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The Effects of Freeze-Thaw Cycles and of Storage Time on the Stability of Proakap4 Polypeptide in Raw Sperm Samples: Implications for Semen Analysis Assessment in Breeding Activities

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Abstract

Evaluation of the concentrations of the sperm macromolecule called proAKAP4, has been successfully introduced as a pertinent sperm parameter to assess sperm quality and high concentrations of proAKAP4 was shown to be highly correlated with sperm motility and fertility in large mammals. The sandwich ELISA kits known as Pig 4MID® Kits allowed the artificial insemination stations to monitor sperm quality more accurately with threshold values qualifying each ejaculate and animal. Introducing new methods and procedures are always challenging and sperm frozen collections have been suggested to standardize sperm assessment in daily routine. We thus have designed an experimental study to assess proAKAP4 stability and integrity in neat frozen ejaculates. Following baseline measurements, fresh ejaculates were aliquoted and stored at -20 °C for stability experiments up to 6 months and following up to 10 freeze-thawing cycles. ProAKAP4 concentrations were assayed at each time point using the Pig 4MID® Kit and western blot. Median or mean changes from baseline concentrations were evaluated statistically. We showed that the frozen storage conditions neither modified the total proAKAP4 concentrations nor changed the degradation rates of the proAKAP4 into mature AKAP4, that should in turn ensures signaling, capacitation and motility. This sperm parameter was shown then to be robust for semen quality analysis on fresh and on frozen neat semen. Taken together, proAKAP4 polypeptide can be considered as a highly stable analyte when kept frozen in raw semen up to the semen quality analysis using the Pig 4MID® Kit.

Keywords: Boar; Proakap4; 4MID®; Fertility; Stability; Precursor; Freeze-thaw cycle; Storage; Semen processing

Abbreviations: AKAP4: A-kinase Anchor Protein 4; PKA: Protein Kinase A; CASA: Computer Assisted Semen Analysis

Introduction

ProAKAP4 concentrations are considered as a new sperm parameter that have been validated by field studies for sperm analysis assessment in large mammals [1-6]. Measurement of proAKAP4 concentrations was thus reported to generate pertinent information to guide the prognosis of sow fertility and prolificity in highly competitive breeding activities [1,4]. This quantitative approach of semen assessment is based on a sandwich ELISA method that allow to compare up to 87 semen simultaneously and is commercialized under the brand name of the 4MID® Kits (4BioDx, France). The Pig 4MID® Kits provide then a reliable and valuable figure reflecting the amount of proAKAP4 in pig ejaculates, with threshold values that are allowing a follow-up of the sperm quality inside and between pig breeding centers.

Structurally, proAKAP4 is a precursor protein and will have to be converted by motile and alive spermatozoa in AKAP4 (A-kinase anchor protein 4) that in turn, coordinate the main transduction signals regulating sperm motility, capacitation and fertility [1,7-11]. ProAKAP4 concentrations has been reported to be correlated with total and progressive motility in stallion, in human and in bull [2,3,6,12,13]. Clearly the proAKAP4 concentrations is a reflect of the sperm motility giving a more objective figure compared to microscopic observations of the spermatozoa that are motile only at the time of analysis. In contrast, with the Pig 4MID® Kit, the more the proAKAP4 concentration is high in the ejaculate, the more the spermatozoa will be motile and efficient to go up to the site of fecundation. They have been evidences that spermatozoa

with few or without proAKAP4 will be less motile or immotile and then infertile [14-18]. Therefore, we considered as essential to determine the stability and integrity of the full-length proAKAP4 in frozen storage conditions before the critical step of the sperm quality analysis. Data concerning the effects of freezing, thawing, and long-term storage effect on sperm proAKAP4 concentrations were not yet available in the literature. In this study, we aimed then to examine the analytical stability of proAKAP4 in fresh boar semen. We then assess the variations of proAKAP4 concentrations and proAKAP4 degradation rates in following freeze-thaw cycles and in long-term storage at minus 20 °C, in a final goal to improve operating procedures for semen analysis in swine breeding centers.

Materials and Methods

Sperm Preparation

Fresh boar sperm samples (Large White strain) were obtained from a boar stud and was first checked for total volume. They were then aliquoted into 1.5-mL polypropylene cryovials for the stability experiment. For stability assessment, the sampling of each ejaculate was then composed of 5 aliquots (2 for freeze-thaw cycle experiment and 3 for long-term storage experiment). Following baseline measurement (T0), they were all maintained frozen until analysis. The remaining boar ejaculates were either processed for the control quality experiment or for proAKAP4 expression controls. Samples stored at -20 °C were kept in a freezer equipped with a temperature recorder.

