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Expression, localization, and concentration of A-kinase anchor protein 4 (AKAP4) and its precursor (proAKAP4) in equine semen: Promising marker correlated to the total and progressive motility in thawed spermatozoa

D. Blommaert [a], N. Sergeant [b, c], M. Delehedde [b], N. Jouy [d], V. Mitchell [b, e], T. Franck [f, *], I. Donnay [g], J.P. Lejeune [a], D. Serteyn [a, h]

[a] LINALUX-MLS, Centre Européen du Cheval, Mont-le-Soie, 1, Vielsalm, 6698, Belgium
[b] SPQI - 4BioDx, 1, Place de Verdun, 59045, Lille, France
[c] Univ. Lille, Inserm UMRS 1172, Place de Verdun, 59045, Lille, France
[d] Univ. Lille, Plateau de Cytométrie, Place de Verdun, 59045, Lille, France
[e] Univ. Lille, EA4308, 59045, Lille, France
[f] Center for Oxygen Research and Development (CORD), University of Liège, Liège, Belgium
[g] Louvain Institute of Biomolecular Science and Technology, Catholic University of Louvain, Belgium
[h] Equine Clinic, Veterinary Medicine Faculty, University of Liège, Liège, Belgium

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ABSTRACT

A-kinase anchor protein 4 (AKAP4) is playing a central role in flagellar structure, chemotaxis, capacitation and sperm motility. In mammals, AKAP4 is expressed during spermatogenesis. AKAP4 is synthesized as a precursor, proAKAP4, which is cleaved into mature AKAP4 during fibrous sheath assembly. The proAKAP4 is a good indicator of sperm quality in humans and boars. The aims of this work were to study the expression, the localization and the concentration of proAKAP4 and AKAP4 in equine semen, and to evaluate the possible correlation between the total and progressive motility and the concentration of proAKAP4 measured by ELISA in post-thawed semen. Frozen sperm from 13 different stallions were used. Semen samples ($n = 17$) were prepared using the INRA Freeze medium to reach a concentration of 150 million spermatozoa/mL, packaged and frozen in 0.5 mL straws. The precursor proAKAP4 and the mature protein AKAP4 both localize to the fibrous sheath of the principle piece of equine sperm flagellum. The concentrations of proAKAP4 were determined in the post-thawed semen using ELISA method (Horse 4MID® kits, 4BioDx, France). The mean concentration of proAKAP4 was then of 7.372 ± 0.79 ng/μL and was significantly correlated with the post-thawed total motility (Pearson coefficient $r = 0.66$, $p = 0.002$) and progressive motility (Pearson coefficient $r = 0.76$, $p = 0.0002$) and the amount of proAKAP4 represent the amount of spermatozoa that expressed proAKAP4. Taken together, these preliminary results confirm the interest to use proAKAP4 concentrations as a promising marker of stallion sperm quality as close correlation was observed between the proAKAP4 concentration and sperm motility parameters.

1. Introduction

In recent years, equine breeding has undergone significant improvements largely due to improvement of stallion semen cryopreservation. Freezing of equine semen allows the long-term semen preservation and a worldwide distribution of the best international genetics while avoiding the risk of disease transmission associated with stallion transport [1]. However, in comparison to other species, the fertilizing ability of frozen equine semen remains low. In addition and despite a good quality of fresh stallion semen, about 20% of ejaculates do not support the freezing and thawing phases [2]. Stallions with a post-thaw progressive sperm motility less than or equal to 35% in 3 or more ejaculates out of 10 were

* Corresponding author.
E-mail address: t.franck@uliege.be (T. Franck).

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considered to be with 'poor' freezing capabalities [3]. These candidates are called "poor freezers".

Mitochondria functionality is essential for sperm motility, and damage related to cryopreservation plays a central role in mitochondrial dysfunction [4,5]. A deficient function of sperm mitochondria impact cyclic AMP (cAMP) and ATP production and could be responsible for low progressive motility [6]. Indeed, cAMP and ATP are essential for the phosphorylation of tyrosine kinase involved in the signaling process of sperm motility [7].

Frozen ejaculates suitable for insemination are mainly selected based on the post-thaw motility assessed by computer-assisted semen analysis. To predict more accurately the fertility of equine semen, additional tests have been developed and consist of the evaluation of DNA integrity, mitochondrial activity, acrosome integrity hypoosmotic resistance, etc. [4,8]. Moreover, research efforts have focused on identifying reliable markers of fertility at either genomics or proteomic levels, in the sperm or the seminal plasma [9–12]. The cellular functions including active movement inside the female genital tract are essential for successful fertilization. Thus, sperm motility is considered as an essential sperm quality marker of semen fertility. Motility is controlled by the sperm tail ensuring the delivery through the female genital tract and penetration of the oocyte, an obligatory step of fertilization [13]. Motility and flagellar motion is under the control of a complex molecular and structural machinery, among which A-kinase anchor protein 4 (AKAP4) is suggested to play critical functions [14].

The *AKAP4* gene belongs to the X-linked member of *AKAP* gene family [15]. AKAP4 protein expression is restricted to germ cells from the round spermatid stage until the mature spermatozoa and mRNAs are found only in post-meiotic phases of spermatogenesis [13]. AKAP4 protein is the most abundant constitutive protein of the sperm fibrous sheath in all mammals, being part of the principle piece of the flagellum [15–18]. This scaffold protein is therefore instrumental in regulating sperm flagellum motion [13]. AKAP4 is phosphorylated by tyrosine kinase which triggers a cascade of protein phosphorylation involved in sperm motility and hypermotility [17,19]. Interestingly, AKAP4 deletion results in a defective fibrous sheath formation and flagellar dysplasia resulting to infertility due to the loss of motility [13]. Initially AKAP4 is synthesized as a precursor protein named proAKAP4. Its activation involves the release of a sequence peptide called prodomain to provide the mature AKAP4 of 666 amino acid, which occurs during sperm differentiation in the testes [15,16,20–22]. The AKAP4 amino acid sequence is highly conserved [23] and in the *equus genus*, AKAP4 protein shares 75% similarity with the other mammals' protein including the human one. The equine *AKAP4* gene has been partially cloned and characterized [24]. It encodes for an 849 amino acid AKAP4 precursor protein with a prodomain of 182 amino acids. To our knowledge, no study was performed on the relationship between the content of proAKAP4 or AKAP4 in the equine semen and the quality of the semen after thawing.

