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ORIGINAL ARTICLE

Influence of sperm cryopreservation on sperm motility and proAKAP4 concentration in mice

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Abstract

Background: The protein proAKAP4 is crucial for sperm motility and has been suggested as an indicator of male fertility. We determined the relationship between proAKAP4 concentration and sperm motility parameters in mice, and investigated the effects of cryopreservation on these variables.

Methods: Computer-assisted sperm analysis and ELISA were applied to determine sperm motility and proAKAP4 concentration in fresh and frozen-thawed epididymal sperm of SWISS, B6D2F1, C57BL/6N, and BALB/c mice.

Results: ProAKAP4 levels ranged between 12 and 89 ng/ml and did not differ between fresh and frozen-thawed samples, or between strains. We found a negative relationship between proAKAP4 levels and some sperm motility parameters. Sperm traits differed between strains, and cryopreservation negatively affected sperm velocity but not sperm direction parameters.

Conclusion: ProAKAP4 levels in epididymal mouse spermatozoa were unaffected by cryopreservation, highlighting the robustness of this parameter as a potentially time-independent marker for sperm motility and fertility. The high individual variation in proAKAP4 levels supports the potential role of proAKAP4 as a marker for sperm quality, though we found no positive, and even negative relationships between proAKAP4 levels and some sperm motility parameters. Future studies have to investigate the significance of proAKAP4 as an indicator for fertility in mice.

KEYWORDS
computer-assisted sperm analysis, cryopreservation, mice, proAKAP4, sperm motility

1 | INTRODUCTION

Laboratory mice are considered the model organism of choice for studying human diseases, as mice share 99% of their genes with humans, and a huge number of disease models can be generated with modern genetic modification techniques. Furthermore, mice are easy to maintain and breed in captivity.[1,2] Today, an enormous number of different inbred, hybrid, and outbred mouse strains—including genetically modified mouse lines—are available and used in research laboratories worldwide. The generation of these models mainly relies on assisted reproductive techniques, like embryo transfer, sperm or embryo cryopreservation, and in vitro fertilization (IVF).

A thorough understanding of the processes underlying male fertility and in particular sperm function will improve the efficiency of these techniques, thereby lowering the number of animals required, which is in line with Russel and Burch's principles of 3R, i.e., the replacement, reduction, and refinement of animal experiments.[3]

In recent decades, epidemiological studies have been reporting a rise in human infertility rates with male subfertility being the reason in approximately 40%–50% of childless couples.[4] Most of the progress in understanding the genetics and pathophysiology of human infertility has come from the analysis of mouse reproductive physiology and genetics.[4,5] Reduced sperm motility (asthenozoospermia) is one of the predominantly contributing factors to male infertility and therefore it is crucial to understand the mechanisms that regulate flagellar movement.[6]

Proteins from the A-kinase anchoring protein (AKAP) family provide the molecular basis for sperm motility. These proteins are responsible for mediating signal transduction downstream of cell surface receptor activation and regulate signal transduction and signal termination in a convergence of signaling pathways.[7,8] Especially the AKAP4 protein (formerly known as p82 and AKAP82) plays a crucial role in sperm movement.[9] AKAP4 is sperm-specific and located in the fibrous sheath, a unique cytoskeletal structure surrounding the axoneme and outer dense fibers in the principal piece region of the flagellum.[10] The fibrous sheath influences the degree of flexibility, the plane of flagellar motion, and the shape of the flagellar beat. In mouse sperm, almost half of the protein isolated from fibrous sheaths is AKAP4, whereas in humans AKAP3 (formerly known as AKAP110) is predominant.[11] Akap4 knockout mice are infertile, the motility of their sperm is poor, the principal piece of the flagellum is reduced in diameter, and the fibrous sheath is incompletely developed.[12,13]

AKAP4 is synthesized as a precursor called proAKAP4 (formerly known as proAKAP82) in round spermatids and processed to the active form AKAP4 in condensing spermatids.[8] It has been hypothesized that proAKAP4 and fibrous sheath proteins are transported to the axoneme where AKAP4 drives the correct fibrous sheath folding.[14] Two clinical studies analyzing the proteomic profiles of spermatozoa in patients with idiopathic infertility, i.e., normospermia according to WHO criteria,[15] but complete failure of fertilization in IVF found that AKAP4 and proAKAP4 were among several proteins down-regulated.[16,17] In a similar study, using bovine spermatozoa as a model for human fertility, Peddinti et al.[18] analyzed the proteome of bull sperm, which was classified as having either a high or low fertilization ability. Among 125 putative biomarkers of fertility, expression of AKAP4 was significantly increased in high fertility spermatozoa. These proteomic studies indicate that AKAP4 and proAKAP4 can potentially be used as biomarkers to study the mechanisms of infertility.[19,20]