Freeze thaw Cycles and Long-term Storage Experiments

After 24 hours, 2 frozen sperm aliquots were thawed at room temperature until completely thawed, and then mixed properly with a micropipette before analysis (freeze-thaw 1). Samples were immediately re-frozen at -20 °C. This cycle was repeated for ten consecutive time points (T1, T2, T3, T4, T5, T6, T7, T8, T9, T10) to yield freeze- thaw processing. A group of 3 semen aliquots were stored at -20 °C for up to 1, 3 and 6 months, and then analyzed for stability at three-time intervals (T1M, T3M, T6M). As described below, the concentrations of proAKAP4 were assessed at each time point using the Pig 4MID® Kit (4BioDx, France). In parallel, proAKAP4 expressions and metabolism of the same aliquot were examined by western blotting. The results were compared with those obtained from the initial analysis of fresh samples. Median or mean changes from baseline (T0) concentrations were evaluated statistically.

Spermatozoa and Seminal Plasma Preparation from Boar Semen

A volume of 500µL of the remaining fresh semen was added in a 1.5mL Eppendorf tube and then centrifuged during 10 min at 2000rpm. The supernatant over the spermatozoa pellet was recovered with a 200µL micropipette and corresponded to the seminal plasma fraction. One volume of Tris Buffer (10 mM Tris HCl pH 6.8) with 2% SDS was added to the seminal plasma and sonicated at 22kHz (15 Watts) for 30 seconds. In parallel, 250µL of Tris Buffer with 2% SDS was added to the spermatozoa pellet, mix thoroughly with a vortex and then sonicated for 30 seconds (22 kHz, 15 Watts). Protein concentrations were determined using the Bradford's method (BioRad, France). Then 50µL of the Tris-SDS sample was added to 1 volume of 2x concentrated NuPAGE LDS Sample Buffer (ThermoFisher, USA) and 10µL of NuPAGE Sample Reducing Agent (ThermoFisher, USA). Samples were vortexed and heated at 80 °C for 10 min.

Analysis of pig ProAKAP4 Expression and Metabolism by Western Blot

Equal protein concentrations of each sperm preparation were loaded on polyacrylamide gel (4-12% NuPage Precast Gels) and then transferred onto 0.45µm nitrocellulose membranes (G&E Healthcare, USA) using the Liquid Transfer System (Life Technologies, USA). Membranes were incubated overnight at 4 °C with the first antibody at a dilution of 1:4000 in 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20 (TNT Buffer), either with the clone 7E10, a monoclonal antibody anti-AKAP4 (4BioDx, 4BDX-1602, France), or with the clone 6F12, a monoclonal antibody anti-proAKAP4 (4BioDx, 4BDX-1701, France). After washing 3 times in TNT (10 min), each membrane was incubated with the secondary anti-mouse antibody coupled to horseradish peroxidase at 1:50000 diluted (Vector Laboratories, Burlingame, CA USA) and revealed with the ECL™ chemioluminescence kit (G&E Healthcare, USA). Images were acquired using the Image Quant™ LAS 4000 system (G&E Healthcare, USA).

The Pig 4MID® ProAKAP4 ELISA Assays

Thawed semen samples (respectively 50, 25 and 12µL) were mix with the Pig Lysis Buffer (450, 475 and 488µL) and then proceeded for ELISA quantification using the Pig 4MID® Kit (4VDX-18K2) according to the manufacturer's instructions (4BioDx, France). Briefly, 100µL of semen lysates was then added to each well of the antibody-coated plate. A solution with conjugated proAKAP4 antibody was then added and after appropriate washing, the complexed sandwich was incubated with a substrate solution. The resulting color intensity was proportional to the amount of proAKAP4 present in each semen sample and could be measured by spectrophotometry at 450nm. A standard curve was determined in parallel for precise concentrations of proAKAP4 in the pig semen sample. Results of proAKAP4 concentrations were always expressed in ng/mL.

Statistical Analysis

Statistical analysis was achieved using Prism 8.2 GraphPad software (GraphPad Software, USA). D'Agostino and Pearson normality tests were performed to determine if the populations were following a Gaussian distribution and Pearson correlation coefficients were determined for each proAKAP4 concentration. The threshold for statistical significance was set to be $p<0.05$. In normally distributed groups, results were presented as mean ± standard deviation. The significant differences from T0 value were determined by a non-parametric paired samples t-test Mann-

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Whitney U-test. Stabilities of proAKAP4 after freeze thaw cycles and after long term storage were assessed by the percentage change from T0 for paired groups (T0-T1, T0 -T2, etc. and T0 – T1M, T0 -T3M, etc.). Bias was calculated by the formula: [(CX - C1)/C1] × 100%, with C1: the mean or median of the T0 sample; and Cx: the mean or median of the experimented sample. For non-Gaussian groups, median variations from T0 were determined by non-parametric Friedman test and Wilcoxon signed rank test.