The aims of this work were then first to study the expression, the localization and the concentration of proAKAP4 and AKAP4 in equine sperm and then to measure the correlation between the total and progressive motility and the concentration of proAKAP4 measured by ELISA in post-thawed semen. We then first examine proAKAP4 expression and localization in stallion spermatozoa by Western blotting, fluorescence and electron microscopy as well as by flow cytometry. Second, we assessed the relationship between proAKAP4 concentration in stallion semen and sperm quality parameters (viability, motility) in a series of 17 semen samples in order to evaluate its potential as pertinent biomarker in routine use.

2. Material and methods

2.1. Analysis of viability and motility of thawed sperm

Frozen sperm in straws from 13 different stallions were used. Some stallions were sampled twice at different date. Semen samples (n = 17) were prepared using the INRA Freeze medium (IMV Technologies, L'Aigle, France) to reach a final concentration of 150 million spermatozoa/mL, packaged and frozen in 0.5 mL straws. The straws were taken in liquid nitrogen storage tanks from the equine reproduction center Linalux/Mont le Soie (Vielsalm, Belgium). Each straw was thawed by immersion in a water bath at 37 °C. The sperm was kept in an incubator at 37 °C and protected from light. The initial concentration of spermatozoa was determined using the NucleoCounter SP-100 solution with the detergent Chemometec (CHEMOMETEC, Allerod, Denmark). The concentration of dead sperm was also determined with the same device for the viability of fresh semen following the manufacturer instructions. Thereafter, a sample of thawed semen was diluted to a concentration of 20×10^6 total spermatozoa/mL in the "INRA Freeze" medium. The diluted sample was incubated for 5 min at 37 °C protected from light. Total and progressive motilities were determined using 20 μL Leja cells in a computer assisted semen analyser (CASA) (IVOS version 12.0, Hamilton Thorne Research, Beverly, MA, USA). CASA settings were as follows: 37 °C, 60 frames per second (60 Hz), sperm size set between 4 and 6 pixels. The total mobility was defined when the Velocity Average Path (VAP) was ≥ than 15 μm/s and progressive motility was defined when the VAP was ≥30 μm/s and straightness (STR) greater than 50%. The analysis was repeated twice in five different fields (10 repetitions for each analysis and a minimum of 600 analysed spermatozoa).

2.2. Semen sample preparation

For total semen lysate, one volume of post-thawed semen was added with one volume of PBS with 2% SDS. The homogenate was rapidly vortexed and further sonicated on ice at 22 kHz, 15 Watt during 30s. The resulting lysate was centrifuged at 14,000×*g* 20 min at 4 °C. For the separation of spermatozoa and seminal plasma, a volume of 500 μL of semen was pipetted in 1.5 mL Eppendorf tube and centrifuged at 2000 rpm at 20 °C during 10 min. The seminal plasma supernatant over the spermatozoa pellet was recovered with a 200 μL pipette. The seminal plasma was added with one volume of PBS with 2% SDS and sonicated on ice at 22 kHz, 15 Watt during 30s. To the spermatozoa pellet 250 μL of PBS with 2% SDS were added. The spermatozoa pellet was resuspended by vortexing the tube and the spermatozoa homogenate was further sonicated on ice at 22 kHz, 15 Watt during 30s. The seminal plasma and spermatozoa protein concentrations were assayed according to the Bradford's method (BioRad, France). Total semen protein lysates were then stored at −80 °C until analysis. Equal protein concentrations were loaded to perform the Western-blot.

2.3. Western-blot

Spermatozoa concentrations were used to load the same number of spermatozoa per well. To that purpose, to the 2% SDS post-thawed semen lysate, a volume of LDS-PAGE lysis buffer was added to reach an equal concentration of spermatozoa in the final lysate (NuPage LDS lysis buffer, Life technologies, USA) and protein concentrations were determined by BCA method (Biorad, USA). Thirty μg of total semen proteins or seminal plasma were then separated by SDS-PAGE using 4–12% NuPage Precast Gels and further transferred onto 0.45 μm nitrocellulose membranes (G&E Healthcare, USA) using the Liquid Transfer System (Life

Technologies, USA). Membranes were reversibly stained with Ponceau Red to verify the quality of the loading and homogeneity of transferred proteins and thereafter blocked in 15 mM Tris-HCl pH 8.0, 140 mM of NaCl and 0.05%; (w/v) of Tween-20 (TNT) added with 5% skimmed milk. Membranes were then incubated with the monoclonal antibody anti-AKAP4 clone 7E10 (4BioDx, 4BDX-1602, France), dilution of 1:10000) or the monoclonal antibody anti-proAKAP4 clone 6F12 (4BioDx, 4BDX-1701, France), dilution of 1:10000) in TNT overnight at 4 °C. After three washes in TNT during 10 min each, membranes were incubated with the detection antibody coupled to horseradish peroxidase (stallion anti-mouse: 1:50000 diluted in TNT (Vector Laboratories, Burlingame, CA USA). Immunocomplexes were incubated with the ECL™ chemioluminescence kit (G&E Healthcare, USA) and exposed to a digital camera LAS-3000 system (G&E Healthcare, USA) Semi-quantification was achieved using ImageJ software (NIH). The integrated optical density (IOD) of positive immune-staining was in arbitrary unit.