Recently, several studies have directly investigated the link between proAKAP4 and sperm quality. In stallions,[21] bulls,[22] and dogs,[23] the proAKAP4 concentration of sperm samples was positively correlated with sperm motility and other sperm kinematic parameters investigated, suggesting that proAKAP4 levels could be uniformly used as a marker for sperm quality and potentially even male fertility.[22,24] The roles of both proAKAP4 and AKAP4 in mice have been studied in the context of oxidative stress,[25] but to our knowledge, no study has investigated the relationship between proAKAP4 concentration and sperm quality in laboratory mice, the most commonly used model organism in biomedical research. Given the broad application of sperm cryopreservation and in vitro fertilization in mice, a reliable sperm quality marker would allow for selecting highly fertile males as sperm donors, thereby reducing the number of females needed and contributing to 3R.

Given that artificial reproduction technologies often use cryopreserved sperm, it is crucial to understand whether and how freezing affects sperm quality and proAKAP4 concentration alike.

Some studies have investigated the effect of sperm freezing on proAKAP4 concentration and found contradicting results. In ram, the proAKAP4 concentration of ejaculated and extended sperm declined with freezing,[26] whereas in boar the proAKAP4 concentration of ejaculated and undiluted sperm did not change after multiple freeze-thaw cycles, indicating that proAKAP4 is a stable protein that is not degraded during the freeze-thaw cycles or when stored frozen.[27] Recently, specific cryopreservation media have been shown to positively affect proAKAP4 concentration and sperm quality in frozen-thawed samples,[28,29] suggesting that proAKAP4 concentration is variable and can depend on cryopreservation conditions. More studies are needed to investigate whether and how cryopreservation affects proAKAP4 concentration in sperm to be able to use proAKAP4 as a reliable fertility indicator.

Here, we determined proAKAP4 concentration and various sperm motility parameters measured by computer-assisted sperm analysis (CASA) in fresh and frozen-thawed mouse sperm samples to (i) assess the relationship between proAKAP4 levels and sperm motility, and (ii) analyze the effect of sperm freezing on both proAKAP4 levels and sperm motility. We performed this study with four commonly used laboratory mouse strains, SWISS, B6D2F1, C57BL/6N, and BALB/c to be able to test for strain-specific differences in proAKAP4 concentration and to gain a better estimate of how proAKAP4 concentration and sperm motility are related in this species.

2 | MATERIAL AND METHODS

2.1 | Experimental animals and ethical statement

Twelve males each from the following mouse strains, RjOrl:SWISS, B6D2F1Rj, C57BL/6NRj, and BALB/cByJRj were purchased as sperm donors from Janvier Labs, France. Males were specific pathogen-free and between 6- and 8-week-olds at arrival. All males were ordered at a specific date and spent a variable habituation period (19–36 days) so that they had the same age (10.7–11.1 weeks) at the time of dissection. Two SWISS mice died during the habituation period for unknown reasons. All animals were kept in pairs in Makrolon® filter top cages type III (425 × 276 × 153 mm)

containing wood granulate bedding (SAFE® select, SAFE Complete Care Competence), nesting material (Pur-Zellin® tissue swabs, Paul Hartmann GmbH) and enrichment (GLP Fun Tunnels Standard, LBS Biotechnology). Housing conditions were a 15L:9D light cycle (lights on 0000–1500) and a constant temperature of 22 ± 1°C with free access to food (mouse maintenance diet, ssniff Spezialdiäten GmbH) and water.

