and progressive motility (PMOT) averaging 25.5% (range, 12 do 55%).

Experiment 2

Measurement of total protein content

Total protein content was measured according to the method proposed by Lowry et al. (1951), with bovine serum albumin (BSA, IBSS BIOMED S.A., Poland) as the standard.

2D-PAGE analysis

Individual samples of SP (16 samples collected during BS and 16 samples collected during the NBS) were subjected to two-dimensional polyacrylamide gel electrophoresis (2D PAGE) according to a previously described method (O'Farrell 1975), with some modifications (Mogielnicka-Brzozowska et al. 2015). Each protein sample was loaded on the gel at least three times. We measured the IOD values of all spots on the each gel and only six protein spots showed marked changes in the IOD values. Protein concentration was calculated at 75 µg per strip and the destained gels were analyzed using PDQuest 7.2 software (BioRad, Laboratories, Herkules, CA, USA). The 2D PAGE digital images were analyzed according to the PDQuest user guide to detect spots, determine integrated optical density (IOD), match spots and calculate the molecular weights (Mr) and isoelectric point (pI) values for each spot.

Protein identification by matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry (MALDI TOF/TOF MS)

Six protein spots showing marked changes in IOD values were excised from the gel and subjected to in-gel trypsin digestion using sequencing-grade modified trypsin (Promega, Madison, WI, USA). Desalting was conducted with ZipTip µC18 resin (Merck Millipore and EMD Millipore, Billerica, MA, USA) (Zakharchenko et al. 2011). Peptides were eluted with 70% acetonitrile (ACN, Merck, Darmstadt, Germany), dried and stored at -80°C until required for mass spectrometry (MS) analysis (Mogielnicka-Brzozowska et al. 2017).

Statistical analysis

The results were analyzed statistically using Statistica 13.1 (Stat Soft Incorporation, Tulsa OK, USA). Pearson's correlation coefficients were calculated between the IOD values (average of three gel analysis) of the six analyzed polypeptides with TMOT and PMOT during the breeding season (BS) or non-breeding season (NBS). The results are presented in scatter plots, and correlations were statistically significant at $p<0.05$. The sperm motility parameters are expressed as mean ± SD.

Results

Protein concentration in the SP of the analyzed samples averaged 28±8 mg/ml. The number of polypeptides of SP samples was analyzed in three molecular weight ranges: low-molecular-weight polypeptides (LMW) – below 20 kDa, medium-molecular-weight polypeptides (MMW) – 20 to 40 kDa, and high-molecular-weight polypeptides (HMW) – above 40 kDa. The total number of polypeptides identified by 2D-PAGE analysis in stallion SP was higher during the BS (77.8±4.8) than during the NBS (61.1±2.3). A similar trend was observed in the group of LMW polypeptides, in which the number of polypeptides was higher ($p<0.05$) during the BS (33.5±2.4) compared with the NBS (24.8±1.1). Furthermore, the number of MMW (21.0±8.5) and HMW polypeptides (24.1±2.8) was higher during the BS compared with the NBS (17.5±1.5 and 18.7 ±1.6, respectively), but the observed differences were not statistically significant ($p>0.05$).

Only the peptides that were detected in the SP of all stallions, and those which showed fluctuations in expression on a seasonal basis were selected for analysis (Fig. 1). The analyzed parameters represented the IOD of six SP polypeptides and their correlations with motility parameters, TMOT and PMOT, during the BS

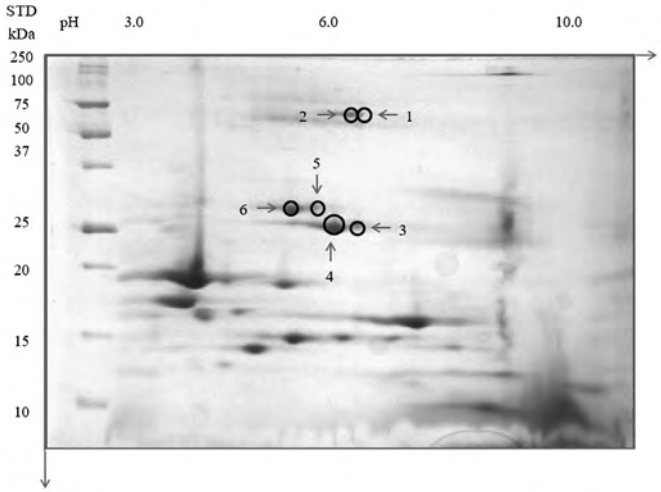

Fig. 1. Two dimensional polyacrylamide gel electrophoresis (2D PAGE) of stallion seminal plasma proteins. Polypeptides were identified using MALDI TOF-TOF MS. 1 – albumin 1 (ALB1), 2 – albumin 2 (ALB2), 3 – kallikrein 1 (KAL1), 4 – kallikrein 2 (KAL2), 5 – kallikrein 3 (KAL3), 6 – kallikrein 4 (KAL4). STD – Precision Plus Protein Standards (Bio-Rad).