Results

ProAKAP4 Expression in Boar Raw Semen

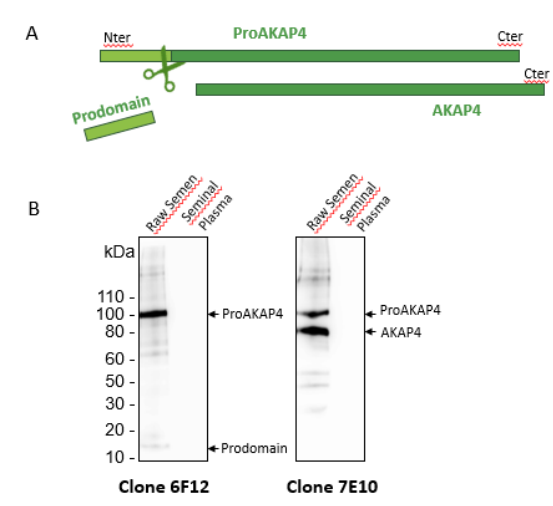

Figure 1: ProAKAP4 Expression and Metabolism in boar.

As observed previously from other mammals [1-3] proAKAP4 was only expressed in spermatozoa preparation and not in the seminal liquid as revealed with the monoclonal antibody (clone 6F12) against proAKAP4 (Figure 1). The proAKAP4 was cleaved in AKAP4 mature protein and the prodomain was released (Figure 1A). This cleavage and metabolism of the precursor proAKAP4 can also be followed by western blotting using specific monoclonal antibodies such as the clone 7E10 which recognized the C-terminus of both proAKAP4 and AKAP4 (Figure 1B). Therefore, in this initial T0 experiment, we observed the same amount of proAKAP4 and AKAP4 in the spermatozoa preparation sample of the fresh pig ejaculate. As expected, we confirmed that proAKAP4 is a spermatozoa specific protein expressed in the flagellum of pig spermatozoa.

Stability of Boar proAKAP4 during Freeze-Thaw Cycles of the Same Aliquot

The concentration of proAKAP4 was measured in the ejaculate using the Pig 4MID® Kit as T0 value for the stability experiments. The initial mean concentration of ProAKAP4 was of 50.7 ± 1.3ng / mL, reflecting a high-quality semen [1]. After semen aliquots have been frozen and thawed up to ten times, there were no statistically significant differences in proAKAP4 concentrations as quantified using the Pig 4MID® Kit from T0 to T10 (Table 1).

Table 1: ProAKAP4 concentrations did not changed significantly during freeze-thaw cycles.

	T0	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
Conc	50.7 ±1.3	48.1±1.1	56.25±4.2	55.66±0.31	55.13±7.5	54.29±3.2	57.81±9	47.32±8	50.21±4.2	47.87±5.4	46.12±5.3
	(49.9-51.6)	(47.6 – 48.5)	(58.3 – 55.1)	(55.5-55.7)	(54.3-55.9)	(51.6-56;9)	(54.3-61.3)	(44.4-50.1)	(46.2-54.2)	(45,3 – 50.4)	(48-44.2)
p	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

All concentrations were in ng /mL and indicated as a mean± SD and median (interquatile ranges). Clearly, the proAKAP4 concentrations were not modified statistically after ten freeze-thaw cycles and the global percentage of variations was at 9.54%. Dilutions of the neat semen (half and quarter dilution factor) had no effect on the recovery of proAKAP4 concentrations as shown graphically on Figure 2. These dilutions highlighted the robustness of the Pig 4MID® Kit to quantify accurately the amount of the proAKAP4 polypeptide in neat pig semen. We checked then the expression and metabolism of proAKAP4 by western blotting (Figure 3). None of proAKAP4 and AKAP4 expressions or metabolisms were altered by the freeze-thaw cycles. Neither the integrity of proAKAP4 or AKAP4 was shown to be altered along the 10 freeze-thawing cycles and proAKAP4 was not further converted

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into AKAP4 showing that proAKAP4 and AKAP4 processing were not modified by freeze thawing cycles. The proAKAP4 was therefore considered as a very stable analyte when kept frozen in raw semen until we performed the Pig 4MID® Kit analysis.