2.2 | Reagents and media

All media were prepared in-house using ingredients purchased from Sigma-Aldrich, Austria, unless otherwise stated. For sperm cryopreservation, a cryoprotective agent (CPA) was used containing 18% raffinose pentahydrate, 3% skim milk (BD BBL™/Difco™, Fisher Scientific, Austria), and 100 mM L-glutamine.[30] To dilute the sperm for sperm quality analysis, a TYH medium was used, a modified Krebs-Ringer bicarbonate solution. TYH consists of 119.37 mM sodium chloride, 4.78 mM potassium chloride, 1.71 mM calcium chloride, 1.19 mM magnesium sulfate heptahydrate, 1.19 mM potassium dihydrogen phosphate, 25.07 mM sodium bicarbonate, 5.56 mM D+ glucose, 0.5 mM sodium pyruvate, Penicillin G (7.5 mg/100 ml), Streptomycin (5.0 mg/100 ml), and polyvinyl alcohol (100 mg/100 ml), and was supplemented with 0.75 mM methyl-beta-cyclodextrin.[30,31]

2.3 | Study design

We determined sperm motility parameters and proAKAP4 concentration in fresh and frozen-thawed mouse sperm samples. First, we performed a pilot trial using SWISS males to establish the workflow for the sperm motility analysis and the commercial ELISA Mouse 4MID® Kit for proAKAP4 assessment. Subsequently, we performed the sperm analysis of 12 B6D2F1 hybrid, 12 C57BL/6N, and 12 BALB/c males balancing sample processing for mouse strain.

To evaluate whether cryopreservation affects proAKAP4 concentration, we measured proAKAP4 in fresh and frozen-thawed sperm samples of the same individual. We determined sperm motility parameters immediately after sperm collection and after thawing of cryopreserved sperm using CASA. In addition, we re-assessed sperm motility parameters in fresh and frozen-thawed sperm samples after a 90 min incubation period (37°C, 5% CO_2) in TYH medium to estimate sperm longevity and to be able to determine whether proAKAP4 concentration can be indicative of sperm survival.

2.4 | Sperm collection

Experimental males were killed by cervical dislocation before both *caudae epididymidis* were dissected from each male and transferred into a 300 µl drop of CPA under paraffin oil (NidOil™, Nidacon, Sweden) in a 35 mm petri dish. We cut each *cauda epididymidis* 3–5 times and allowed the spermatozoa to swim out for 10 min. Afterwards, we homogenized the sperm suspension by carefully rotating the dish, before preparing the different aliquots for cryopreservation, proAKAP4 analysis, and sperm motility assessment by CASA.

2.5 | Sperm cryopreservation

The sperm cryopreservation method was based on the protocol published by Takeo and Nakagata[32,33] and modified for the purposes of this study. Four 0.25 ml plastic semen straws (Minitüb, Germany) were filled consecutively with about 100 µl TYH as ballast, one 20-µl, and one 10 µl drop of sperm suspension, all fractions separated by air. The straws were closed by a heat sealer (Polystar®, Rische und Herfurth GmbH), before being placed into a floating device in the vapor phase of liquid nitrogen for 10 min. Then, the straws were plunged into liquid nitrogen and kept there for 4 h until being thawed the same day for analysis.

For thawing, the frozen straws were placed into a water bath (37°C) for 10 min. Then, the sealed ends were cut open and the two sperm drops were released with the aid of a metal rod. One drop was then used for CASA, the other for proAKAP4 assessment in frozen-thawed sperm.

2.6 | Computer-assisted sperm analysis (CASA)

We used CASA (Sperm Class Analyzer®, Microptics SL) to determine sperm motility parameters. The system consists of a microscope (Nikon Eclipse 2000, Nikon) with a 10× objective (Nikon) under negative phase contrast, a high-speed digital camera (Basler acA1300-200uc), and a PC containing the analysis software (SCA® Version 6.5.0.15). Each sperm suspension was diluted 1:20 with TYH medium to reach optimal density for sperm analysis. Three microliter were then loaded on a prewarmed (37°C) Leja® slide (20 µm deep; Leja Products BV) on a stage warmer, before eight randomly selected microscopic fields were captured (frame rate 50 fps; 25 images per field) and analyzed by the software. The total magnification was 494×. All setup parameters were chosen according to the recommendations of the manufacturer. The frame rate is considered as a compromise between an optimal representation of the "real track" and the increased storage requirements along with higher frame rates (up to 200 fps), which have been proposed by some authors.[34,35] The following CASA parameters were measured: curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), path linearity (LIN, linearity of the actual sperm track), path wobble (WOB, departure of actual sperm track from average path), path straightness (STR, linearity of the average path), average lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz). In addition, the motility, defined as the percentage of motile spermatozoa (VCL > 10 µm/s), was determined.