2.4. Immunofluorescence and confocal microscopy

Semen spermatozoa were isolated by centrifugation at 350×*g* and then resuspended in Tris buffered saline (TBS) with 2% paraformaldehyde for 15 min at room temperature (RT). After centrifugation at 350×*g* during 10 min, the sperm pellet was resuspended in TBS and washed twice by centrifugation and finally resuspended in 100 μL of TBS. Using a Pasteur pipette a drop was placed on a Superfrost® slide (Menzel-Glazer, Germany) and layed using the coverslip. Slides were then dried for 2 h and immerged in ice cold acetone for 5 min and then washed twice in TBS (5 min). Slides were incubated with a blocking solution containing 0.2% BSA in TBS for 1 h at room temperature. The blocking solution was discarded and replaced by 100 μL of the monoclonal antibody anti-proAKAP4 clone 6F12 or the antibody anti-AKAP4 clone 7E10 (4BioDx, 4BDX-1701 and 4BDX-1602 France, diluted 1:500 in TBS). The slides were incubated at 4 °C in a humid chamber overnight. After washing twice the slides 5 min in TBS, they were incubated 1 h at RT with an Alexa 568 secondary antibody (Invitrogen, USA) diluted in TBS (1:500). The slides were washed twice in TBS and mounted with Vectashield Mounting Medium containing 4′,6′-diamidino-2-phenylindole (H-1200, Vector Laboratories, USA). Observation and image acquisition were performed with an LSM 710 confocal microscope (Zeiss, Jena, Germany). Acquisition parameters were made in sequential mode and image were analysed with Zeiss Efficient Navigation confocal software (Zeiss). To test for non-specific binding, monoclonal antibodies against AKAP4 or proAKAP4 (4BioDx, France) were omitted from control incubation.

2.5. Electron microscopy

Isolated spermatozoa were fixed in a solution of 0.1 M phosphate buffer (pH 7.4) containing 2% (v/v) paraformaldehyde and 0.05% glutaraldehyde (v/v) for 1 h at 4 °C, dehydrated in an increasing concentration series of ethanol and further embedded in LR White Resin (EMS, USA). Grids were incubated in a blocking TBS buffer containing 1% (v/v) of normal goat antiserum and 1% (w/v) of bovine serum albumin for 30 min at room temperature. They were subsequently incubated with the monoclonal anti-proAKAP4 antibody clone 6F12 or clone 7E10 (4BioDx, 4BDX-1701 and 4BDX-1602 France) diluted at 1:50 in the blocking buffer. Grids were washed three times in TBS and incubated with 12 nm colloidal gold conjugated to a goat anti-mouse IgG (H + L) antibody (Jackson Immunoresearch Laboratories, USA) diluted 1:50 in the blocking buffer. Control grids were incubated in colloidal conjugated antibody alone. Following three washes in TBS, all grids were fixed and counterstained with 2% uranyl acetate. Grids were observed and photographed using a Zeiss transmission 900 electron microscope.

2.6. Flow cytometry

After thawing the semen in a water bath at 37 °C, the semen was diluted with three volumes of RPMI medium (Thermofisher) and was centrifuged at 700×*g* during 10 min at RT. The supernatant was discarded, and the sperm pellet was resuspended in 100 μL of RPMI and gently re-homogenized. Cell death was assessed using the Zombie NIR™ Die (BioLegend). 0.5 μL of Zombie NIR™ Die was added to 0.5 10^6 spermatozoa. For proAKAP4 and AKAP4 labelling, a total volume of 2 mL of paraformaldehyde at 3% (v/v) in PBS was progressively added drop by drop and gently mixed. Fixation was achieved after incubation at RT during 20 min in the dark. After centrifugation at 500×*g* during 10 min, spermatozoa were rinsed twice in 100 μL of PBS and after the last centrifugation spermatozoa pellet was gently homogenized with 200 μL of PBS. Numeration was determined using a Malassez slide and a max of 0.4 million spermatozoa/test were used. Primary antibodies anti-proAKAP4 (clone 6F12) (4BioDx, 4BDX-1701, France) and anti-AKAP4 (4BioDx, 4BDX-1602, France) were used and diluted in PBS with 0.3% Triton X-100 at a final dilution of 1 μg/mL. Spermatozoa were incubated for 45 min at room temperature and further centrifuged at 500×*g* in 1 mL of PBS during 5 min. Spermatozoa were resuspended in 1 mL of PBS Triton X-100 at 0.3% supplemented with the secondary antibody (Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Thermofisher) at a final dilution of 0.25 μg/mL. After 30 min of incubation, spermatozoa were rinsed twice by centrifugation in PBS. The final pellet of spermatozoa was resuspended in 400 μL of PBS. Flow cytometry acquisition was performed in a FS/SS mode with UV filter at 488 nm (excitation) and emission at 530 nm with the Cyan ADP (Beckman – Coulter Flow Cytometer and Summit Software). The gating strategy was designed to avoid any aggregates and to analyze specifically the spermatozoa cells separated from cell debris. After gating the single cells from aggregates, a second gating selection was performed on the FCS and FITC staining with the area on width parameter (Supplementary Fig. 1A) to gate only the single cells stain with proAKAP4, and therefore only analysed the singulet events (Supplementary Fig. 1A). After proAKAP4 labelling, four quadrant were then defined, the A–, the A-+, the A + - and the A++ (Supplementary Fig. 1A). No overlap between the secondary antibody and proAKAP4 labelling was observed (Supplementary Fig. 1B). The Zombie NIR™ probe allowed also to identify cell debris (including tails, heads from broken cells), viable and dead cells (Supplementary Fig. 2).

2.7. ProAKAP4 ELISA assays

Semen was first thawed on ice. Then 50 μL semen suspension were added to 450 μL of lysis buffer and proceeded for ELISA quantification using the proAKAP4 ELISA kit (4BioDx, 4VDX-18K3, France). The samples and standards were processed using commercial buffers and according to the manufacturer's instructions (4BioDx, 4VDX-18K3, France). Briefly, 50 μL of semen are added to each well of the antibody-coated plate. The proAKAP4 antibody is covalently coupled to horseradish peroxidase. After washing, the substrate solution is then added to each well and the resulting color intensity is proportional to the amount of proAKAP4 present in semen samples. The color reaction is stopped using a stop solution and the color intensity is measured by spectrophotometry at 450 nm. A standard curve is determined in parallel for concentrations of proAKAP4 comprised between 3 ng/μL to 0.04 ng/μL. Results of proAKAP4 concentrations in the stallion semen were expressed in ng/μL.