Table 1. Identification of proteins in stallion seminal plasma using MALDI TOF-TOF mass spectrometry.

Protein No	Protein name	pI (2D-PAGE)	pI calculated	M.W (kDa) (2D-PAGE)	M.W (kDa) calculated	Mascot score	Cover (%)
1	albumin 1 (ALB 1)	6.2-7.0	5.89	61.0-75.2	70.4	128	9
2	albumin 1 (ALB 2)	6.1-6.8	6.05	60.9-68.2	71.1	75	4
3	kallikrein 1 (KAL 1)	6.1-6.9	5.44	21.4-26.7	21.9	93	12
4	kallikrein 2 (KAL 2)	5.9-6.6	5.44	20.0-25.6	21.9	121	12
5	kallikrein 3 (KAL 3)	5.5-6.3	5.44	24.5-30.6	21.9	60	12
6	kallikrein 4 (KAL 4)	5.2-5.8	5.44	25.9-28.5	21.9	72	12

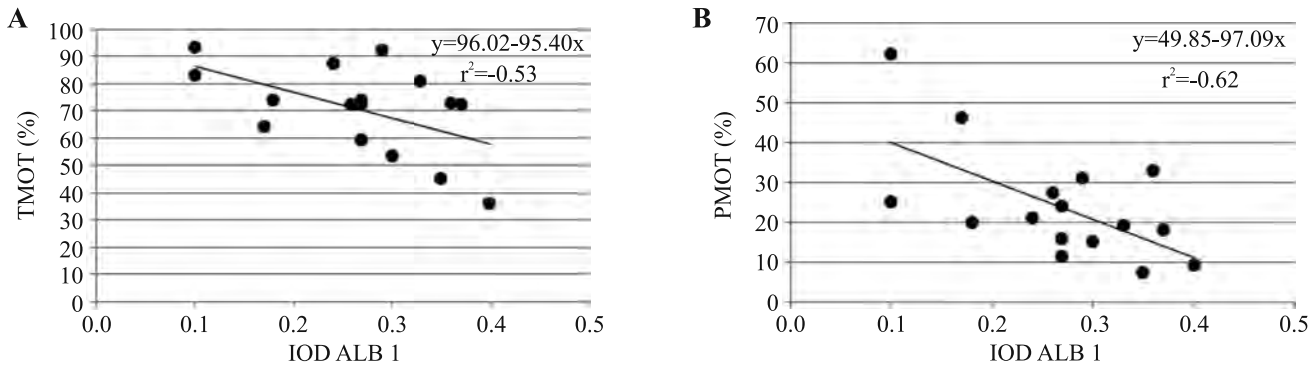

Fig. 2. Regression model showing variations in the (A) total motility (TMOT) and (B) progressive motility (PMOT) of stallion spermatozoa during the breeding season as related to the integrated optical density (IOD) of seminal plasma albumin 1 (ALB 1) ($p \leq 0.05$).

and NBS. The molecular weights (Mr) and isoelectric points (pI) of the analyzed polypeptides were as follows: albumin 1 (ALB 1) – 61.0-75.2 kDa, pI 6.2-7.0, albumin 2 (ALB 2) – 60.8-68.2 kDa, pI 6.1-6.8, kallikrein 1 (KAL 1) – 21.4-26.7 kDa, pI 6.1-6.9, kallikrein 2 (KAL 2) – 20.0-25.6 kDa, pI 5.9-6.6, kallikrein 3 (KAL 3) – 24.5-31.6 kDa, pI 5.5-6.3, and kallikrein 4 (KAL 4) – 25.9-28.5 kDa, pI 5.2-5.8 (Fig. 1, Table 1).

