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SHORT COMMUNICATION: CAN proAKAP4 BE USED AS A PREDICTOR OF RABBIT SPERM FREEZABILITY?

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Abstract: This study aimed to evaluate the suitability of proAKAP4 —a structural protein associated with flagellar integrity— as a potential biomarker for predicting sperm quality after thawing, in the context of establishing a gene bank for Slovak national rabbit breeds. A total of 48 samples from 8 males of the Chrabran rabbit breed were collected and analysed. ProAKAP4 concentration was measured using ELISA (Rabbit 4MID Kit) in fresh ejaculates prior to cryopreservation. Native and post-thaw sperm quality was assessed via computer-assisted sperm analysis, focusing on total (TM) and progressive (PM) motility, as well as secondary motility parameters. To support motility-based findings, flow cytometry was additionally applied, employing DRAQ7, YO-PRO-1, Caspase 3/7, and FITC-PNA to evaluate sperm viability, apoptotic activity and acrosomal integrity. ProAKAP4 concentration in fresh semen showed a moderate negative correlation with post-thaw motility traits (r=–0.67 for % TM after freezing and thawing (F/T); r=–0.59 for % PM F/T). These findings suggest that proAKAP4 may hold potential as a predictor of sperm freezability in rabbits, but its relationship to post-thaw quality appears to differ from that observed in other species. The recorded moderate negative correlation implies a species-specific mechanism, possibly linked to differences in sperm structure or response to cryostress. Further research is needed to clarify the functional role of proAKAP4 in rabbit sperm preservation.

Key Words: rabbit, sperm, cryopreservation, biomarker, genetic resource conservation, biodiversity.

INTRODUCTION

The preservation of genetic resources in livestock is a key element in maintaining biodiversity. One of the most effective strategies for conserving animal genetic diversity is sperm cryopreservation, enabling the long-term storage of male gametes in gene banks. However, the selection of high-quality ejaculates for cryobanking can be particularly challenging in genetically valuable but low-yielding local breeds. Therefore, identifying reliable biomarkers that can efficiently predict post-thaw sperm quality is essential.

In rabbits, sperm cryopreservation protocols remain inconsistent across studies, with post-thaw motility and viability outcomes ranging widely —from 10% to 60% for total motility (TM) and 5% to 45% for progressive motility (PM)— highlighting the need for improved selection criteria and standardised procedures (Di Iorio *et al.*, 2024, 2025). Work on Slovak native breeds further emphasises that breed- and male-dependent variability affects post-thaw sperm performance, and that objective assessments of fresh semen (e.g., total motility) may help predict frozen-thawed quality (Kulíková *et al.*, 2017).

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In this search for predictive markers, the sperm structural protein proAKAP4 has emerged as a promising candidate. Thus, proAKAP4, the precursor of AKAP4 (a major component of the fibrous sheath in sperm flagella), plays a vital role in motility, and its levels correlate with sperm competence across several species (Carracedo *et al.*, 2022; Dordas-Perpinyà *et al.*, 2022). In particular, studies in bulls have demonstrated strong positive correlations between proAKAP4 concentrations, post-thaw motility parameters and fertility outcomes (e.g., non-return rate) (Bastan & Akcay, 2021; Dordas-Perpinyà *et al.*, 2022). Conversely, in laboratory mice, this relationship was absent or even negative, underscoring possible species-specific differences in proAKAP4 dynamics (Boersma *et al.*, 2022).

Given these inter-species discrepancies, the present study investigates the utility of proAKAP4 as a predictive biomarker for post-thaw sperm quality specifically in rabbits, within the broader framework of creating a sperm gene bank for Slovak national breeds. We hypothesised that proAKAP4 levels measured in fresh ejaculates might correlate with sperm motility and structural integrity after thawing, and thus compared their potential with traditional CASA and cytometric markers.

MATERIAL AND METHODS

Animal management

Eight clinically healthy Chrabran rabbit bucks, aged 12 to 18 mo, were included in the experiment. The animals were housed individually at the breeding facility of the NPPC – Research Institute for Animal Production (RIAP), Nitra, Slovak Republic, under controlled environmental conditions. All rabbits were fed a standard commercial pelleted diet and water was provided *ad libitum*. Animal handling and experimental procedures were carried out in compliance with the Slovak Animal Protection Regulation RD 377/12, harmonised with EU Directive 2010/63/EU and approved by the Ministry of Agriculture and Rural Development of the Slovak Republic (Permit No. SK U 18016).