2.8. Statistical analysis

All the experiments reported here were replicated twice using independent samples. Statistical analysis was performed using Prism 7 GraphPad. First, we performed a D'Agostino and Pearson normality test in order to determine if the population follow a Gaussian distribution then Pearson correlation coefficient was determined between sperm parameters and proAKAP4 concentrations. Pearson correlation between the proAKAP4 concentrations and the values of total and progressive motility respectively were calculated using the Graphpad Prism 7 Statistical Software. The threshold for statistical significance was set to $p < 0.05$.

3. Results

3.1. Analysis of AKAP4 and proAKAP4 expression by western blotting

Expression of proAKAP4 in stallion spermatozoa was first analysed by immunoblotting using specific AKAP4 antibodies against the prodomain of the AKAP4 precursor (anti-proAKAP4 clone 6F12) and an anti-AKAP4 antibody (clone 7E10) against an epitope at the C-terminus of the protein. Stallion semen, isolated spermatozoa and seminal liquid were loaded onto SDS-PAGE. ProAKAP4 staining with anti-proAKAP4 clone 6F12 showed a single band at 100 kDa in the neat semen and in isolated spermatozoa. In addition to this 100 kDa band an additional band at 20 kDa was detected in isolated spermatozoa, corresponding to the cleavage product, also called the prodomain (Fig. 1A).

Immunostaining with the AKAP4 antibody clone 7E10 consisted of two bands at 100 kDa and a more intense band at 82 kDa corresponding respectively to the AKAP4 precursor and the mature protein lacking the 182 aa of the prodomain (Fig. 1A). Those two bands were observed in the semen sample and in isolated spermatozoa but never in the seminal plasma. Neither the precursor proAKAP4 nor the mature AKAP4 are detected in the seminal plasma showing that both proteins are specific to spermatozoa (Fig. 1A and B).

3.2. Localization of proAKAP4 and AKAP4 in stallion spermatozoa by microscopy

Both antibodies anti-proAKAP4 clone 6F12 and anti-AKAP4 clone 7E10 were then used to localize the precursor and the mature AKAP4 protein by fluorescence microscopy and electron microscopy on fixed spermatozoa. As expected, AKAP4 is one of the main constituent of the spermatozoa flagellum. Only the principle piece of the flagellum was stained by both antibodies (Fig. 1B). The head and intermediate piece were never labelled, as previously described by others. Some spermatozoa flagella were lightly or not stained with either the proAKAP4 or AKAP4 monoclonal antibodies. Thus there are spermatozoa that are not expressing either proAKAP4 or AKAP4 as a mature form (see Fig. 5). By electron microscopy (Fig. 2A and B) both the mature protein AKAP4 and the precursor were localized to the fibrous sheath of the principle piece of equine sperm flagellum. The proAKAP4 (Fig. 2A) and AKAP4 (Fig. 2B) were localized in the transverse rib and longitudinal columns of the principal piece of the flagellum. They were neither observed in the outer dense fibers or in the axoneme.

3.3. Variations of expression of proAKAP4 and AKAP4 by western blotting in stallion ejaculates

We then investigated by western blotting the expression of proAKAP4 and AKAP4 in 8 stallion ejaculates. The same amounts of spermatozoa proteins were loaded from sample lyzed in a same volume of lysis buffer as verified by post-transfer reversible Ponceau Red staining (data not shown). As both used monoclonal antibodies are able to detect the proAKAP4, we first described that level of expression of the precursor of AKAP4 varies from one sperm sample to another as shown in Fig. 3A and B. The prodomain was also observed in different amounts in samples and more intensively in samples 12–13 and 16–17 while in decreasing amounts in samples 11, 14–15 and 10 (Fig. 3). In contrast as shown with the monoclonal 7E10 antibody (Fig. 3), the AKAP4 expression was much less heterogeneous and less variable from a sample to another except for sample 10 for which the AKAP4 band was less intensively stained when compared to the other samples.

The analysis of AKAP4 and proAKAP4 expression by western blotting indicated that the AKAP4 staining may also be reduced in semen samples exhibiting concomitantly a lower band intensity of the precursor proAKAP4 (e.g. Fig. 3B. Samples 10, 14–15). In contrast, proAKAP4 expression was significantly higher in semen samples number 12,13 as well as in number 16 and 17 (Fig. 3A and B).

3.4. ProAKAP4 concentration variations in stallion semen

The proAKAP4 concentration was further assessed using a quantitative sandwich ELISA assay that quantifies specifically the proAKAP4 in stallion semen. The concentrations of proAKAP4 were determined based on a standard curve. As reported in Table 1, the proAKAP4 concentrations were variable and ranging from a minimum 3.45 ± 0.1 ng/μL (concentration obtained in the stallion # 10) to a maximum 13.22 ± 0.68 ng/μL. The mean concentration ±SEM was at 7.372 ± 0.79 ng/μL of stallion semen, the median, 25% and 75% percentile were at 5.78, 4.87 and 10.84 respectively.

Motility parameters were then compared to proAKAP4 concentrations determined by the proAKAP4 ELISA assay. The total and progressive (PR) motility and the proAKAP4 concentration (in ng/μL of semen) were reported in Table 1. The total and progressive motility was ranging between 0/0 for stallion 10 to 58/42 for stallion 13 respectively. The mean ± SEM was at 32.88 ± 4.37 for the total motility and at 22.12 ± 3.27 for the progressive motility.

The values of total motility, PR motility and concentration of proAKAP4 passed the D'Agostino & Pearson normality test and enable the use of the Pearson correlation test. We then further assessed the potential correlation between motility, progressive motility and the concentration of proAKAP4 in sperm samples using the Pearson correlation coefficient calculation. The proAKAP4 concentration was significantly correlated with total motility (Pearson coefficient $n = 17$, $r = 0.7693$, $p = 0.0003$) and progressive motility (Pearson coefficient $n = 17$, $r = 0.6648$, $p = 0.0036$) as illustrated in the (Fig. 3A and B).