The IOD values of **ALB 1** of the SP were negatively correlated with TMOT (r^2=-0.53) and PMOT (r^2=-0.62) during the BS (Fig. 2A and 2B, respectively). It was found that the IOD values of **ALB 2** were negatively correlated ($p<0.05$) with TMOT (r^2=-0.50) and PMOT (r^2=-0.52) (Fig. 3A and 3B, respectively). The IOD values of **KAL 1** were negatively correlated with TMOT and PMOT (r^2= -0.53 and r^2=-0.43, respectively) during the BS (Fig 4 A and B, respectively). Nega-

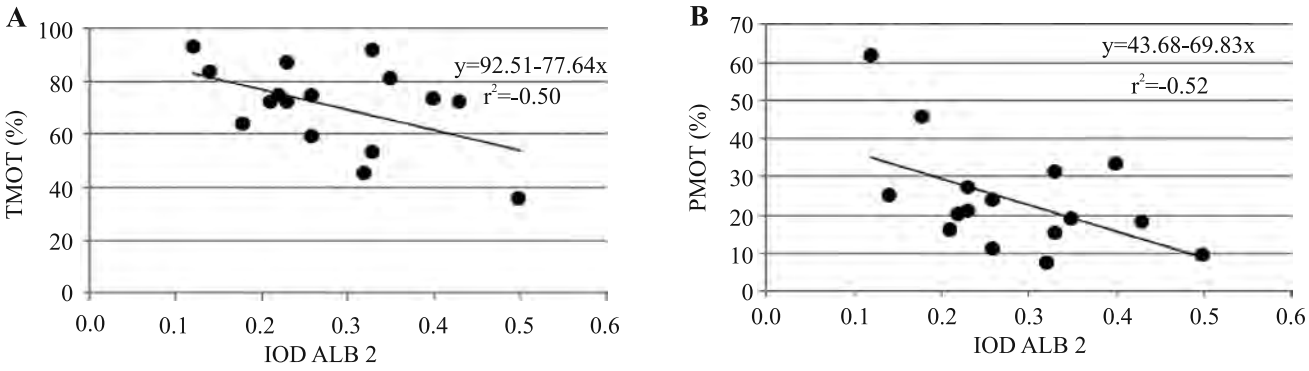

Fig. 3. Regression model showing variations in the (A) total motility (TMOT) and (B) progressive motility (PMOT) of stallion spermatozoa during the breeding season as related to the integrated optical density (IOD) of seminal plasma albumin 2 (ALB 2) ($p \leq 0.05$).

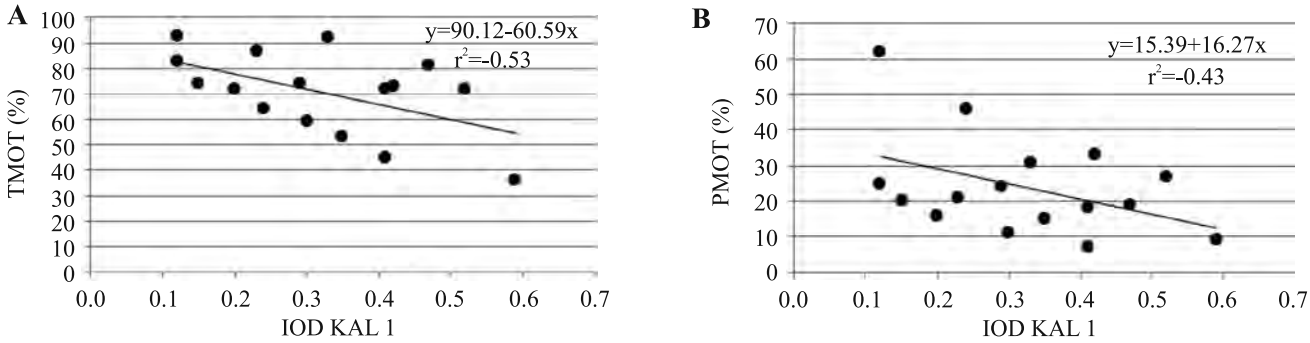

Fig. 4. Regression model showing variations in the (A) total motility (TMOT) and (B) progressive motility (PMOT) of stallion spermatozoa during the breeding season and as related to the integrated optical density (IOD) of seminal plasma kallikrein 1 (KAL 1) ($p \leq 0.05$).