2.7 | ProAKAP4 assessment

ProAKAP4 concentration (ng/ml) in sperm samples was analyzed by sandwich ELISA using the Mouse 4MID Kit (4BioDx) according to the manufacturer's protocol. From each sample, we selected approximately 0.5×10^6 spermatozoa for the analysis. The reagents necessary for sperm lysis and the sandwich ELISA were supplied with the kit. Briefly, freshly prepared or thawed sperm samples were lysed and the sperm lysate was stored at −20°C for up to 1 week. For the ELISA, sperm lysates were thawed and added to 8-well strips precoated with the capture antibody. Following a 3 h incubation with sperm lysate, the strips were washed and incubated with detection antibody for 1 h. Strips were washed again and incubated with a developing and stop solution. The optical density of samples was determined at 450 nm using a Power Wave HT microplate spectrophotometer (BioTek Instruments), and sample concentration was calculated using the Gen5TM software (BioTek Instruments). Samples, controls, and the standard curve were all analyzed in duplicates.

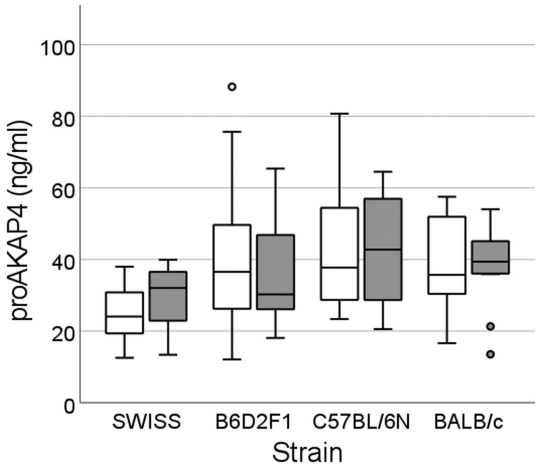

FIGURE 1 ProAKAP4 level in sperm samples before and after cryopreservation. ProAKAP4 concentration in fresh (white boxes) and frozen-thawed (gray boxes) sperm samples of SWISS ($N = 10$), C57BL/6N ($N = 12$), B6D2F1 ($N = 12$), and BALB/c ($N = 12$) mice. Circles (○) refer to mild outliers (Q1/Q3 ± 1.5xIQR).

2.8 | Statistical analysis

All statistical analyses were performed in IBM SPSS Statistics 25 and 27. To test for strain-specific differences in proAKAP4 levels in fresh and frozen-thawed sperm samples respectively, we run ANOVAs. To test for differences in the proAKAP4 level between fresh and frozen-thawed samples, we applied a Wilcoxon signed-rank test. To assess the relationships between proAKAP4 and specific sperm traits across all strains in fresh and frozen-thawed samples, we applied Partial correlations and included strain as controlling factor. We confirmed that all data were normally distributed using Kolmogorov–Smirnov tests and transformed data if necessary. We performed Benjamini–Hochberg corrections for multiple testing and accepted a 20% false discovery rate for the respective sets of correlations. Similar to other proAKAP4 studies[36,37] and based on the distribution of proAKAP4 concentrations in our sperm samples, we classified sperm in groups as having a low (below the 20th percentile), medium (between 20th and 80th percentile), or high (above the 80th percentile) proAKAP4 concentration. To test whether sperm traits differ between proAKAP4 concentration groups, we pooled data from strains and run ANOVAs in fresh and frozen-thawed samples, respectively. We applied Tukey HSD as a post hoc test.

To test how strain, sperm freezing, and the 90 min incubation period affected sperm motility parameters, we first performed a Principal Component Analysis (PCA) on the 9 sperm parameters (i.e., motility, VCL, VSL, VAP, STR, LIN, WOB, ALH, and BCF) determined in all samples ($N = 184$ analyses). The Kaiser–Meyer–Olkin measure of sampling adequacy was 0.611, and the Bartlett's test of sphericity was significant ($p < 0.001$), indicating that correlations between sperm measurements were sufficiently large for performing a PCA. Only factors with eigenvalues ≥1 were considered[38,39] and we retained two principal components (PC), which accounted for 80.5% of the total variance. Among the factor solutions, the varimax-rotated two-factor solution yielded the most interpretable solution, and all items loaded highly on only one of the two factors. We then performed a general linear mixed-effects models where we included the scores from the first and second PC as dependent variables and strain, sample type (fresh or frozen), and time of analysis (after swim out or after 90 min of incubation) as fixed factors. Male IDs were included as random factors to control for the repeated measurements within individual sperm samples. Tukey HSD was used as post hoc test. We confirmed that model residuals were normally distributed using the Kolmogorov–Smirnov test and transformed data when necessary.