3.5. ProAKAP4 expression as assessed by flow cytometry in equine sperm

We then analyze the expression of proAKAP4 by flow cytometry in 3 stallion semen. The proAKAP4 concentrations were first determined by ELISA and showed incremental concentrations ranging from 3.2 ± 0.2 (Fig. 5A), 5.0 ± 0.1 (Fig. 5B) and 9.4 ± 0.9 ng/μL of semen (Fig. 5C). No overlap between the secondary antibody and proAKAP4 labelling were observed (Supplementary Fig. 1B). Isolated spermatozoa were stained with proAKAP4 6F12 monoclonal antibody and analysed by flow cytometry. The sperm-specific events were positively gated on the basis of their forward scatter and side scatter distribution (Supplementary Fig. 1A). Viable and dead spermatozoa were stained both by the Dead cells Zombie NIR™ probe and proAKAP4 monoclonal antibody. The cells debris

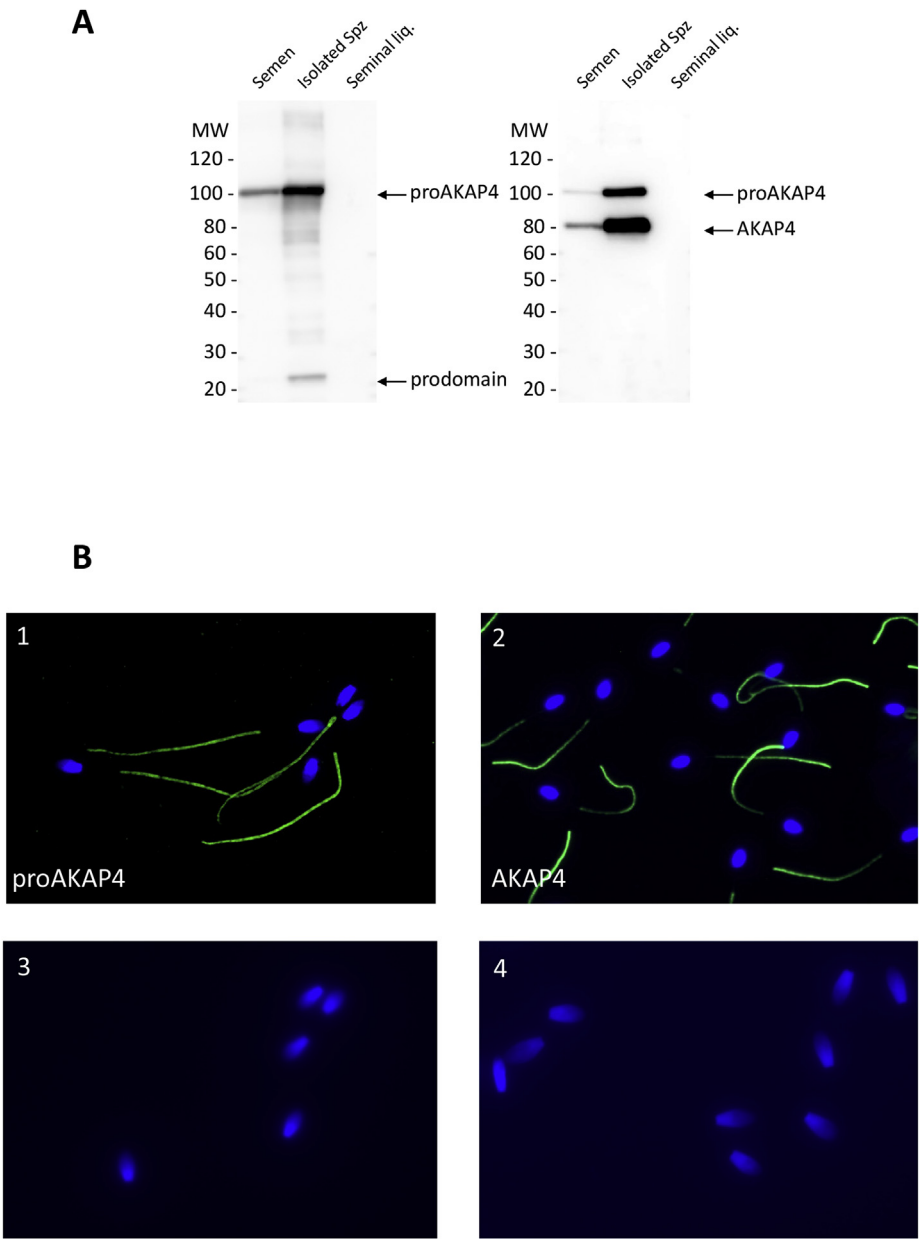

Fig. 1. ProAKAP4 and AKAP4 are specifically expressed in spermatozoa. (A) Western blot analysis of proAKAP4 and AKAP4 expression in total protein from semen and isolated spermatozoa (spz) lysates and from seminal liquid after thawing. Protein were separated using (4–12%) SDS-PAGE gels. (B) Fluorescence microscopy for expression of proAKAP4 and AKAP4 in equine spermatozoa. ProAKAP4 and AKAP4 (green fluorescence) are localized in the principle piece of the flagellum. The intermediate piece was never labelled (B1, B2). In the absence of antibodies anti-proAKAP4 (B3) anti-AKAP4 (B4) no green fluorescence was observed from the flagellum. Images were acquired with an objective lens of 60x.

(as detected by Zombie NIR™ violet probe) were not labelled with the proAKAP4 antibody and were then excluded from the analysis (Supplementary Fig. 2). Quadrants and gates were subsequently defined for each test according to the side scattering, the proAKAP4 immunostaining of stallion spermatozoa and cell debris were excluded. Density plots are represented with the side scattering and the fluorescence staining (Fig. 5). The region A++ correspond to the FITC signal of proAKAP4 with 2 different granularities (SSC) observed in all sperm samples. Interestingly, proAKAP4 immunostaining was not observed in all spermatozoa and we detected stallion spermatozoa without proAKAP4 expression (Fig. 5A–C windows A-+). The FITC signal intensity was principally comprised between 10 and 100 (Fig. 5A–C), suggesting that proAKAP4 labelling was homogeneous. An RFU overlay was performed to compare the overall expression in horse frozen semen samples (Supplementary Fig. 2B). However, the RFU overlay did not provided the proportion of unlabeled spermatozoa and therefore, the proportion of proAKAP4 spermatozoa is only observed with RFU (x-axis) and side scatter (y-axis). The staining and therefore the expression of proAKAP4 by spermatozoa was very homogenous and consequently represented a percentage of all spermatozoa expressing proAKAP4. Interestingly, this percentage was directly associated to the amount of proAKAP4 as determined by ELISA and Western-blotting. In the semen with 3.2 ± 0.2 ng/μL of proAKAP4 the percentage of spermatozoa detected was of 83.14% whereas in the sample with 5.0 ± 0.1 or 9.4 ± 0.9 ng/μL the percentage of stained spermatozoa reached 92.05 and 97.09% respectively. Then, our data suggest that the proAKAP4 concentration in semen reflects a proportion of spermatozoa expressing the proAKAP4 precursor as an homogenous population in stallion semen.