Semen collection, processing and initial assessment

Semen was collected twice weekly, using an artificial vagina (IMV Technologies, L'Aigle, France) filled with water at approximately 50°C, and collected into sterile tubes. Samples exhibiting visible contamination were excluded from further processing. Ejaculates were transported to the laboratory at room temperature (RT), where volume, sperm concentration and motility parameters were promptly evaluated using a computer-assisted sperm analysis (CASA) system.

Following the motility assessment, each ejaculate was divided into aliquots: one portion was used for proAKAP4 quantification via enzyme-linked immunosorbent assay (ELISA), a second for flow cytometric analysis, and the remaining volume was diluted at a 1:4 ratio (sample:medium) using OptiXcell cryoprotective medium (IMV Technologies, L'Aigle, France). The diluted semen was subjected to an equilibration period of 1 hour at 5°C (in a refrigerator). Cryopreservation was carried out by exposing the equilibrated straws to liquid nitrogen vapour in a specialised freezing box, ensuring gradual cooling before immersion in liquid nitrogen for long-term storage. After thawing, sperm samples underwent repeated CASA analysis and flow cytometric evaluation to assess post-thaw functional and structural sperm quality.

Computer-Assisted Sperm Analysis

Sperm motility and kinematic parameters were assessed using a CASA system (CASA; SpermVision, Minitube, Tiefenbach, Germany) in combination with a phase contrast microscope (AxioScope A1, Zeiss, Oberkochen, Germany) at 200× magnification and a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Prior to analysis, semen samples were diluted with saline (0.9% NaCl; Braun, Nuaille, Germany) in a 1:20 (sample/saline) ratio and analysed under standard conditions recommended by the software for rabbit semen.

The system provided measurements of total motility (TM), progressive motility (PM) and sperm concentration, along with secondary motility parameters (curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), etc.).

Flow cytometry

Flow cytometric analysis was used to evaluate sperm membrane integrity, apoptotic activity and acrosomal status. All assays were performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon ion laser and red-diode (635 nm) laser. For each sample, 1×10^6 spermatozoa were suspended in 100 µL phosphate-buffered saline (PBS) and incubated with specific fluorescent probes. Early apoptotic changes were detected using YO-PRO-1 iodide nuclear green dye (0.1 µM; Thermo Fisher Scientific, Waltham, MA, USA), with samples incubated for 15 minutes at RT in the dark. Apoptotic activity was further evaluated using the Caspase-3/7 (CellEvent™; Thermo Fisher Scientific, Waltham, MA, USA); spermatozoa were incubated with 2 µM reagent for 15 minutes at 37 °C in the dark. The acrosomal status was determined using 10 µg/mL FITC-conjugated peanut agglutinin (FITC-PNA; Thermo Fisher Scientific, Waltham, MA, USA), with incubation carried out for 15 minutes at RT °C in the dark. To assess membrane-compromised or dead spermatozoa, samples were stained with DRAQ7, a far-red fluorescent nucleic acid dye (3 µM; BioStatus Limited, Shepshed, UK) (after the incubation with primary dyes) and incubated for 10 minutes at RT in the dark. Cells positive for YO-PRO-1, caspase 3/7 and FITC-PNA were evaluated separately, but also together as "damaged cells".

Immediately after staining, the samples were analysed on the flow cytometer (at least 10 000 events per sample). Appropriate gating strategies were applied to exclude debris and sperm aggregates. The data were processed using the Cell Quest Pro™ software (BD Biosciences, San Jose, CA, USA).

