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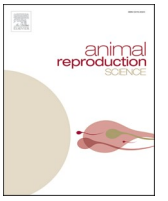

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ABSTRACT

ProAKAP4 is identified within the flagellum of spermatozoa in various mammalian species, serving as a structural protein associated with motility parameters. This investigation focuses on the presence of proAKAP4 in donkey sperm, elucidating its localization, molecular characteristics, and its correlation with motility descriptors and mitochondrial membrane potential. Twelve ejaculates from Catalan donkeys were analyzed in this study. The initial steps involved proAKAP4 sequencing and detection through Western blotting and immunofluorescence. Post-thaw assessments were conducted at 0, 1, and 3 h, encompassing proAKAP4 levels, sperm motility analyzed via Computer-Assisted Sperm Analysis (CASA), and mitochondrial membrane potential determined by flow cytometry using the JC-1 stain. The findings reveal that proAKAP4 in donkeys exhibits a characteristic localization at the principal piece of the flagellum, consistent with observations in other mammals. The molecular weight of proAKAP4 is determined to be 100 kDa. Significantly, a positive correlation ($p \leq 0.05$) is established between proAKAP4 concentration and both total and progressive motility. The presence of cryoprotectant is associated with a lower proAKAP4 concentration. Notably, proAKAP4 experiences a substantial decrease ($p \leq 0.05$) during the initial hour post-thawing. In conclusion, proAKAP4 is identified in donkey sperm, akin to its presence in other mammals. It exhibits a positive correlation with total and progressive motility, its concentration is notably affected by the presence of cryoprotectant with significant consumption observed during the initial hour following thawing. These findings contribute to our understanding of proAKAP4 dynamics in donkey sperm, providing insights that may have implications for semen preservation and reproductive technologies in equids.

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1. Introduction

Traditionally serving as a traction animal, the donkey (Equus asinus) continues to play an important role in underdeveloped countries (Camillo et al., 2018; Canisso et al., 2019), whereas many European breeds encounter endangerment (DAD-IS, 2017). However, there is a burgeoning interest in diversifying the use of donkeys for purposes, such as milk, cosmetics, meat, and skin production, as well as in leisure or sport activities like mule production (Camillo et al., 2018). The imperative to comprehend the reproductive physiology of donkeys and implement reproductive technologies has grown significantly. This is not only to preserve endangered breeds (Rota et al., 2012) also to optimize production outcomes (Camillo et al., 2018; Canisso et al., 2019; Oliveira et al., 2016).

While semen manipulation protocols designed for stallions have been adapted for use in donkeys, it is imperative to recognize the physiological distinctions between these phylogenetically close yet distinct species. The procedures for semen collection, processing, and freezing adhere to similar protocols (Canisso et al., 2019; Monteiro et al., 2022). However, certain studies highlight potential toxicity concerns associated with extender components utilized in horses when applied to donkeys (Rota et al., 2012; Trimeche et al., 1998). Moreover, chemical ejaculation induction with imipramine, effective in stallions, proves ineffective in donkeys (Canisso et al., 2019; McDonnell, 2001). A noteworthy distinction lies in the size ofthe male genital tract's sexual glands (bulbourethral, prostate, and ampullas of the vas deferens) in donkeys, which are significantly larger than those in stallions. This results in a substantial volume of seminal plasma characterized by higher protein content and enhanced antioxidant enzymes activity, like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (Gacem et al., 2020; Papas et al., 2019). Computerized sperm movement analysis (CASA) reveals distinct kinematic descriptors between donkey and horse sperm, with donkey sperm displaying higher speed and linearity (Canisso et al., 2019; Gacem et al., 2021). The kinematic descriptors, particularly speed and linearity, significantly differ between donkey and horse sperm when analyzed using computerized systems (CASA) (Canisso et al., 2019; Gacem et al., 2021). Notably, donkey sperm demonstrates significantly greater speed and linearity compared to horse sperm. (Gacem et al., 2021). This underscores the importance of species-specific considerations in reproductive technologies for equids.

Furthermore, ProAKAP4, a protein involved in the sperm movement and specific to the sperm flagellum (Johnson et al., 1997; Sergeant et al., 2021; Carracedo et al., 2022) was identified across mammals studied (bull, stallion, mouse, rat, human, dog, pig, buck, camel) (Carracedo et al., 2022; Malo et al., 2021). Notably, it has also been discovered in non-mammalian species such as crocodile (Nixon et al., 2019) and wall lizards (Sarkar et al., 2016). Tian et al. (2020) specifically identified ProAKAP4 in donkey sperm.

This AKAP4 precursor has been described as an anchoring protein exclusive to spermatozoa that demostrate correlation with total and progressive motility in bulls (Dordas-Perpinyà et al., 2022a) and stallions (Blommaert et al., 2021; Dordas-Perpinyà et al., 2022b). While it holds potential as a fertility biomarker due to its association pregnancy rates in bulls (Dordas-Perpinyà et al., 2022a), it is notably absent in men with unexplained fertility problems (Xu et al., 2012; Delehedde et al., 2020) and is also underregulated in hybrids such as Cattle yaks (Wu et al., 2020).

This study aims to characterize and label proAKAP4 in donkeys and correlate their concentration with motility characteristics and mitochondrial activity in thawed semen and its evolution over time.