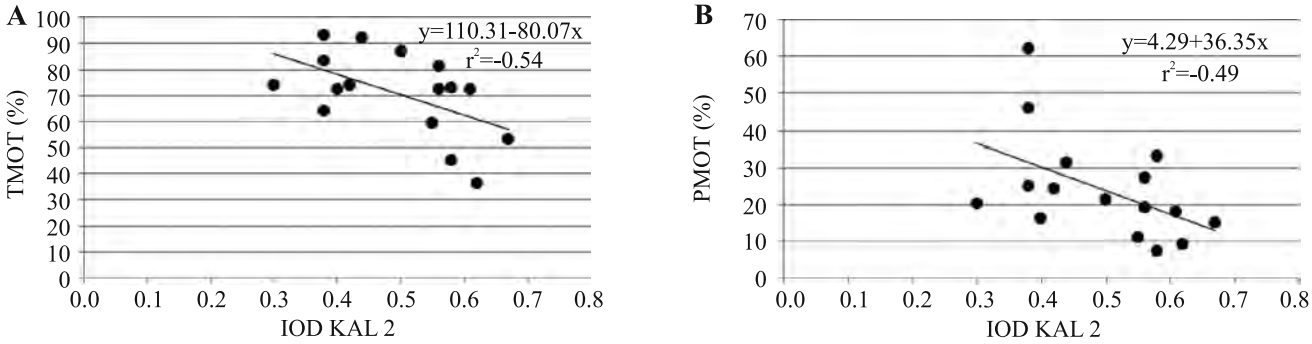

Fig. 5. Regression model showing variations in the (A) total motility (TMOT) and (B) progressive motility (PMOT) of stallion spermatozoa during the breeding season as related to the integrated optical density (IOD) of seminal plasma kallikrein 2 (KAL 2) ($p \leq 0.05$).

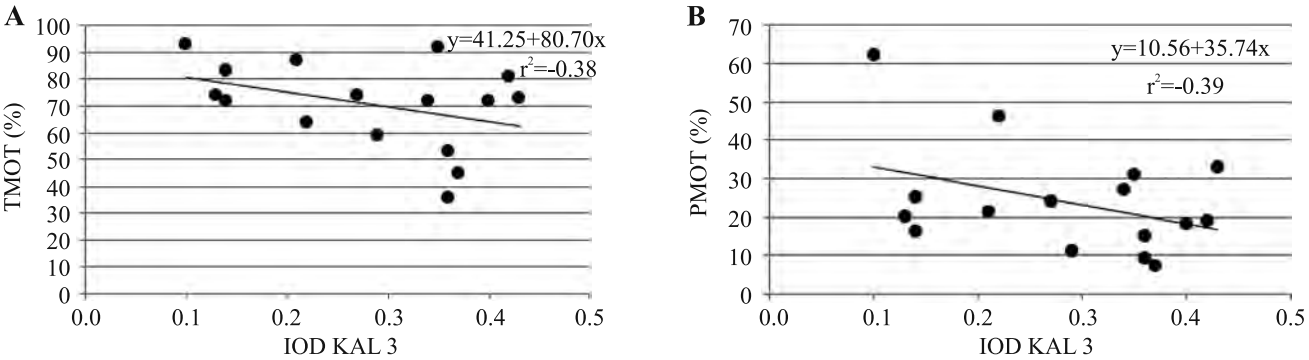

Fig. 6. Regression model showing variations in the (A) total motility (TMOT) ($p \leq 0.05$) and (B) progressive motility (PMOT) ($p \geq 0.05$) of stallion spermatozoa during the breeding season as related to the integrated optical density (IOD) of seminal plasma kallikrein 3 (KAL 3).