ELISA (proAKAP4 quantification)

ProAKAP4 concentration in fresh ejaculates was quantified using the Rabbit 4MID® ELISA kit (4BioDx, Lille, France) according to the manufacturer's instructions. After initial CASA assessment, aliquots corresponding to around 50×10^6 spermatozoa per mL were pelleted by centrifugation, washed in PBS and lysed in the kit-provided extraction buffer to obtain sperm protein extracts. Lysates were stored at –80°C until analysis. All samples were assayed in duplicate on microplates supplied with the kit. Optical densities were read at 450 nm and concentrations interpolated from a standard curve. Final results are reported as ng proAKAP4 per 10 million spermatozoa (ng/10M) after correcting for extraction volume and dilution factors.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Associations between predictive markers (proAKAP4 levels and motility parameters in fresh semen) and sperm quality parameters after the cryopreservation process were evaluated using Spearman's rank correlation coefficient, based on a dataset of 48 semen samples. Results were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Correlation analysis revealed no statistically significant association between proAKAP4 concentration and motility parameters in fresh semen. In contrast, a significant moderate negative correlation was observed between proAKAP4 concentration and motility after cryopreservation (TM F/T: $r=–0.6694$, $P<0.0001$ and PM F/T: $r=–0.5896$, $P<0.0001$; Figure 1). This opposite trend compared to most other species suggests a possible species-specific mechanism in rabbits, which may limit the direct practical use of proAKAP4 for routine sample selection without further validation. Among secondary motility parameters, a significant negative correlation with proAKAP4 was detected only for VCL F/T($r=–0.5574$, $P=0.0002$) and VSL F/T ($r=–0.5089$, $P=0.0008$). Correlations for other secondary motility traits were weak or absent (data not shown). In comparison, progressive motility prior to freezing showed positive moderate association with post-thaw PM ($r=0.6560$, $P<0.0001$) and positive moderate association with post-thaw TM ($r=0.5542$, $P=0.0003$; Figure 2.).

Flow cytometric assessment revealed several significant associations between proAKAP4 concentration, CASA motility traits and sperm functional parameters. In fresh semen, proAKAP4 levels showed a moderate positive correlation with the proportion of sperm with damaged acrosomes detected by FITC-PNA ($r=0.5573$, $P=0.0047$). Fresh TM and PM were positively correlated with the proportion of viable spermatozoa (TM: $r=0.4846$, $P=0.0164$;

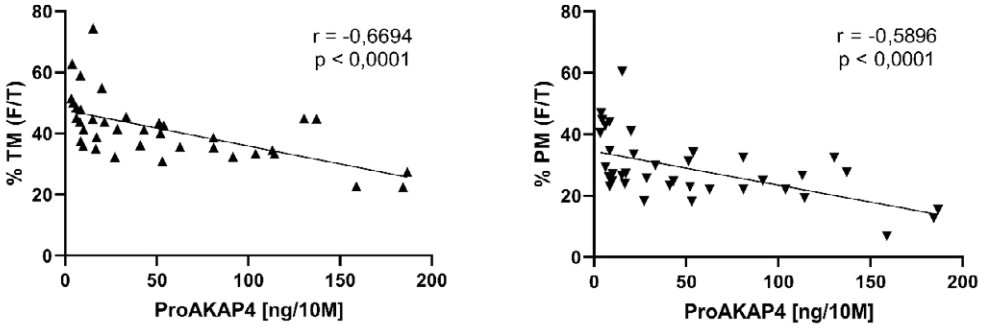

Figure 1: Correlation between proAKAP4 concentration and motility in post-thawed rabbit semen. ProAKAP4 concentration in ng per 10 million of spermatozoa (ng/10M) and percentage of total and progressive motility (TM and PM) in frozen/thawed (F/T) sperm. Statistically significant at $P<0.05$.

PM: r=0.4789, P=0.0179), and negatively correlated with both PNA-positive cells (TM: r=–0.6691, P=0.0004; PM: r=–0.5738, P=0.0034) and damaged sperm (TM: r=–0.6064, P=0.0017; PM: r=–0.5361, P=0.0069). When post-thaw changes were considered, proAKAP4 concentration in fresh semen was moderately negatively correlated with the proportion of viable sperm (r=–0.4499, P=0.0274) and positively correlated with the proportion of DRAQ7-positive (non-viable) cells (r=0.4751, P=0.0190) after thawing. All significant relationships are depicted in Figure 3.

Together, these findings support PM as a robust, positive predictor of post-thaw sperm quality —more intuitive for field use— while proAKAP4 shows a significant but inverse trend. This suggests a species-specific mechanism in rabbits, where elevated proAKAP4 may signal vulnerability to cryostress, contrasting with its role in other species.