2. Material and methods

2.1. Animals

Two ejaculates from each of six jackasses of Guarà Català (Catalan donkey endangered breed) were collected. Donkeys are owned by the Reproduction Service of the Universitat Autonòma de Barcelona (UAB, Bellaterra, Cerdanyola del Vallès, Spain) for research and breed conservation purposes. The selected jackasses, aged from 3 to 12 years old, had proven fertility and good body condition, were free of Equine Arteritis, Infectious Anemia, and Contagious Metritis (CEE health requirements), and were following a regular semen collection. For each male, two ejaculates were collected and processed for cryopreservation between the years 2015 and 2021 and 3 straws from each ejaculate were dedicated to this research study. The study was approved by the Ethics Committee, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain; Cose: CEEAH 1424, 23.04.2018).

2.2. Semen collection

The collection of donkey ejaculates was done at the Equine Reproduction Service of the UAB which is a European centre approved with authorization code ES09RS01E.

Semen collection was achieved using a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an inline nylon filter. After collection, the ejaculate was immediately diluted 1:5 in Kenney extender prewarmed at 37 °C in 50 mL Corning tubes. All ejaculates were evaluated for semen parameters before freezing: total volume, sperm concentration by a haematocytometer (Neubauer chamber, Paul Marienfeld GmbH and Co. KG; Lauda-Königshofen, Germany), motility by a computer-assisted sperm-analysis system (CASA, Section 2.5), and morphology using the eosin-nigrosin staining (Bamba, 1988).

2.3. Sperm cryopreservation

Before cryopreservation, the seminal plasma was removed. Briefly, each extended semen sample was centrifuged at 600 x g at 20 °C for 15 min (Medifriger BL-S, JP Selecta S.A., Barcelona, Spain). Supernatants were discarded and pellets were resuspended in a

commercial freezing medium (Botucrio®, Botupharma Animal Biotechnology; Botucatu, Brazil) to a concentration of 200 × 10^6 of viable spermatozoa per mL, being re-evaluated sperm concentration and viability, (Neubauer chamber, Paul Marienfeld GmbH and Co. KG; Lauda-Königshofen, Germany). Samples were packaged into 0.5 mL straws and cryopreserved using a controlled-rate freezer (IceCube 14 S; Minitüb, Tiefenbach, Germany). Briefly, cryopreservation was achieved following three successive freezing steps: 1) cooling from 20 °C to 5 °C at the rate of − 0.25 °C/min for 60 min. 2) freezing from 5 °C to − 90 °C at a rate of − 4.75 °C/min for 20 min. 3) freezing − 90 °C to − 120 °C at a rate − 11.11 °C/min for 2.7 min. Straws were then stored in liquid nitrogen in appropriate tanks.

2.4. Semen motility analysis

Sperm motility was evaluated using a CASA system (IVOS II ®; Hamilton Thorne; IMV Technologies; L'Aigle, France). Samples were kept at 37 °C before the analysis, and 3 μL of sperm samples were placed into a Leja® slide (IMV Technologies; L'Aigle, France) prewarmed at 37 °C. Sperm motility analysis was repeated three times per sample. Analysed sperm motility descriptors were: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, μm) and frequency of head displacement (BCF, Hz).

CASA settings were: magnitude of x200 with 10 randomly fields; 60 images captured per second; 30 sequences taken in each analysis; particle area 3 pixels; connectivity: 6; minimum number of images to calculate the ALH: 10. Cut-off value for motile spermatozoa was VAP ≥ 20 μm/s, and for progressive motile spermatozoa was STR ≥ 80% and VAP ≥ 30 μm/s.

2.5. Mitochondrial membrane potential measurement

The measurement of mitochondrial membrane potential was performed using the Guava® Easycyte II™ flow cytometer (Guava Technologies Inc., Hayward, CA, USA, distributed by IMV Technologies, l'Aigle, France) and the EasyKit™2 (IMV Technologies, l'Aigle, France). The EasyKit™2 consists of a 96-well plate containing the JC-1 fluorochrome, specific for mitochondrial activity. In each well, 190 μL of EasyBuffer® (IMV Technologies, l'Aigle, France), 10 μL of DMSO, and 1.75 μL of diluted semen solution were deposited at T0. Then, the plate was incubated for 30 min at 37 °C in the dark. The wells were then analyzed using the Guava® Easycyte II™ flow cytometer, previously calibrated with the Easy Check® kit (Guava Technologies Inc., Hayward, CA, USA), and the Express Pro® software (IMV, l'Aigle, France). The EasyKit™2 fluorochrome emits maximum fluorescence of 525 nm (green) and 596 nm (red). For each well, 5000 sperm were analysed. The fluorescent marker JC-1 is validated as a marker of the intermediate piece of spermatozoa with high membrane potential and low membrane potential mitochondria. Polarized, active, mitochondria are coloured orange while depolarized, inactive, mitochondria are coloured green according to the defined parameters. The results are expressed as a percentage of "polarized" mitochondria (strongly or partially), and as a percentage of "depolarized" mitochondria. In our study, mitochondrial activity is expressed by the percentage of "polarized" so-called "active" mitochondria.