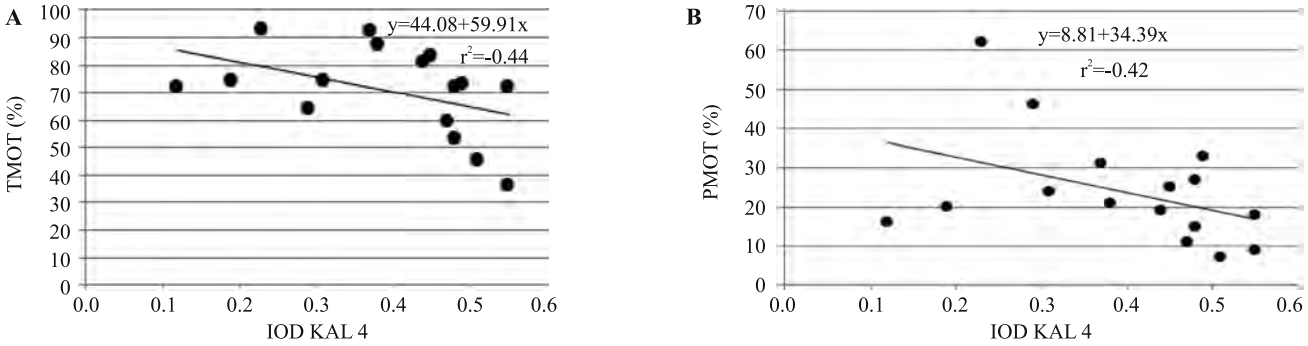

Fig. 7. Regression model showing variations in the (A) total motility (TMOT) ($p \leq 0.05$) and (B) progressive motility (PMOT) ($p \geq 0.05$) of stallion spermatozoa during the breeding season as related to the integrated optical density (IOD) of seminal plasma kallikrein 4 (KAL 4).

tive correlations were found between the IOD values of **KAL 2** and TMOT (r^2=-0.54) and PMOT (r^2=-0.49) (Fig 5A and 5B, respectively). However, only the correlation between the IOD of KAL 2 and TMOT was statistically significant ($p<0.05$) (Fig. 5 A). In addition, negative correlations were observed between the IOD values of **KAL 3** and TMOT (r^2=-0.38) and PMOT (r^2=-0.39) (Fig. 6A and 6B, respectively), and between the IOD values of **KAL4** with TMOT (r^2=-0.44) and PMOT (r^2=-0.42) (Fig. 7A and 7B, respectively). During the NBS the IOD values of ALB1, ALB2, KAL1, KAL2, KAL3 and KAL4 were slightly positively correlated with TMOT and PMOT, but the observed differences were not statistically significant ($p>0.05$) (unpublished data).

Discussion

Selection and fertility prediction in the stallion are routinely based on the analysis of seminal and behavioural parameters. The parameters used to assess semen quality include sperm motility, morphology, of chromatin and membrane integrity, and analysis of SP composition (Magstrini et al. 1996). The SP contains protein and non-protein components, however, the protein components deserve particular attention because they participate in key processes before fertilization, such as capacitation, acrosome reaction and sperm-oocyte interactions (Töpfer-Petersen et al. 2005). Stallions exhibit libido throughout the year, but considerable differences can be found in the composition of the SP (Tejerina et al. 2009). Sperm motility plays a major role in fertilization success, and is often used to assess the semen quality and sperm viability in the stallion (Tejerina et al. 2009). The assessment of the microenvironment surrounding the sperm cell at the time of ejaculation has potential benefits because it defines the function and health of the spermatozoa (Caballero et al. 2012). It is commonly accepted that SP proteins play an important role in several sperm functions such as epididymal maturation, metabolism, motility, capacitation and acrosome reaction, and are implicated in the modification of the sperm membranes and interaction with the oviductal epithelium (Mogielnicka-Brzozowska and Kordan 2011, Caballero et al. 2012, Mogielnicka-Brzozowska et al. 2015).

It is possible that the identification of proteins occurring in the SP and on the sperm surface could probably detect unknown interactions between the SP components and spermatozoa, which could affect sperm motility. However, our study is based on the analysis of SP proteins, which were not bound to spermatozoa at ejaculation. Some of these proteins showed differences in their expression level in the SP and correlations with the sperm motility parameters. The integrated optical density (IOD) parameter is routinely used for the measurement of protein expression level and is often used in proteomic evaluation of SP and spermatozoa of human and different animal species (Zahn et al. 2006, Rahman et al. 2013). In the literature, there is a lack of information about the correlations between individual SP proteins and sperm motility parameters in the stallion. Also, the effect of seasonal variations in the proteome of stallion SP has also not been fully investigated.

Our study revealed correlations between the expression (IOD) of ALB 1 (61.0-75.2 kDa, pI 6.2-7.0) and ALB 2 (60.9-68.2 kDa, pI 6.1-6.8) and motility parameters of stallion spermatozoa. Albumins are represented by polypeptides with a molecular weight of around 65 kDa. These proteins play several important functions, such as regulation of oncotic pressure and transportation of active molecules (Elzanaty et al. 2007). Furthermore, albumins possess antioxidant properties, scavenge reactive oxygen species, prevent oxidative stress and could regulate the concentrations of Zn^{2+}, Ni^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+} and Cu^{2+} ions (Lu et al. 2008, Roche et al. 2008). The SP albumins are secreted by the testes, epididymides and the prostate, and they are actively involved in the sperm-egg fertilization process (Elzanaty et al. 2007). Moreover, albumins increase the fluidity of the sperm cell membrane during capacitation, and they