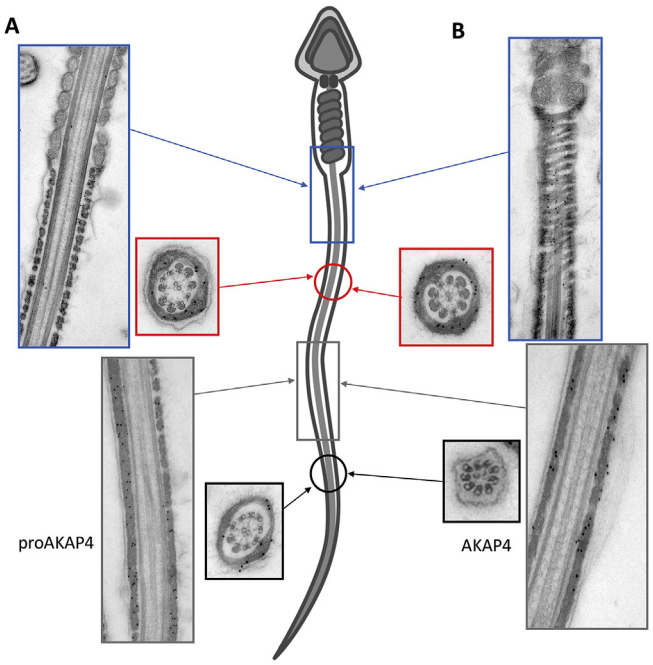

Fig. 2. Schematic representation of an equine spermatozoa showing a longitudinal section head, midpiece, principal piece and terminal piece. A. Electron microscopy images of proAKAP4 staining, in a longitudinal section of the proximal part and medial part of the principal piece of the flagellum (in blue and in grey) and coronal sections of the flagellum which position is indicated on the spermatozoa picture. B. AKAP4 labelling in longitudinal section and coronal section of an equine spermatozoa. The color of the sections and their respective positions are indicated on the spermatozoa picture. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In the present study, the precursor protein of AKAP4 called proAKAP4 was analysed in frozen stallion semen and compared to the sperm parameters as determined after thawing. We showed first that proAKAP4 concentrations in semen were variable

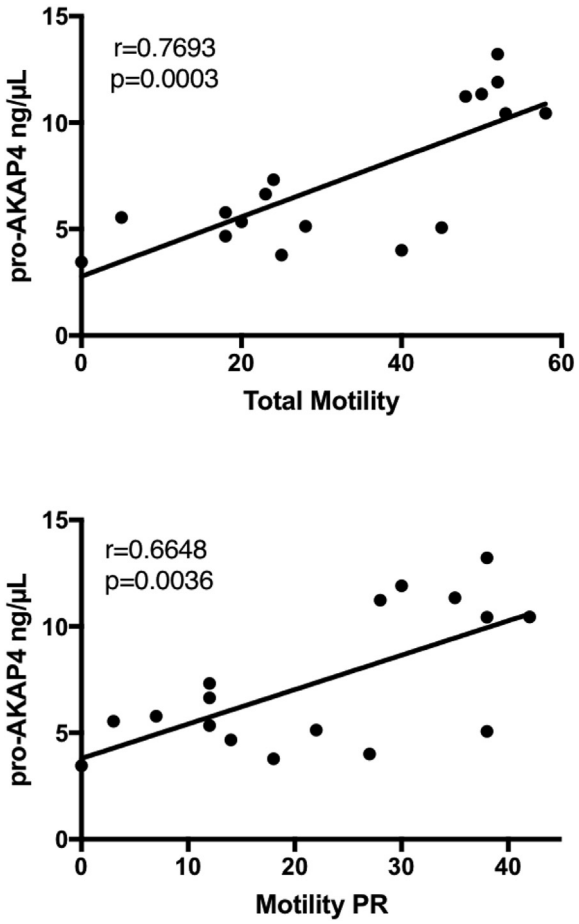

Fig. 4. Correlation between proAKAP4 concentration (ng/µL) measured by ELISA in sperm samples and total motility (A) or progressive (PR) motility (B) using the Pearson correlation coefficient calculation (n = 17).