3 | RESULTS

We did not find any difference in the proAKAP4 concentration of SWISS, B6D2F1, C57BL/6N, and BALB/c mice, neither in fresh ($F = 2.440$, $p = 0.078$, $N = 46$, Figure 1) nor in frozen-thawed ($F = 2.104$, $p = 0.114$, $N = 46$, Figure 1) sperm samples. Also, we found no difference in the proAKAP4 level of fresh and frozen-thawed sperm samples ($Z = 0.508$, $N = 46$, $p = 0.611$, Figure 1). Overall, the proAKAP4 level of fresh sperm samples was in the range of 12.1 to 88.3 ng/ml (mean ± SD: 37.6 ± 17.3 ng/ml), and frozen-thawed samples ranged between 13.4 and 65.4 ng/ml (mean ± SD: 37.0 ± 13.4 ng/ml).

Across strains, the proAKAP4 concentration of fresh samples was significantly associated with specific sperm swimming traits measured immediately after sperm swim out (see Table 1). In particular, we found a significantly negative relationship between proAKAP4 concentration and VSL ($r = -0.328$, $p = 0.028$, $N = 46$, Figure 2A), VAP ($r = -0.297$, $p = 0.048$, $N = 46$), LIN ($r = -0.334$, $p = 0.025$, $N = 46$, Figure 2B), and WOB ($r = -0.335$, $p = 0.024$, $N = 46$, Figure 2C). In frozen-thawed samples, we only detected

TABLE 1 Relationship between proAKAP4 concentration and sperm motility parameters in mice. Correlation coefficients and *p*-values of partial correlations between proAKAP4 concentration and different sperm motility and direction parameters in fresh and frozen-thawed sperm samples measured immediately after swim out. Data from SWISS ($N = 10$), B6D2F1 ($N = 12$), C57BL/6N ($N = 12$), and BALB/c ($N = 12$) mice were pooled as strain-specific effects were statistically controlled

Sample	Statistic	MOT	VCL	VSL	VAP	STR	LIN	WOB	ALH	BCF
Fresh	Cor. coefficient	−0.182	−0.184	−0.328	−0.297	0.260	−0.334	−0.335	−0.181	0.047
	p-value	0.230	0.227	0.028*	0.048*	0.084	0.025*	0.024*	0.234	0.760
Frozen-thawed	Cor. coefficient	0.056	−0.103	−0.299	−0.215	−0.378	−0.29	−0.222	−0.088	0.031
	p-value	0.715	0.501	0.046*	0.156	0.011*	0.053	0.142	0.566	0.841

Abbreviations: ALH, average lateral head displacement; BCF, beat-cross frequency; LIN, path linearity; MOT, sperm motility; STR, path straightness; VAP, sperm average path velocity; VCL, sperm curvilinear velocity; VSL, sperm straight line velocity; WOB, path wobble.*Statistically significant correlation after Benjamini–Hochberg correction for multiple testing.