remove cholesterol and other sterols from the plasma membrane prior to fertilization (Macıas-Garcıa et al. 2015). Arroteia et al. (2014) found that the sperm membrane contains albumin, which is involved in the penetration of the zona pellucida of egg. The influence of exogenous (but not endogenous) albumin or bovine serum albumin (BSA) on sperm function has been more widely researched, and BSA has been used as a component of sperm capacitation medium. Macias-Garcia et al. (2015) demonstrated that, unlike in other mammals, BSA does not serve as a cholesterol acceptor when used for the capacitation of stallion spermatozoa. In the current study, the correlations observed between the SP albumin and sperm motility parameters are somewhat difficult to interpret. It could be suggested that the analyzed proteoforms of endogenous albumins (ALB 1 and ALB 2) might play a protective role by adhering to sperm cells, resulting in a reduction of albumin level in the SP. It is likely that such protective influence of albumins on the sperm plasma membrane could improve sperm motility. Additionally, the analyzed endogenous albumins can also influence sperm motility, in combination with zinc ions.

The SP kallikreins are the second group of proteins whose expression levels were correlated with sperm motility. The content of KAL 1 (21.4-26.7 kDa, pI 6.1-6.9) and KAL 2 (20.0-25.6 kDa, pI 5.9-6.6) polypeptides was correlated with the motility parameters of stallion spermatozoa. Kallikreins are present in the blood serum and in body tissues, and are involved in the coagulation of human semen (de Lemirande 2007). Little is still known about the physiological role of kallikreins in the semen of various animal species or the role of tissue kallikreins. In the human SP the levels of various forms of kallikrein are correlated with sperm motility (de Lemirande 2007). However, it should be noted that KAL 3 is a prostate-specific antigen (PSA) and is the main enzyme responsible for the proteolysis of semenogelin I and II, semen liquefaction and sperm motility (de Lemirande 2007). The amino acid sequence of horse prostate kallikrein (HPK), isolated from the SP, is highly similar to that of human PSA (Carvalho et al. 2002). The mRNA expression of HPK is androgen-dependent (Henttu and Vihko 1994).

The function of kallikrein in animal semen has been analyzed only in the kallikrein-kinin system in the bull (Somlev et al. 1996, Somlev and Subev 1997). It has been assumed that kallikrein, produced by the prostate, induces the proteolytic degradation of kininogen in the SP, resulting in the formation of kinins including bradykinin. Kininases deactivate kinins through proteolytic degradation (Schill and Miska 1992). Exogenous and endogenous kallikrein seems to exert a different influence on sperm motility. Exogenous kallikrein and bradykinin stimulate the sperm motility apparatus in humans (Bratanov et al. 1978). It has been suggested that bradykinin influences phosphofructokinase and energy metabolism in sperm cells (Bratanov et al. 1978). A previous study showed that the motility of human and bovine spermatozoa increased *in vitro* after the addition of exogenous bradykinin and kallikrein (Bratanov et al. 1978). In another study it was shown that exogenous bradykinin increased the motility of fresh and cryopreserved ram spermatozoa (Muller et al. 1991), and had a stimulating effect on motility of bull spermatozoa (Barakat et al. 2015). However, to our knowledge, there is no information in the literature about the regulatory mechanism of exogenous kallikrein on the motility of stallion spermatozoa. We can conclude that endogenous kallikrein seems to exert varying effects on sperm function compared with its exogenous form. According to Novak et al. (2010), the content of endogenous kallikrein (KLK 2) in the stallion SP was negatively correlated with fertility parameters. In the current study, the high expression levels of endogenous kallikrein proteoforms (KAL 1 and KAL 2) were negatively correlated with sperm motility during the breeding season, which is directly related to stallion fertility. Similar results were reported by Somlev et al. (1996) who observed that high activities of endogenous kallikrein in bull SP were associated with reduced sperm motility. Likewise, Jobim et al. (2011) demonstrated that high concentrations of kallikrein (26.7 kDa, pI 5.5) in stallion SP were associated with low semen freezability. We suggest that KAL 3 (24.5-30.6 kDa, 5.5-6.3 pI) and KAL 4 (25.9-28.5 kDa, pI 5.2-5.8) proteoforms, detected in stallion SP, could be related to kallikrein, reported in the study of Jobim et al. (2011), which confirmed that high concentrations of SP kallikrein could compromise the quality of post-thaw semen.