2.6. ProAKAP4 assessment

2.6.1. proAKAP4 sequence homology analysis

ProAKAP4 protein sequence homology comparison was determined using the repository protein sequence (Uniprot: Horse ProAKAP4 protein F6Y0B1 848AA; Donkey ProAKAP4 protein A0A8C4L657 832AA) using the multalin protein sequence comparison algorithm (Corpet al, 1988).

2.6.2. Gel electrophoresis and western-blotting

The expression of proAKAP4 in donkey spermatozoa was first analysed by SDS polyacrylamide protein gel electrophoresis Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) followed by immunoblotting using either the specific proAKAP4 mouse monoclonal antibody against the prodomain of the AKAP4 precursor (anti-proAKAP4 clone 6F12, 4BioDx, Lille, France) or the mouse monoclonal anti-AKAP4 antibody (clone 7E10, 4BioDx, Lille, France) directed against an epitope located at the C-terminus of the AKAP4 protein. The antibody was obtained using the amino-terminal sequence of the human prodomain of AKAP4. The conserved sequence is yet unknown, but immunoreactivity with proAKAP4 from humans, bulls, horses, mice, rats, and rams was established using western blotting. Donkey semen protein lysate was obtained following the addition of 90 μL of SDS-PAGE lysis Buffer (10 mM Tris HCl pH 6.8, 30% Glycerol, 2% SDS) to 10 μL of donkey semen sample. After determining protein concentrations using the Bradford method, an equivalent of 25 μg added 1x volume of the NuPAGE Sample Reducing Agent (ThermoFisher, Waltham, MA, USA). Samples were vortexed and heated at 80 °C for 10 min 10 μg of semen lysate protein was then loaded on polyacrylamide gel (4–12% NuPage Precast Gels) and run for up to 45 min under constant tension of 100 volts per gel. Using the Liquid Transfer System (Life Technologies, Waltham, MA, USA), the gel was transferred onto a 0.45 μm nitrocellulose membrane according to the manufacturer's instructions (G&E Healthcare, North Richland Hills, TX, USA). The membrane was then incubated at 4 °C overnight with the first antibody (either the anti-proAKAP4 or anti-AKAP4 mouse monoclonal antibodies, SPQI, 4BioDx, Lille France) at a dilution of 1:4000 in 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% (*v/v*) Tween 20 (TBS-T Buffer). After appropriate washing steps (3 times 10 min in TBS-T), the membrane was then incubated with a secondary anti-mouse antibody coupled to horseradish peroxidase at 1:50,000 dilution (Vector Laboratories, Burlingame, CA, USA) and further revealed with the ECL™ enhanced chemiluminescence kit (G&E Healthcare, North Richland Hills, TX, USA). Images were acquired using the Image Quant™ LAS 4000 system (G&E Healthcare, North Richland Hills, TX, USA).

2.6.3. Immunofluorescence

Donkey 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* for 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, Braunschweig, Germany) and laid using the coverslip. Slides were then dried for 2 h immersed 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, Waltham, MA, 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 -DAPI- (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 images 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.

2.6.4. ELISA assays

The semen concentration of ProAKAP4 was analyzed by quantitative ELISA at ONIRIS Laboratory in Nantes (France) using the Stallion 4MID® kit (SPQI, Lille, France), a quantitative ELISA sandwich assay to specifically detect and quantify the proAKAP4 in equine semen samples. A 7 points standard solution was provided to make a reference curve and determine the concentration of proAKAP4 present in each donkey semen sample. Sample preparation was achieved following the manufacturer's instructions for cryopreserved semen samples.

The samples selected for the analysis were taken from the different experimental groups described in Section 2.4. 40 μL of diluted semen from each group was diluted with 160 μL of the Horse-specific lysis buffer. After 1 min of vigorous mixing with a vortex, 200 μL of dilution buffer was added and rapidly mixed before loading in the 96-well-coated plate. After 2 h of incubation under gentle agitation with an ELISA-plate horizontal shaker and 3 successive washing steps, the horseradish conjugated detection antibody was added to each well and further incubated for 1 h. The colour reaction was achieved by the addition of the TMB substrate allowing after 10 min the appearance of colour levels quantitatively proportional to the amount of proAKAP4 present in each donkey semen sample. The colour reaction was stopped by a stop solution added to each well and colour intensities were acquired using a spectrophotometer (BioTek®, Vermont, US) equipped with a 450 nm filter. Optical densities were further used to determine proAKAP4 concentration using the Calculation sheet provided by the manufacturer (SPQI, 4BioDx, Lille France). Briefly, the proAKAP4 concentration in ng per 10 million spermatozoa was determined using the following formula: proAKAP4 ng/ 10 M spz = (proAKAP4 in ng/mL /spz M/mL) x 10 × 16 x (2/3). The concentration of proAKAP4 in ng/mL was determined with the Horse 4MID® kit calculation sheet. The 10x factor is applied to provide the final concentration in 10 million spermatozoa. The 10x dilution factor correspond to post-thawed sample dilution factor during the sample preparation procedure. The 2/3 factor correspond to the dilution volume loaded in each well of the 96-well plate of the Horse 4MID® assay.