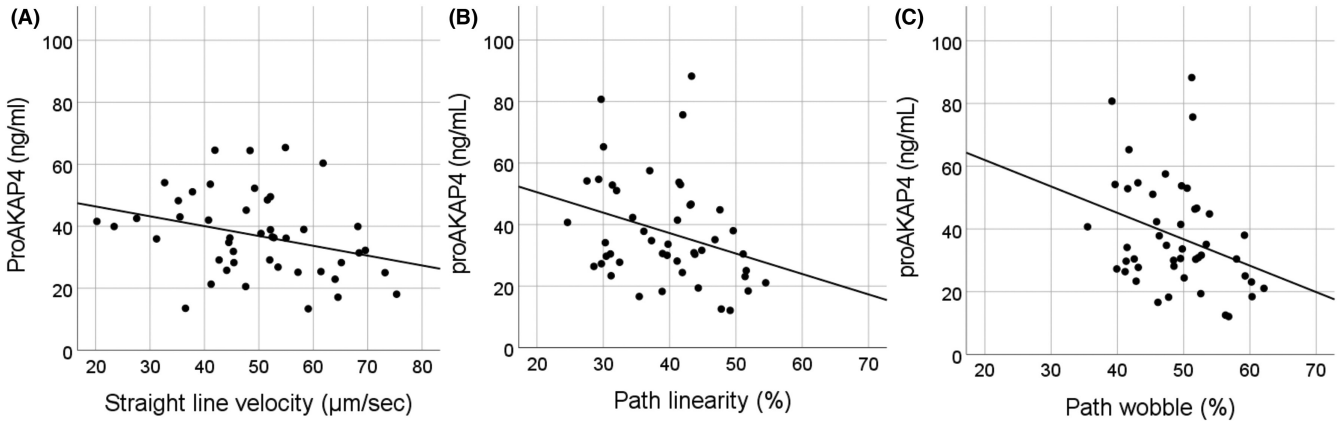

FIGURE 2 Relationship between proAKAP4 level and sperm direction parameters. Relationship between proAKAP4 concentration and (A) straight line velocity (µm/sec), (B) path linearity (%), and (C) path wobble (%) in fresh sperm samples measured after swim out in SWISS ($N = 10$), C57BL/6N ($N = 12$), B6D2F1 ($N = 12$), and BALB/c mice ($N = 12$).

TABLE 2 Relationship between proAKAP4 concentration and sperm motility parameters in mice. Correlation coefficients and *p*-values of partial correlations between proAKAP4 concentration and different sperm motility and direction parameters in fresh and frozen-thawed sperm samples measured 90 min after swim out. Data from SWISS ($N = 10$), B6D2F1 ($N = 12$), C57BL/6N ($N = 12$), and BALB/c ($N = 12$) mice were pooled as strain-specific effects were statistically controlled

Sample	Statistic	MOT	VCL	VSL	VAP	STR	LIN	WOB	ALH	BCF
Fresh	Cor. coefficient	−0.123	−0.222	−0.154	−0.15	0.068	0.136	0.153	−0.204	−0.014
	p-value	0.422	0.143	0.312	0.326	0.655	0.374	0.317	0.180	0.926
Frozen-thawed	Cor. coefficient	0.079	−0.015	−0.092	−0.015	−0.05	0.028	0.059	0.027	−0.004
	p-value	0.604	0.921	0.547	0.923	0.746	0.857	0.698	0.860	0.981

Abbreviations: ALH, average lateral head displacement; BCF, beat-cross frequency; LIN, path linearity; MOT, sperm motility; STR, path straightness; VAP, sperm average path velocity; VCL, sperm curvilinear velocity; VSL, sperm straight line velocity; WOB, path wobble.

two significant correlations between proAKAP4 concentration and sperm swimming traits (Table 1). Again, VSL correlated negatively with proAKAP4 concentration ($r = -0.299$, $p = 0.046$, $N = 46$), and so did STR ($r = -0.378$, $p = 0.011$, $N = 46$). LIN was marginally non-significant ($r = -0.29$, $p = 0.053$, $N = 46$). We found no significant relationship between proAKAP4 concentration and any of the recorded sperm traits after 90 min of incubation, neither in fresh, nor in frozen-thawed sperm samples (see Table 2).

Based on the distribution of individual proAKAP4 concentrations (Figure 3), we classified samples as having either a low (<25 ng/ml), medium (25–50 ng/ml), or high (>50 ng/ml) concentration. In fresh samples, we found that sperm LIN ($F = 4.61$, $p = 0.015$, $N = 46$, Figure 4A), STR ($F = 3.31$, $p = 0.046$, $N = 46$, Figure 4B), and WOB ($F = 4.76$, $p = 0.013$, $N = 46$, Figure 4C) differed significantly between proAKAP4 groups, and low concentration groups always showed significantly higher values compared to high concentration groups (all $p < 0.015$, Figure 4A–C). No differences were detected between the medium and high concentration groups (all $p > 0.115$). Sperm VSL ($F = 3.11$, $p = 0.055$, $N = 46$) and VAP ($F = 2.90$, $p = 0.066$, $N = 46$) were marginally nonsignificant and showed the same distribution

pattern as the sperm direction parameters. In frozen-thawed samples, we found no significant difference between concentration groups in any of the recorded sperm traits.