Interestingly, Elzanaty et al. (2007) demonstrated that albumin concentrations were correlated with the kallikrein concentrations of human semen. It could be suggested that, similarly to albumins, kallikreins bind to the surface of spermatozoa and protect their membranes. It seems likely that low levels of SP kallikreins could be indicative of their binding activity on the surface of spermatozoa, which could have a positive effect on sperm motility. The stimulatory effect of kallikreins on sperm motility could be analyzed in a broader context based on the influence of zinc ions on their activity. Our previous studies on canine prostate-specific esterase (CPSE) (which belongs to the kallikrein family) demonstrated that the binding of kallikrein to zinc ions could regulate the effect of CPSE on the motility of canine spermatozoa (Mogielnicka-Brzozowska et al. 2014, Mogielnicka-Brzozowska et al. 2015). We sug-

gest that further research is needed to establish the presence of such relationships in stallion semen. The physiological role of different forms of kallikreins, and the mechanisms that regulate their activity in the SP of stallions and other animal species have not yet been fully elucidated. Interestingly, albumins also bind to zinc ions in the SP (Mogielnicka-Brzozowska et al. 2015), suggesting that zinc ions could play an important role in the regulatory effects of albumins and kallikreins on sperm motility.

The findings of the current study show that the analyzed proteoforms of albumins and kallikreins exerted significant effects on sperm motility only during the breeding season. This phenomenon seems to be related to the high androgen levels during the breeding season, which influence kallikrein synthesis (Henttu and Vihko 1994). Presently, there is a lack of literature to corroborate the observation of our study. The formation of kinins, the ubiquitous presence of SP protease inhibitors, and the amounts of kallikrein bound to spermatozoa and occurring in the SP should be considered as factors affecting the analysis. Also, the form of kallikrein bound to spermatozoa and its specific effect on sperm motility, and SP zinc ions are also important factors that should be considered.

This study demonstrated that selected proteoforms of albumins and kallikreins affect the motility of stallion spermatozoa, suggesting that the analyzed polypeptides may belong to the group of fertility associated proteins (FAPs). We suggest that these substances could be used as additional markers for the assessment of the reproductive performance of stallions based on motility evaluations. However, further research is needed to elucidate the role of the various forms of SP albumins and kallikreins in the regulation of the motility of stallion spermatozoa.

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References

Abou-Ahmed MM, El-Belely MS, Ismail ST, El-Baghdady YR, Hemeida NA **(1993)** Influence of age and season on certain biochemical constituents of seminal plasma of Arabian horses. Anim Reprod Sci 32: 237-244.

Arroteia KF, Barbieri MF, Souza GH, Tanaka H, Eberlin MN, Hyslop S, Alvares LE, Pereira LA **(2014)** Albumin is synthesized in epididymis and aggregates in a high molecular mass glycoprotein complex involved in sperm-egg fertilization. Plos One 9: e103566.

Ashrafzadeh A, Karsani SA, Nathan S **(2013)** Mammalian sperm fertility related proteins. Int J Med Sci 10: 1649-1657.

Barakat IA, Danfour MA, Galewan FA, Dakhil MA **(2015)** Effect of various concentrations of caffeine, pentoxifylline and kallikrein on hyperactivation of frozen bovine semen. BioMed Res Int 2015: 94-101.

Bratanov K, Somlev B, Doycheva M, Tornyov A, Efremova V **(1978)** Effect of kallikrein on bull sperm motility in vitro. Int J Fertil 23: 73-75.

Caballero I, Parilla I, Almiñana C, del Olmo D, Roca J, Martínez EA, Vázquez JM **(2012)** Seminal plasma proteins as modulators of the sperm function and their application in sperm biotechnologies. Reprod Domest Anim 47: 12-21.

Carvalho AL, Sanz L, Barettino D, Romero A, Calvete JJ, Romão MJ **(2002)** Crystal structure of a prostate kallikrein isolated from stallion seminal plasma: a homologue of human PSA. J Mol Biol 322: 325-337.

De Lemirande E **(2007)** Semenogelin, the main protein of the human semen coagulum, regulates sperm function. Semin Thromb Hemost 33: 60-68.

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

Elzanaty S, Erenpreiss J, Becker C **(2007)** Seminal plasma albumin: origin and relation to the male reproductive parameters. Andrologia 39: 60-65.