2.7. Experimental design

Three straws per ejaculate were thawed in a water bath at 37 °C for 50 s. Subsequently, the contents of These straws were transfered into separate Eppendorf tube previously filled with 500 μL of INRA96® previously warmed at 37 °C. The content of each Eppendorf tube was gently agitated before being divided in two Eppendorfs. One of these Eppendorfs tubes has been centrifuged to remove the cryoprotectant and the other has been kept the cryoprotectant. To remove the cryoprotectant, the Eppendorf tube was centrifuged for 10 min at 600 x g and the pellet was resuspended with 500 μL of INRA96®. Once we had one Eppendorf tube with cryoprotectant and another without it, they were kept inside the incubator at 37 °C; different analyses were done at time 0 h, 1 h and 3 h after thawing.

Seminal analyses done in both groups and at different time points included motility descriptors via CASA (Section 2.4), proAKAP4 concentrations (Section 2.6.3) and mitochondrial membrane potential (Section 2.5).

2.8. Statistical analysis

The R statistical package (V 4.0.3, R Core Team; Vienna, Austria) was used to analyze the experimental data and GraphPad Prism software (V 8.4.0, GraphPad Software LLC; San Diego, CA, USA) to create the graphs of results. Previous to the statistical analysis, the Shapiro-Wilk test was used to verify the normality of the data and Levene's test to check the homoscedasticity of variances. In case of requiring a normal distribution, the data were transformed by applying the function arcsin $\sqrt{x}$. The concentration of proAKAP4, the total motility and progressive, and the mitochondrial membrane potential of donkey spermatozoa were compared with a mixed generalized linear model of repeated measures in the time. Incubation time (0 h, 1 h and 3 h) was the within-subjects factor, the treatment (centrifugation and no centrifugation) was the fixed effect factor and the donkey was the random effects factor. On the other hand, the correlation coefficients (r) between the concentration of ProAKAP4 with the parameters of spermatic motility and with the mitochondrial membrane potential of donkey spermatozoa in each treatment and incubation time were obtained using a Pearson correlation.

The minimum level of statistical significance was set at $P \leq 0.05$ for all analyses. Results are expressed as mean ± standard error of

the mean (SEM).

3. Results

3.1. ProAKAP4 sequentiation

The Donkey (*Equus asinus asinus*) proAKAP4 amino acids sequence differs from the Horse proAKAP4 sequence (*Equus caballus*) by 17 amino acid stretch insertion of 17 amino acids at position 34 of the horse proAKAP4 sequence (Fig. 1). The Donkey amino acids sequence also differs by the proAKAP4 starting sequence with cysteine-leucine before the first methionine. Apart from these two major protein sequence differences, the overall protein sequence homology is higher than 90% showing a high conservation of proAKAP4 protein sequence homology between these two species and then suggesting a preserved PKA-docking function of proAKAP4 and AKAP4 in Donkey.

3.2. Western-blot

ProAKAP4 and AKAP4 were observed by western blotting (Fig. 2) with the detection of a band at 100 kDa for proAKAP4 (clone6F12 antibody) and at 80 kDa for AKAP4 (clone 7F10 antibody). A slightly lowered apparent molecular weight is observed for proAKAP4 and AKAP4 in donkey semen samples when compared to the horse semen sample. However, the detection of both proAKAP4 and AKAP4 in donkey semen samples showed that the conversion cleavage process of the proAKAP4 into AKAP4 is preserved in Catalan donkey sperm. The prodomain at 20 kDa released following the cleavage of proAKAP4 produce the mature AKAP4 (Fig. 2A), at a ratio 1:1 between proAKAP4 and AKAP4 polypeptide reflecting a conserved proAKAP4 metabolism in Catalan donkeys (Fig. 2B).

3.3. Immunofluorescence

By immunofluorescence, proAKAP4 and AKAP4 labelled in green the principal piece of the flagellum (Fig. 3) of donkey spermatozoa. Interestingly not all donkey spermatozoa were labelled highlighting a differential expression and/or metabolism in individual post-thaw spermatozoa from donkey and stallion spermatozoa (not shown).