We found that most measured sperm parameters after swim out were affected by the cryopreservation procedure and declined over the 90 min incubation period (Table 3). Also, strain-specific differences became apparent in some of the observed sperm traits (Table 3). As sperm traits were strongly correlated, we performed a PCA to reduce the dimensionality of the data. We retained two principal components, which accounted for 80.5% of the total variance (PC1: 53% and PC2: 27.5%). PC1, where sperm motility, VCL, VSL, VAP and ALH were heavily loading, was significantly affected by the cryopreservation procedure ($F = 119.204$, $p < 0.001$, $N = 184$, Figure 5B) and the incubation period ($F = 49.368$, $p < 0.001$, $N = 184$, Figure 5C), as both factors significantly reduced the PC scores. Also, PC1 scores differed significantly between strains ($F = 38.102$, $p < 0.001$, $N = 184$, Figure 5A) and BALB/c mice had significantly lower scores than any other strain (all $p < 0.001$). SWISS and B6D2F1 hybrids showed the highest scores and did not differ from each other ($p = 0.233$). C57BL/6N mice were in-between and had scores significantly higher than BALB/c ($p < 0.001$) and significantly lower than SWISS ($p < 0.001$) and B6D2F1 hybrids ($p < 0.001$). PC2, where sperm STR, LIN, WOB, and BCF were heavily loading, was neither affected by strain ($F = 1.780$, $p = 0.153$, $N = 184$, Figure 6A), nor the cryopreservation procedure ($F = 0.946$, $p = 0.332$, $N = 184$, Figure 6B). However, the 90 min incubation time significantly affected sperm traits loading on PC2, as PC2 scores were significantly higher prior to incubation compared to postincubation ($F = 78.847$, $p < 0.001$, $N = 184$, Figure 6C).

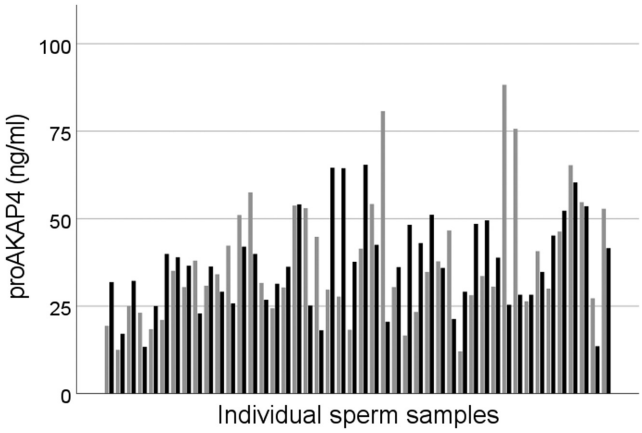

FIGURE 3 ProAKAP4 levels in individual sperm samples before and after cryopreservation. Individual proAKAP4 concentrations in fresh (gray bares) and frozen-thawed (black bars) sperm samples of SWISS ($N = 10$), C57BL/6N ($N = 12$), B6D2F1 ($N = 12$), and BALB/c ($N = 12$) mice. Samples containing proAKAP4 levels below 25 ng/ml were categorized as low concentration; samples containing 25–50 ng/ml as medium concentration and samples above 50 ng/ml were categorized as high concentration.

4 | DISCUSSION

In this study, we describe to our knowledge for the first time the relationship between various sperm motility parameters and proAKAP4 concentration in fresh and frozen-thawed epididymal sperm samples of four commonly used laboratory mouse strains. The range of proAKAP4 concentration was 12.1–88.3 ng/ml in fresh, and 13.4–65.4 ng/ml in frozen-thawed samples. In ejaculated and frozen-thawed bull semen, the proAKAP4 concentration (determined with the same commercially available ELISA kit) ranged between 15.15 and 80.2 ng/ml, with large variation between straws, ejaculates, and bulls.[36] Similarly, in ejaculated sperm of different dog breeds, proAKAP4 concentration ranged between 12.46 and 61.44 ng/ml.[23] This shows that proAKAP4 concentration in epididymal mouse sperm lies in the same range as ejaculated sperm of bulls and dogs. We did not find any strain-specific differences between the proAKAP4 level of SWISS, B6D2F1, C57BL/6N, and BALB/c mice, neither in fresh nor in frozen-thawed sperm samples. This result can likely be explained by the high variation in proAKAP4 levels among individuals within