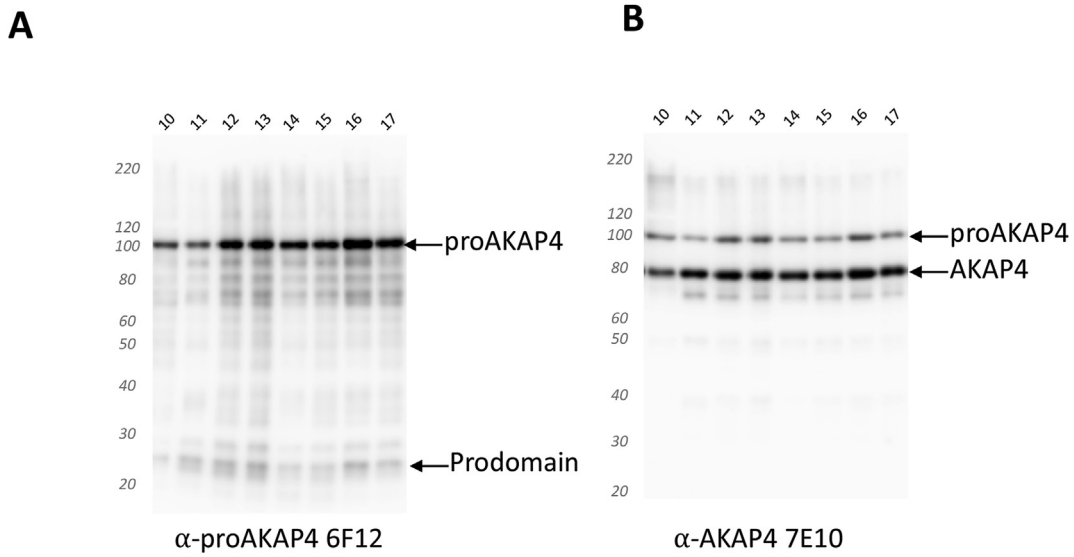

Fig. 3. Western blot analysis of the expression of both ProAKAP4 and AKAP4 in total protein semen lysates obtained from 8 stallion ejaculates. Protein were separated using SDS-PAGE gels. A. ProAKAP4 precursor was detected with the anti-proAKAP4 mouse monoclonal antibody clone 6F12. B. ProAKAP4 and AKAP4 were labelled with the anti-AKAP4 mouse monoclonal antibody clone 7E10.

Table 1
Mean concentration values of proAKAP4 related to the post-thaw total and progressive motilities of spermatozoa of 13 stallions. Some stallions were sampled twice at different date.

Stallion	Total motility (%)	Progressive motility (%)	pro-AKAP4 mean ± SD (ng/μL)
#1	52	30	11.91 ± 0.04
#2	48	28	11.23 ± 0.7
#3	5	3	5.54 ± 0.7
#4	20	12	5.35 ± 0.35
#5	23	12	6.6 ± 0.21
#6	28	22	5.13 ± 0.21
#7	18	14	4.67 ± 0.21
#8	50	35	11.35 ± 0.23
#9	52	38	13.22 ± 0.68
#10	0	0	3.45 ± 0.1
#11	25	18	3.78 ± 0.1
#12	53	38	10.43 ± 0.05
#13	58	42	10.45 ± 0.55
#14	18	7	5.79 ± 0.22
#15	24	12	7.31 ± 0.13
#16	45	38	5.07 ± 0.07
#17	40	27	4.00 ± 0.05

between stallions. These variations in proAKAP4 concentration were positively correlated to both total motility and progressive motility and reflected a proportion of spermatozoa expressing the precursor protein proAKAP4.

Progressive motility is today considered to be a predictive marker of successful fertilization and fertility outcome (especially for intra-uterine insemination) [25–27]. Previous markers were studied in stallion seminal plasma. Novak et al. [9] observed a positive relationship between protein involved in carbohydrate metabolism (citrate synthase, fumarate hydratase, malate dehydrogenase) while a negative relationship with four seminal plasma proteins such as kallikrein, clusterin SP1, SP2. Several studies have focused on candidate genes for fertility including ACE, CRISP1, CRISP2, CRISP3, FKBP6, FSHB, INHBA, PLCz1, PRLR, SPATA1 and SP17 [10–12,28–30]. In the present study, we investigate the proAKAP4 expression not in seminal plasma but directly in spermatozoa. AKAP4 is indeed a known marker involved in flagellum structuration and motion which was first described as associated with the sperm flagellum [17] and highly conserved as already described in mouse [16], in bull [31], human [15] and stallion spermatozoa [24]. Members of the AKAP family, including AKAP4, have been demonstrated to anchor cAMP-dependent protein kinase-A (PKA) to a variety of subcellular locations. AKAP4 anchors PKA to the fibrous sheath of the spermatozoa where the kinase is likely to be required for the regulation of spermatozoa motility and interacts with a number of other proteins in the fibrous sheath [20]. AKAP4 is a major fibrous sheath protein, that acts as a scaffold for both glycolytic enzymes and members of the phosphorylation signaling cascades that are positioned to provide a localized source of ATP and to play a role in the regulation of flagellar motion and therefore sperm motility and hypermotility [20,32,33]. Multiple glycolytic enzymes are tightly bound to the fibrous sheath at the principal piece of sperm flagellum and are required for sperm motility [32,34].

Interestingly, AKAP4 is synthesized as a precursor called proAKAP4 during spermatogenesis from the round spermatids and processed to a the full mature AKAP4 in condensing spermatids [35]. In our study, we wanted to investigate whether the expression and the concentration of proAKAP4 and AKAP4 in equine sperm could be related to the total or progressive motility. We used the newly designed and specific monoclonal antibodies, respectively called anti-proAKAP4 clone 6F12 and anti-AKAP4 clone 7E10 monoclonal antibodies which are the only monoclonal antibodies