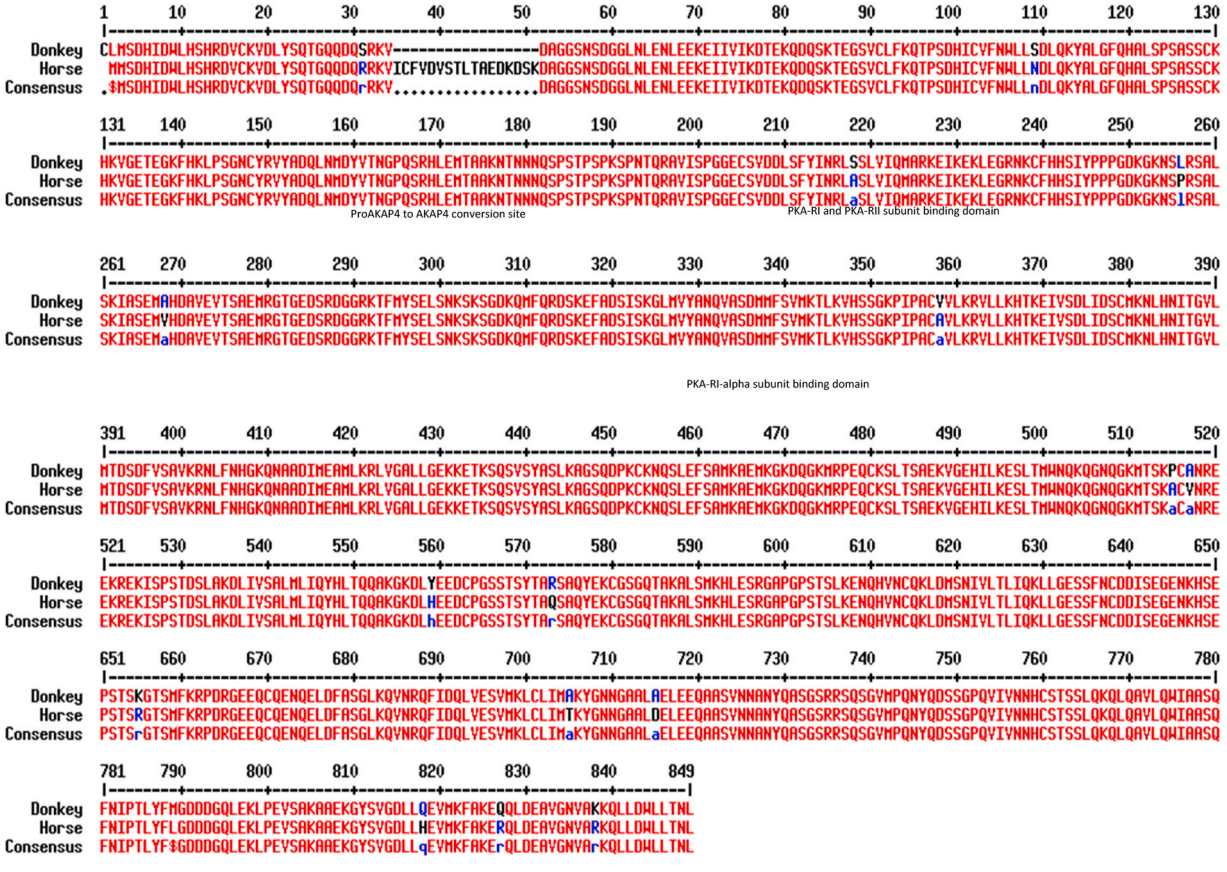

Fig. 1. ProAKAP4/AKAP4 sequence homology.

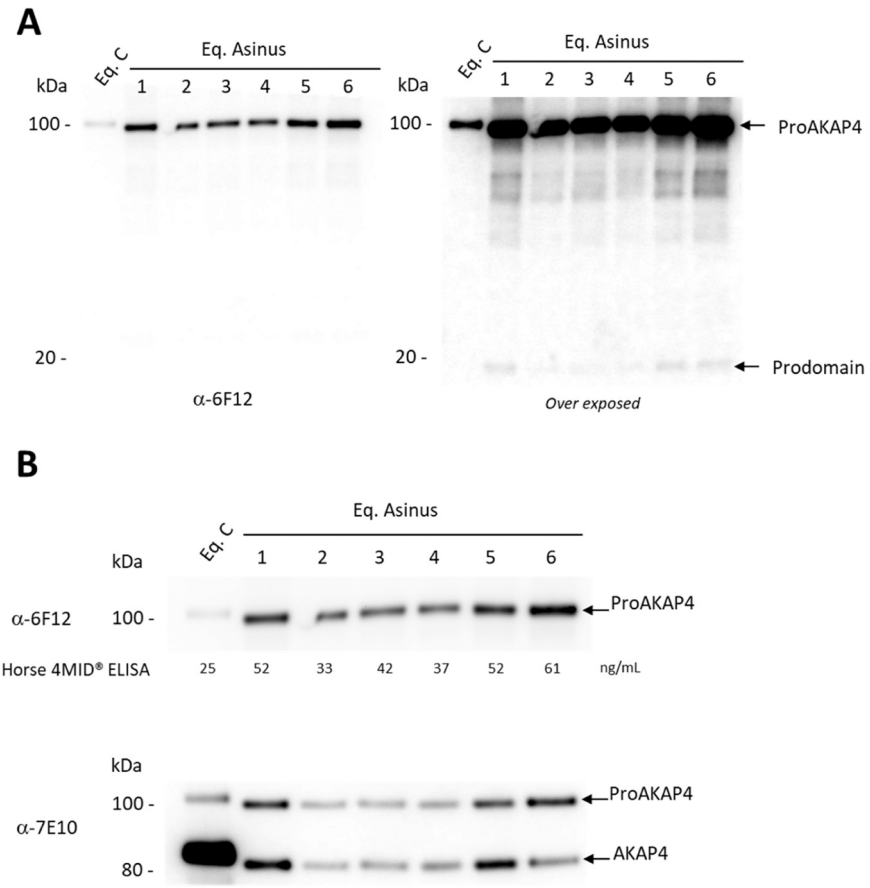

Fig. 2. Images of Western-blot gels. A: proAKAP4 labelling. B: ProAKAP4 and AKAP4 semi-quantitative expression in Donkey semen samples 1 to 6 compared to the Horse semen sample.