Indeed, in bulls, stallions or dogs, proAKAP4 positively correlates with post-thaw motility parameters and fertility outcomes (Blommaert *et al.*, 2019; Dordas-Perpinyà *et al.*, 2022; Siena *et al.*, 2025). In mice or cats, however, proAKAP4 showed no positive relationship with motility or fertility and even exhibited negative associations with some motility traits (Boersma *et al.*, 2022; Prochowska *et al.*, 2024). Our rabbit data appear to align more with the latter trend, underscoring the importance of species-specific validation.

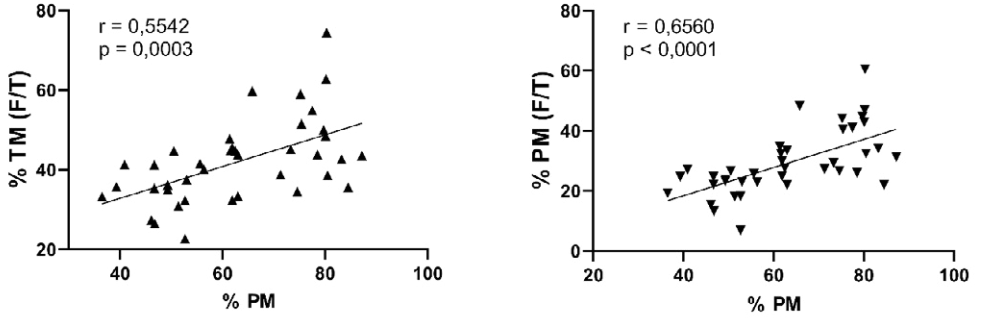

Figure 2: Correlation between progressive motility before freezing (PM) and motility parameters (total (TM) and progressive motility) in frozen/thawed (F/T) sperm. Statistically significant at $P<0.05$.

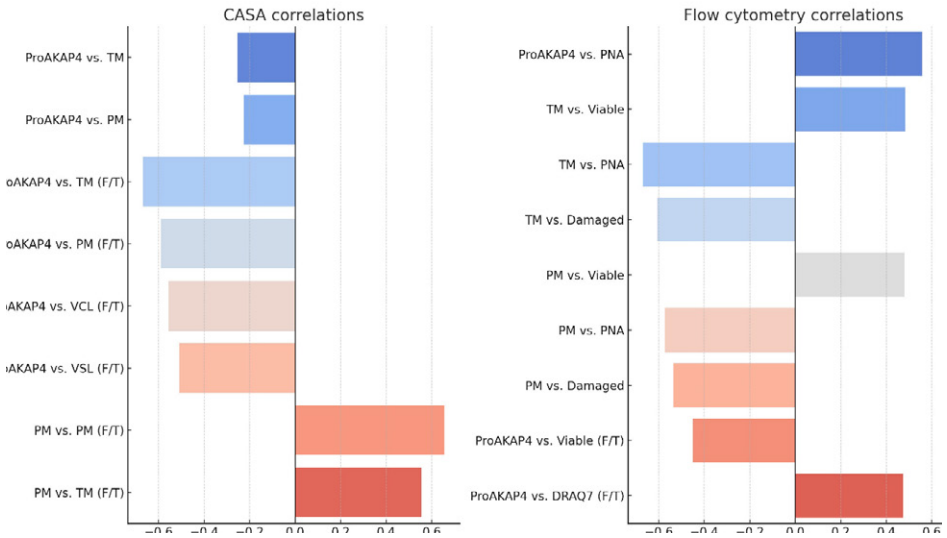

Figure 3: Depiction of all significant correlations. ProAKAP4 concentration in ng per 10 million of spermatozoa (ng/10M), percentage of total and progressive motility (TM and PM) in fresh and frozen/thawed (F/T) sperm, curvilinear velocity (VCL), straight-line velocity (VSL). Statistically significant at $P<0.05$.

CONCLUSION

Progressive motility before freezing is a reliable and intuitive predictor of post-thaw sperm quality in rabbits, with positive correlations to post-thaw motility. While proAKAP4 exhibits significant but inverse associations, its standardised and rapid ELISA-based measurement could be advantageous in large-scale screening programmes, where detailed motility analysis for every sample is impractical. Thus, proAKAP4 may serve as a preliminary selection tool in conservation-oriented cryopreservation, complementing motility-based assessments. Future studies should confirm these findings across breeds and link them to fertility outcomes.

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Conflict of interest: The authors declare no conflict of interest.

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