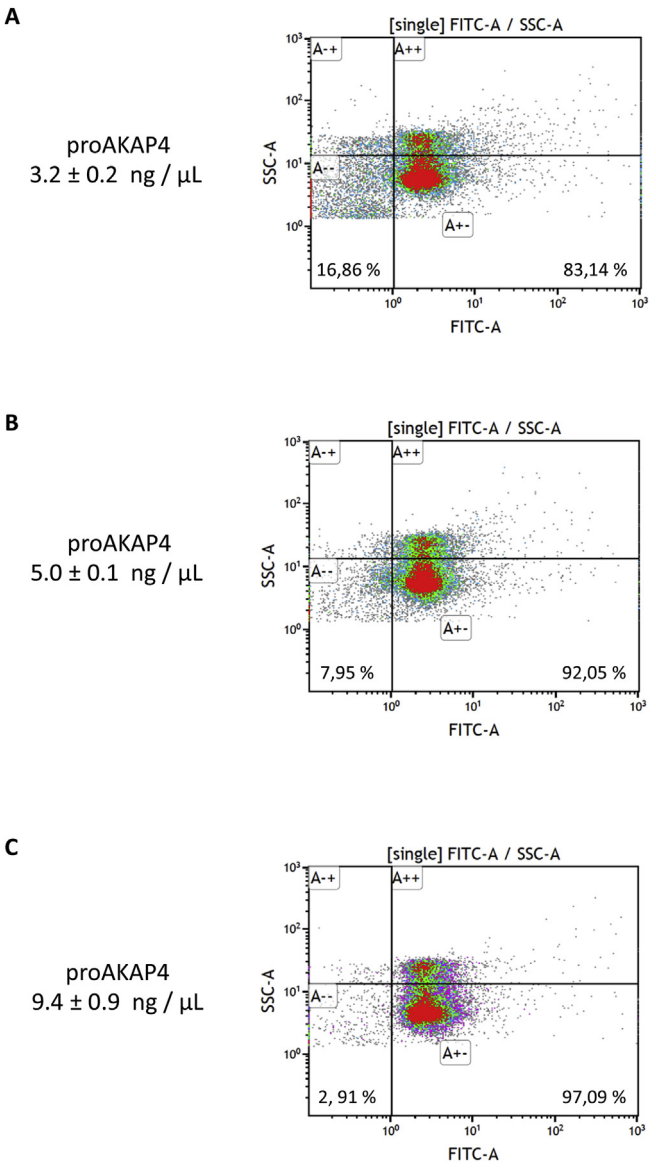

Fig. 5. Expression of proAKAP4 in stallion semen as assessed by flow cytometry. Spermatozoa from frozen semen of three stallions with proAKAP4 concentrations at 3.4, 5.0 and 9.4 ng/μL of semen were isolated and stained with the proAKAP4 (clone 6F12) mouse monoclonal antibody before analysis by flow cytometry. Events of spermatozoa are represented as the side scattering (SSC-A y-axis) and the proAKAP4 immunostaining of stallion spermatozoa (FITC-A x-axis). The four quadrants are defined according to the side scattering and proAKAP4 staining. Quadrants A++ and A + - correspond the proAKAP4 stained spermatozoa and quadrants A–, A- + are spermatozoa events without proAKAP4.

to recognize proAKAP4 and AKAP4 in stallion semen. Using these tools, we first demonstrated by immunostaining methods that neither the precursor proAKAP4 nor the mature AKAP4 are detected in the seminal plasma showing that both are specific to spermatozoa, and then indicative of the presence of spermatozoa with full-flagellum (Fig. 1). Furthermore, the head, the midpiece and the end piece of spermatozoa were never labelled by the antibodies. The mature protein and its precursor were always both localized to the fibrous sheath of the principle piece of equine sperm flagellum (Figs. 1 and 2). These results confirm previous observations made in stallion spermatozoa using fluorescence microscopy with another anti-AKAP4 antibody [24]. In human spermatozoa, Rahamim Ben-Navi et al. [36] recently shown by immunofluorescence that

AKAP4 is localized to the principal piece under basal conditions and in the principal piece, mid-piece and the post-acrosomal region after PMA stimulation that mimics capacitation and activated spermatozoa. The proAKAP4 was previously described principally in epididymal sperm in the mouse and in the bull, whereas the mature AKAP4 was only found in ejaculated sperm in these two species [31]. By immunofluorescence and subcellular fractionation, proAKAP4 was also shown to localize to the periacrosomal membranes in pig semen [37]. Previously, the proAKAP4 precursor was shown with designed constructs, to be soluble and unassembled to the fibrous sheath [35]. In contrast, the mature AKAP4 was described to be insoluble and structure within the fibrous sheath in close proximity to the microtubules of the axoneme [35].

Since AKAP4 and its precursor proAKAP4 were found specifically in the spermatozoa (Figs. 1 and 2), we hypothesized that their immunological detection can be representative of their full content in stallion spermatozoa. Our data confirmed that, behind the conserved sequence, the proAKAP4 and AKAP4 localization and expression are conserved between species [22,38]. The western blot analysis on 8 thawed stallion semen samples showed different expression levels of proAKAP4 whatever the antibody used (Fig. 3). Strikingly enough, the stallion semen samples showing the lower AKAP4 levels also exhibit a low density band for the proAKAP4 expression. As the precursor proAKAP4 is processed to release the mature protein, it represents a kind of « functional reservoir » of the AKAP4 protein.

The absence of AKAP4 prevent signal transduction and the association of glycolytic enzymes involved in motility, as the calcium ($Ca2+$) pathway, the PKA pathway were shown to be important metabolic and signaling pathways involved in the regulation of flagellar motion and sperm motility [19]. On mice models, sperm motility was shown to be completely disrupted in the absence of AKAP4 expression [13]. Interestingly, the interference with the PKA/AKAP4 interaction using PKA inhibitory compounds results in immotile bovine sperm [39] highlighting the crucial role of proAKAP4/AKAP4 in regulating motility. Indeed, if the mature AKAP4 is largely described to be crucial to spermatozoa motility [13,20,40–42], much less is known about the proAKAP4 precursor in stallion semen. To our knowledge, the relationship between the content of proAKAP4 or AKAP4 in the stallion semen and the quality of the semen after thawing has been very little studied. Turner et al. [24] showed in cryopreserved and thawed stallion semen that a reduce motility was not likely associated with a reduce binding of PKA to AKAP4.

Herein, by using a commercial sandwich ELISA assay to capture, detect and precisely quantify the amount of proAKAP4 in stallion semen, we described for the first time that the concentrations of the precursor proAKAP4 are correlated to both the total and the progressive motility in post-thawed stallion semen samples suggesting that proAKAP4 and the mature AKAP4 contribute to regulate flagellar motion and sperm motility (Fig. 4). Thus the proAKAP4 concentrations in thawed semen could be used as a marker of sperm quality. Furthermore, by combining this ELISA assay with flow-cytometry we clearly demonstrated for the first time that the proAKAP4 concentration in semen reflects a proportion of spermatozoa expressing the proAKAP4 precursor in an homogenous population of stallion (Fig. 5). Some spermatozoa do not express either proAKAP4 or AKAP4 as a mature form. In the future, it could be interesting to see if these spermatozoa are still motile. According to Miki et al. [13], spermatozoa lacking proAKAP4 and AKP4 expression are immotile and infertile.

To conclude, our present results bring today new evidences that not only the mature AKAP4 but also the precursor proAKAP4 is involved in the regulation of equine sperm motility. Findings herein presented led to consider sperm proAKAP4 as a potential marker of stallion sperm quality but can be used also in the understanding to the pathophysiology underling the loss of motility and viability of stallion sperm samples. Further studies are needed to show the impact of freezing method, and to compare feezing medium on the proAKAP4 content in stallion semen.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2019.03.011.

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