3.4. Correlation between proAKAP4, motility descriptors and mitochondrial membrane potential

ProAKAP4 correlated positively with progressive motility at time 0 h after thawing. At time 3 h after thawing, proAKAP4 concentration correlated negatively with VSL, VAP and BCF (Table 1).

Mitochondrial activity did not show correlation with proAKAP4 concentration at any time of the experiment.

Table 2 shows the correlation (r) between the concentration of ProAKAP4 with the sperm motility parameters and mitochondrial membrane potential (JC1) of donkey spermatozoa in each experimental group (with or without cryoprotectant). The concentration of ProAKAP4 in centrifuged samples (without cryoprotectant) showed a positive correlation with total motility and progressive motility and a negative correlation with the following motility descriptors: VCL, VSL, VAP, STR, ALH. On the other hand, in samples with cryoprotectant (non-centrifuged) the concentration of ProAKAP4 shows a negative correlation at 1 h with ALH and a positive correlation with the percentage of total and progressive motile spermatozoa and LIN.

3.5. Evolution of proAKAP4, motility descriptors and mitochondrial membrane potential over time

ProAKAP4 (Fig. 4A) concentration showed a significant difference between the centrifuged and non-centrifuged group in the 3 analyzed times, being significantly ($p < 0.05$) higher in centrifuged samples. However, the decrease of the proAKAP4 concentration is statistically significant ($p < 0.05$) during the period from 0 to 1 h after thawing and it is not significant ($p > 0.05$) from 1 to 3 h post-thawing.

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The analysis of total motility (TM)(Fig. 4B) and Fig. 4C for progressive motility (PM)(Fig. 4C), evidenced no significant differences between groups with or without cryoprotectants. Both groups, TM and PM, showed the same evolution over time. During the period from 0 to 1 h post-thawing the decrease of the motility was not significant. However, between 1 and 3 h after thawing, the decrease of the motility was significant ($p < 0.05$) for the centrifuged (without cryoproctectant) group.

The mitochondrial activity was maintained over the time showing no difference between groups and between the hours.

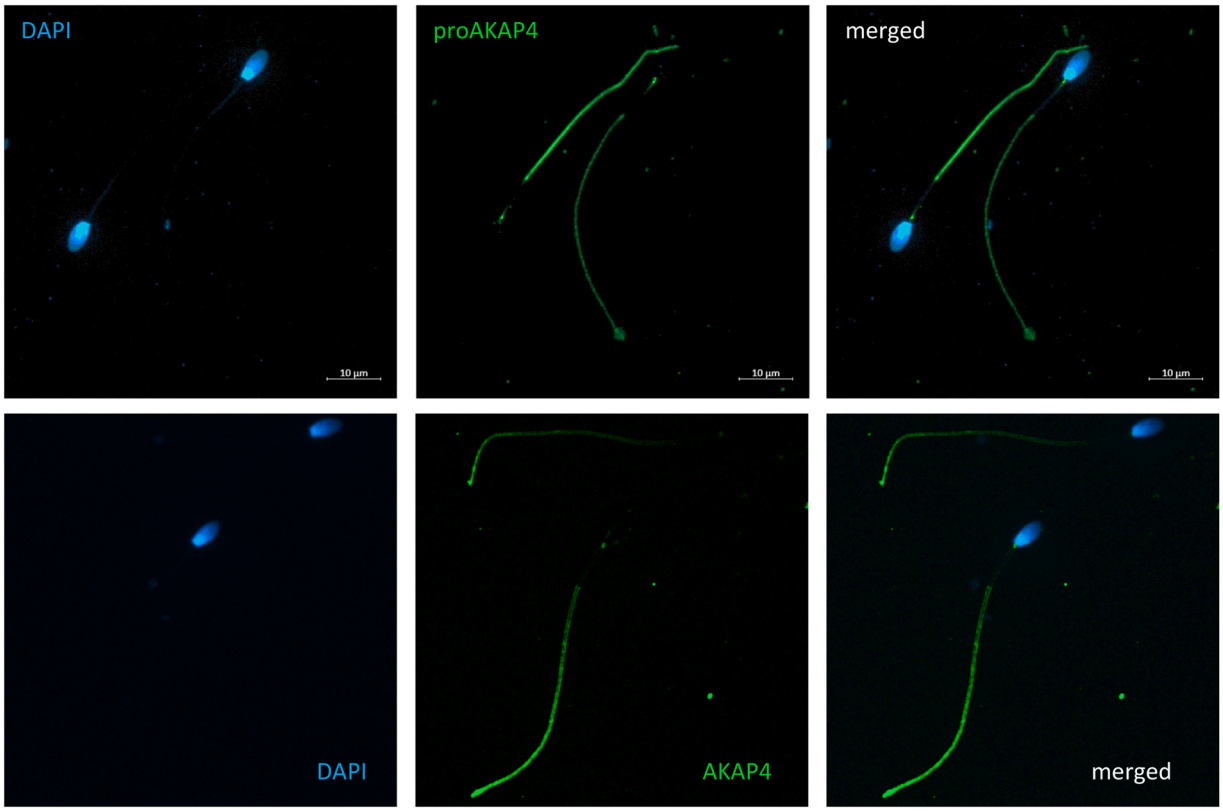

Fig. 3. Confocal microscopy images with spermatozoa labelled with AKAP4 (green) and proAKAP4 (green) antibodies.