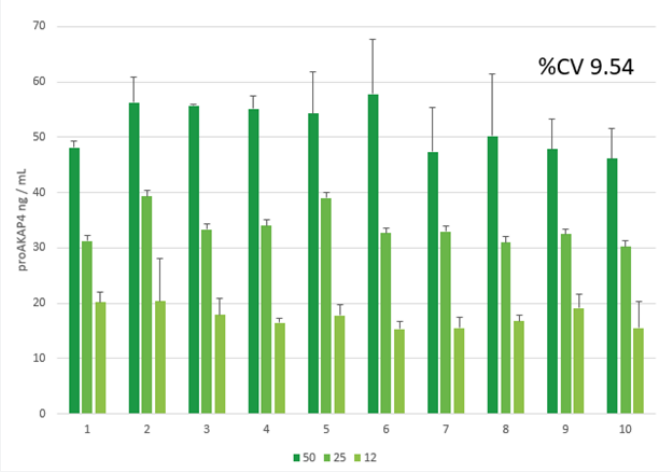

Figure 2: : ProAKAP4 concentrations are stable in semen up to 10 freeze-thaw cycles as measured using the Pig 4MID® Kit – No dilution effect was observed.

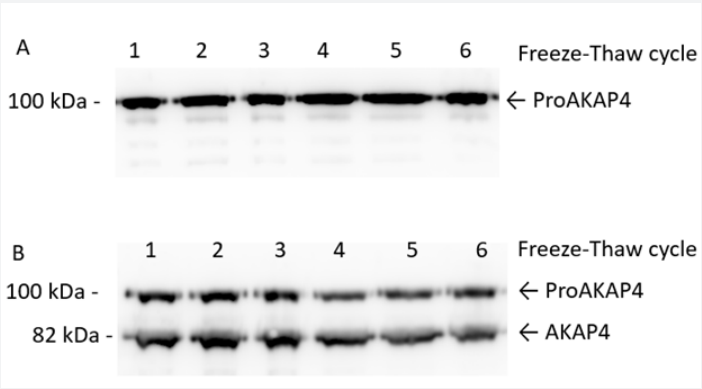

Figure 3: ProAKAP4 stability over 6 freeze-thaw cycles as observed by western blot.

Stability of the Frozen proAKAP4 Polypeptide in long-term Storage Conditions

They were no significant variation in proAKAP4 concentrations as measured with the Pig 4MID® Kit for fresh pig sperm when stored until 6 months at -20 °C (Table 2). No variations were obtained when stored at - 80 °C (data not shown). Statistical significances were evaluated as described in the Materials and methods section. Our results showed that total proAKAP4 concentrations were clearly stable up to six months of storage at -20 °C with the variation in proAKAP4 concentration always below 5%. The western-blot analysis displayed no degradation of the sample stored at -20 °C from up to 6 months highlighting the robustness of the protein when kept frozen in raw semen (data not shown).

Table 2: Stability of frozen proAKAP4 in raw semen up to 6 months. Means and interquartile intervals as well as the % of variations with statistical significance are indicated.

	T0	T1M	T3M	T6M
ProAKAP4 Concentration in ng /mL	50.7 (49.9-51.6)	51 (48.6-53.4)	49.8 (45.9-53.7)	52.1 (48.8-55.4)
% variations	-	0.6% (ns)	-1.77% (ns)	2.7% (ns)

Intra-assay and Inter-Assay of the Pig 4MID® Kit

We further assess the robustness of the Pig 4MID® Kit by evaluation of the intra-assay and inter-assay CV's on the Pig 4MID® Kit with neat pig semen as in the design of our study. These intra-assay and inter-assay CV's were performed with two different ejaculates of the same animal (Table 3). Inter-assay variation was assessed from 10 determinations (with 2 aliquots each day) on ten consecutive study days, and intra-assay variation was calculated from eight sequential determinations obtained from the first day of the study period.

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Table 3: Intra and inter-assay coefficient of variations of the Pig 4MID® Kit.

	Product Name	Method	Intra-assay CV (%)	Inter-assay CV (%)
Pig ProAKAP4	Pig4MID® Kit	sandwich ELISA	2.1	6.2

Discussion

This study examined the storage effects and repeated freeze-thaw cycles on pig proAKAP4 sperm protein integrity in pre-analytical conditions (meaning before the 4MID® Kit procedures) to evaluate the robustness of this new parameter in daily routine of semen analysis for swine breeding activities. We clearly show that proAKAP4 polypeptide is highly stable when frozen at minus 20 °C, for a long time period (up to 6 months) and will not be altered by multiple freeze-thaw cycles in neat semen. These data are of importance as they highlighted for the first time, that specimens of one ejaculate can be aliquoted and kept at minus 20 °C until their analysis and shipped from AI stations to central laboratories without loss of proAKAP4 integrity.