Henttu P, Vihko P **(1994)** Prostate-specific antigen and human glandular kallikrein: two kallikreins of the human prostate. Ann Med 26: 157-164.

Jobim MI, Trein C, Zirkler H, Gregory RM, Sieme H, Mattos RC **(2011)** Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their relation with semen freezability. Theriogenology 76: 765-771.

Kareskoski AM, del Alamo MM, Guvenc K, Reilas T, Calvete JJ, Rodriguez-Martinez H, Andersson M, Katila T. **(2011)** Protein composition of seminal plasma fractionated stallion ejaculates. Reprod Domest Anim 46: e79-e84.

Kareskoski AM, Katila T **(2008)** Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity. Anim Reprod Sci 107: 249-256.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ **(1951)** Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

Lu J, Stewart AJ, Sadler PJ, Pinheiro TJ, Blindauer CA **(2008)** Albumin as a zinc carrier: properties of its high-affinity zinc-binding site. Biochem Soc Trans 36: 1317-1321.

Macıas-Garcıa B, Gonzalez-Fernandez L, Loux SC, Rocha AM, Guimaraes T, Pena FJ, Varner DD, Hinrichs K **(2015)** Effect of calcium, bicarbonate, and albumin on capacitation-related events in equine sperm. Reproduction 149: 87-99.

Magistrini M, Vidament M, Clement F, Palmer E **(1996)** Fertility prediction in stallions. Anim Reprod Sci 42: 181-188.

Mogielnicka-Brzozowska M, Dziekonska A, Strzezek R, Załecki M, Majewska A, Tołscik K, Kordan W **(2014)** Effect of seminal plasma zinc-binding proteins on motility and membrane integrity of canine spermatozoa stored at 5°C. Bull Vet Inst Pulawy 58: 163-168.

Mogielnicka-Brzozowska M, Kordan W **(2011)** Characteristics of selected seminal plasma proteins and their application in the improvement of the reproductive processes in mammals. Pol J Vet Sci 14: 489-499.

Mogielnicka-Brzozowska M, Kowalska N, Fraser L, Kordan W **(2015)** Proteomic Characterization of Zinc-Binding Proteins of Canine Seminal Plasma. Reprod Domest Anim 50: 1017-1021.

Mogielnicka-Brzozowska M, Słowińska M, Fraser L, Wysocki P, Strzeżek R, Wasilewska K, Kordan W **(2017)** Identification and characterization of non-phosphorylcholine-binding and phosphorylcholine-binding proteins of canine seminal plasma. Ann Anim Sci 17: 771-786.

Muller K, Heder G, Nehring H, Siems WE, Wolf R **(1991)** Investigations on the influence of bradykinin upon the motility of ram spermatozoa. Andrologia 23: 357-362.

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.

O' Farrell PH **(1975)** High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007-4021.

Rahman MS, Lee JS, Kwon WS, Pang MG **(2013)** Sperm proteomics: road to male fertility and contraception. Int J Endocrinol 2013: 36-47.

Roche M, Rondeau P, Singh NR, Tarnus E, Bourdon E **(2008)** The antioxidant properties of serum albumin. FEBS Lett 582: 1783-1787.

Schill WB, Miska W **(1992)** Possible effects of the kalikrein-kinin system on male reproductive functions. Andrologia 24: 69-75.

Somlev B, Helili K, Karcheva V **(1996)** Tissue kallikrein activity in seminal plasma of bovine ejaculates with different quality. Theriogenology 45: 471-475.

Somlev B, Subev M **(1997)** Dynamics of exogenous kallikrein-stimulated bovine sperm motility in the presence of seminal plasma kallikrein. Theriogenology 47: 1087-1092.

Tejerina F, Morrell J, Petterson J, Dalin AM, Rodriguez-Martinez H (**2009**) Routine assessment of motility of ejaculated stallion spermatozoa using a novel computer-assisted motility analyzer (Qualisperm™). Anim Reprod 6: 380-385.

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.

Zahn FS, Papa FO, Melo CM **(2006)** Blood serum, seminal plasma and sperm membrane protein profiles in stallions: are they correlated to semen freezability? Anim Reprod Sci 94: 64-66.

Zakharchenko O, Greenwood C, Lewandowska A, Hellman U, Alldridge L, Souchelnytskyi S **(2011)** Meta-data analysis as a strategy to evaluate individual and common features of proteomic changes in breast cancer. Cancer Genomics and Proteomics 8: 1-14.