Table 1
Correlation between proAKAP4 concentration (ng/10 M spz – millions of spermatozoa-) with sperm motility and mitochondrial activity.

	0 hours		1 hours		3 hours	
	r	p	r	p	r	p
TM	0.438	0.032*	0.401	0.050*	0.167	0.436
PM	0.467	0.022*	0.388	0.061	0.043	0.841
VCL	0.087	0.685	-0.156	0.468	-0.375	0.071
VSL	0.023	0.914	-0.235	0.270	-0.463	0.023*
VAP	0.031	0.886	-0.238	0.262	-0.442	0.030*
LIN	-0.082	0.702	-0.230	0.280	-0.286	0.176
STR	-0.017	0.936	-0.001	0.996	-0.386	0.063
ALH	0.267	0.208	0.098	0.649	0.338	0.106
BCF	-0.248	0.242	-0.148	0.490	-0.469	0.021*
JC1	0.343	0,9 0.101	0.113	0.598	0.017	0 0.937

Total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, µm) and frequency of head displacement (BCF, Hz) and mitochondrial activity: JC1 (% of gated spermatozoa) at different incubation times (0, 1, 3 h). * means that correlation is considered significant when $p \leq 0.05$. The table contains the analytics of all samples without discriminate if the simple has been centrifuged or not.

4. Discussion

For the first time, proAKAP4 and AKAP4 protein has been characterised in donkeys using Western blot and immunofluorescence techniques, following its detection by Tian et al. (2020). Both proteins, AKAP4 and its precursor proAKAP4, exhibit a high degree of conservation across mammals (Carracedo et al., 2022; Sergeant et al., 2019). Our research on donkeys has confirmed this conservation, as evidenced in the Western blot, which revealed proAKAP4 at 100 kDa and AKAP4 at 80 kDa, along with its prodomain at approximately 20 kDa, like other species (Carracedo et al., 2022). Notably, in this case, donkey semen samples, both proAKAP4 and AKAP4 displayed a slightly reduced apparent molecular weight compared to the horse semen samples. Small specific differences were observed in the proAKAP4 amino acids sequence between jackasses and stallions.

Table 2
Pearson correlation coefficients (r) between the concentration of ProAKAP4 with sperm motility parameters and with the mitochondrial membrane potential of donkey spermatozoa in each treatment.

	ProAKAP4 concentration (ng/10 M spz)			
	Without cryoprotectant		With cryoprotectant	
	r	p	r	p
TM (%)	0.487	0.003**	0.440	0.007**
PM (%)	0.452	0.006**	0.324	0.050
VCL (μm/s)	-0.330	0.049*	-0.311	0.065
VSL (μm/s)	-0.198	0.247	-0.130	0.448
VAP (μm/s)	-0.196	0.253	-0.120	0.487
LIN (%)	0.105	0.542	0.344	0.040*
STR (%)	-0.191	0.263	-0.052	0.764
WOB (%)	-0.316	0.060	-0.485	0.003**
ALH (μm)	-0.212	0.215	-0.219	0.198
JC-1	0.277	0.102	-0.060	0.730

Total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), amplitude of lateral head displacement (ALH, μm) and frequency of head displacement (BCF, Hz) and mitochondrial activity: JC1 (% of gated spermatozoa).

Like in other males like bull (Bastan and Akcay, 2021; Dordas-Perpinyà et al., 2022a; Marques de Almeida et al., 2022), stallion (Blommaert et al., 2019; Dordas-Perpinyà et al., 2022b; Griffin et al., 2020; Sergeant et al., 2020), boar (Sergeant et al., 2020), dogs (Le Couazer et al., 2019), ram (Riesco et al., 2020), buck (Fatet et al., 2022), camels (Malo et al., 2021) and humans (Jumeau et al., 2018; Sigala, 2016) in jackass proAKAP4 concentration is significantly correlated with total and progressive motility after thawing.

Cryoprotectants and extenders have been developed to preserve semen fertilization capacity (Bustani and Hassan, 2021), and they also can impact post-thaw semen quality (Imrat et al., 2012) in various aspects. Cryoprotectant agents may also affect proAKAP4 levels (Carracedo et al., 2022). This effect can be explained by two hypotheses: the toxic effect on the spermatozoon and the promotion of faster conversion of proAKAP4 into AKAP4 by the cryoprotectant in the extender compared to its absence. The cryopreservation process and cryoprotectant induce changes in the sperm motility patterns in donkey (Flores et al., 2008). In our study, significant changes in sperm motility patterns were observed depending on the maintenance of cryoprotectant and alterations in the proAKAP4 concentration. ProAKAP4 concentration decreased more in the presence of cryoprotectant (samples without centrifugation) than in samples where the cryoprotectant was removed by the centrifugation process. Samples without cryoprotectants exhibited higher proAKAP4 concentration and retained it for a longer duration. The effect of different extenders has been explored by other researchers. Kowalsky et al. (2022)) evidenced that the addition of Holothuroidea extract to the extender increased proAKAP4 levels in bovine semen. However, proAKAP4 consumption in post-thaw conditions depends on the cryoprotectant agent (Sergeant et al., 2020). In stallions, four hours after thawing, one extender, Sperlin®, exhibited better motility parameters and higher proAKAP4 concentration than INRA Freeze® (Blommaert et al., 2021). Further studies are necessary to evaluate the effects of different extenders and/or cryoprotectants.