The reason of this stability could be due to the localization and the inherent functionality of the proAKAP4 itself. As shown on Figure 1, the proAKAP4 is a sperm specific protein that is neither found on the membrane nor released in the seminal plasma. The proAKAP4 polypeptide is inside the spermatozoa, more precisely in the fibrous sheath of the principle piece of the flagellum [19-22] and will need to be released from the fibrous sheath to be further quantified using the 4MID® assay. ProAKAP4 has been shown to be strictly localized to the principal piece of the flagellum and not in other spermatozoa compartments [20-21], tightly anchored to the fibrous sheath, along the longitudinal columns and ribs of the sperm tail [2,3,20-21].

According to the Pig 4MID® assay procedure, the proAKAP4 has then first to be extracted from the spermatozoa. Proteins markers described in sera or in seminal fluids [1,23] are frequently reported to suffer from the shear stress induced in buffered solutions and from long-term storage conditions. In contrast of what we reported with sperm proAKAP4, proteins in buffer solution can be fragile and they may even acquire conformations susceptible to degradation during frozen and post-thawed conditions. Clearly, proAKAP4 concentrations appears to be stable as long as the polypeptide is maintained in neat semen within the spermatozoa flagellum, with the fibrous sheath bringing stability for proAKAP4 integrity. The maintenance of proAKAP4 as a full-length precursor is then important for the aliquot processed for the initial quality assessment of the ejaculate and at further steps, for the quality control during dose processing in AI stations. High proAKAP4 concentrations in the ejaculate and then in doses, will ensure to have enough motile and functional spermatozoa populations in the hours post the artificial insemination.

The total amount of proAKAP4 per spermatozoa is fully synthetized within the testis and before ejaculation. Therefore, an aliquot of the ejaculate could be frozen immediately after semen collection in boar studs as this will represent the exact picture of the long-term motility of the spermatozoa. Freezing of an aliquot of ejaculate at collection point will then facilitate the analysis of semen (related to the proAKAP4 concentration) and favors also transport of such aliquot up to external laboratories. Our results clearly showed that degradation rates of the proAKAP4 were not impacted by frozen storage conditions of the aliquot and are in favor of such collection for delocalized sperm quality assessments.

Furthermore, proAKAP4 stability when stored in aliquots in sperm frozen collections, will allow to better take in account technical and logistical constraints such as i) delays in shipping frozen aliquot when in need to analyze hypofertile animal; ii) being less dependent of any power cut or voltage fluctuations of the low-cost freezers; or the use of frost-free freezer that goes through numerous defrost cycles, as may happen in small breeding centers.

In boar stud, the storage of frozen aliquoted samples could also be convenient to process all the semen in the same time to compare ejaculates of different animals at the end of collection time. The dose semen processing will then not be impacted as the 4MID® analysis will be completely run in 2 hours. The amount of proAKAP4 as a read out of sperm quality should add marketing values for AI stations by ensuring high quality semen. In swine industry, there is also a real interest to identify the best male and then to follow up the sperm production during exploitation. Boars are usually kept from 6 to 9 months in the AI stations. That will be of importance to have a stable parameter to follow animal along all his career and keeping a safe measure of the initial quality of the first ejaculate after quarantine. In this context, the storage of frozen aliquoted samples may allow likewise to identify genetical traits of interest in a particular pig strains, such as fertility, death at birth or litter size, that may be related to proAKAP4 levels of expression [1,24-27].

Finally, keeping frozen aliquots of pig semen could allow to reanalyze the same samples stored to confirm previous results or to perform additional analysis, establishing new path for boar sperm preservation investigations. Better understanding proAKAP4 stability allows now to compare ejaculates at different collection points and compared to extended semen which is being shipped and used many days later. The storage capacity of extenders should be then further explored in relation with proAKAP4 consumption and degradation rates, during several days and in chilled conditions, when spermatozoa will stay alive.

Conclusion

One of current challenge of the swine industry is to standardize the semen processing procedures within boar studs. The proAKAP4 parameter have been initially introduced to facilitate the identification of ejaculates of inferior motility and quality, that were not identified by classical sperm parameters, and that

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could then be withheld before their release into the field. Having a stable sperm parameter such as proAKAP4 that can be kept stable as frozen up to the analysis time should be further interesting for quality check control and to follow up this parameter evolution during all the boar career. Taken together, the proAKAP4 parameter stability present then multiple advantages in favor of harmonizing sperm quality assessment between laboratory and AI centers.

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Conflict of Interest

NS and MD are co-founders of SPQI, a spin-off company (Lille, France).

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