In our study, proAKAP4 was evaluated over a 3-hour period, revealing a distinct consumption pattern compared to total and progressive motility ProAKAP4 consumption is more pronounced in the first hour, followed by a significant decrease in its concentration.; Protein consumption occurs rapidly but its degradation is noticeable later in motility parameters. In stallions, Blommaert et al. (2021), observed a significant decrease in proAKAP4 at 4 h post thawing, although they did not differentiate the consumption pattern. Total and progressive motility remains relatively stable during the first hour post-thawing but experiences a noticeable decline between the first and third hour. When considering all parameters, proAKAP4 exhibits a similar reduction pattern as total and progressive motility if examined only at 0 and 3 h post-thawing. However, the manner in which they decrease differs, suggesting that the decrease in proAKAP4 is not synchronized with the decline in total and progressive motility. ProAKAP4 is consumed first, and its effects on motility (total and progressive) become apparent later.

Our study reveals a positive and significant correlation between proAKAP4 concentration and progressive motility at time 0 h, consistent with previous studies (Bastan and Akcay, 2021; Blommaert et al., 2019; Carracedo et al., 2022; Dordas-Perpinyà et al., 2022a, 2022b; Fatet et al., 2022; Griffin et al., 2020; Le Couazer et al., 2019; Malo et al., 2021; Marques de Almeida et al., 2022; Riesco et al., 2020; Sergeant et al., 2020). However, a negative correlation is observed with speeds (VCL, VSL, VAP) and BCF after 3 h. ProAKAP4 correlates positively with progressive motility is attributed to its role as a reservoir (Delehedde et al., 2019). As proAKAP4 is consumed over time, spermatozoa lose speed due to the depletion of proAKAP4, necessitating its presence to maintain forward movement, even if the initial correlation is not significant.

Regarding mitochondrial activity assessed by the JC-1 probe and its correlation with proAKAP4 levels, no significant differences were observed, however the trend of decrease over time is evident when examining Table 1. Further studies are necessary to confirm this observed tendency.

5. Conclusion

ProAKAP4 is identified within the fibrous sheath of donkey spermatozoa, akin to findings in other investigated mammals. Similar to

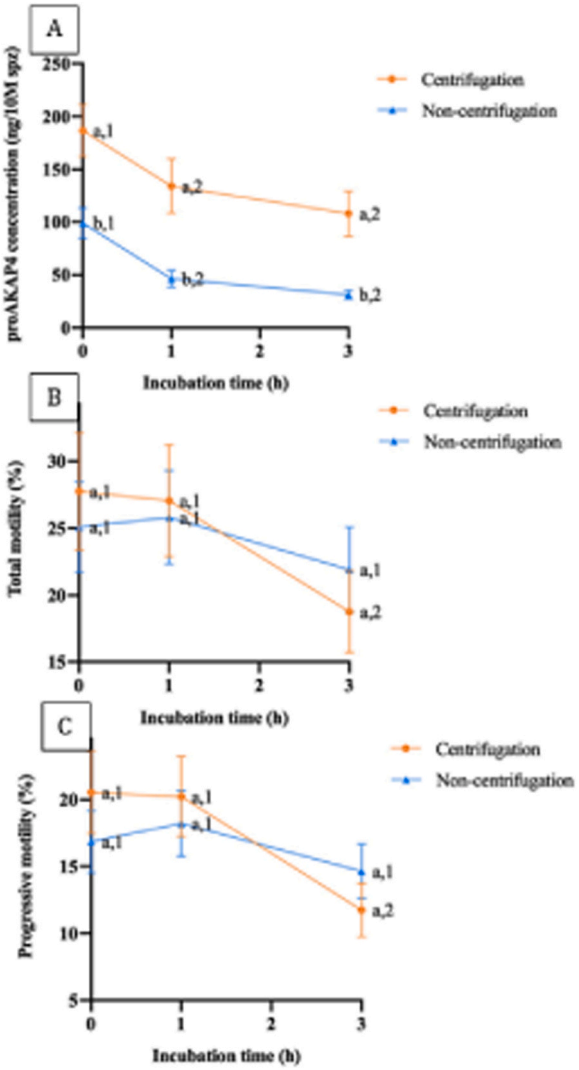

Fig. 4. Evolution of proAKAP4 (A), total motility (B) and progressive motility (C) through the time after thawing maintained at 37 °C expressed by mean ± SEM. Different letters (a,b) show significant differences ($p < 0.05$) between centrifuged (without cryoprotectant) and non-centrifuged (with cryoprotectant) samples inside every time. Different numbers (1,2) show significant differences ($p < 0.05$) between times in the centrifugation.

various species, its presence in donkeys correlates with motility parameters.

In donkey, proAKAP4 functions as a motility reservoir, with a substantial portion of the protein consumed shortly after thawing to sustain motility. However, the decline in motility initiates when proAKAP4 undergoes degradation.

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Declaration of Competing Interest

Maryse Delehedde and Nicolas Sergeant, co-authors of this manuscript, are cofounders of SPQI.

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