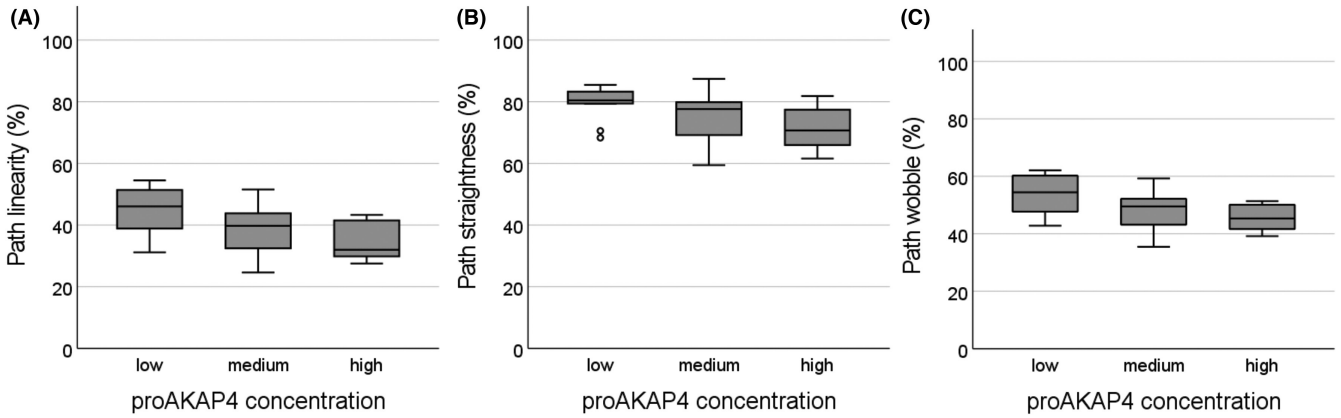

FIGURE 4 Differences in sperm direction parameters depending on proAKAP4 levels. (A) Path linearity (%), (B) path straightness (%), and (C) path wobble (%) in fresh sperm samples of SWISS ($N = 10$), C57BL/6N ($N = 12$), B6D2F1 ($N = 12$), and BALB/c ($N = 12$) mice containing either a low (<25 ng/ml), medium (25–50 ng/ml), or high (>50 ng/ml) proAKAP4 concentration.

TABLE 3 Sperm motility parameters and proAKAP4 concentration in fresh and frozen-thawed sperm samples in four different mouse strains. Mean (±SD) sperm motility and direction parameters in fresh and frozen-thawed sperm samples determined immediately after swim out (T0) and after 90 min of incubation (T1) in SWISS ($n = 10$), B6D2F1 ($n = 12$), C57BL/6N ($n = 12$), and BALB/c ($n = 12$) mice

Strain	Sample	Time	MOT (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	STR (%)	LIN (%)	WOB (%)	ALH (μm)	BCF (Hz)	proAKAP4 (ng/ml)
SWISS	Fresh	T0	50.0 ± 9.3	190.1 ± 28.2	92.9 ± 13.3	107.8 ± 15.1	82.3 ± 2.5	49.3 ± 3.5	57.4 ± 3.5	5.6 ± 1.0	18.3 ± 1.4	25.4 ± 8.0
		T1	37.2 ± 7.4	191.6 ± 14.5	48.4 ± 8.4	74.5 ± 11.4	59.8 ± 4.4	25.4 ± 4.0	39.4 ± 4.1	6.2 ± 0.7	17.0 ± 2.1	-
	Frozen-thawed	T0	13.4 ± 2.7	189.3 ± 26.7	60.7 ± 8.8	80.4 ± 10.5	68.3 ± 4.2	32.0 ± 4.8	43.8 ± 3.9	6.0 ± 0.8	17.3 ± 1.6	29.4 ± 9.3
		T1	10.8 ± 3.3	163.8 ± 24.1	40.4 ± 10.7	61.4 ± 13.0	60.3 ± 8.8	24.6 ± 6.5	37.9 ± 6.5	5.3 ± 0.6	16.9 ± 1.3	-
B6D2F1	Fresh	T0	59.6 ± 6.4	227.9 ± 12.0	95.7 ± 9.9	113.9 ± 9.8	81.2 ± 2.6	42.9 ± 3.0	51.1 ± 2.6	6.9 ± 0.5	18.5 ± 1.2	41.2 ± 22.6
		T1	44.4 ± 6.5	186.6 ± 14.0	49.3 ± 7.7	76.9 ± 7.2	61.1 ± 3.0	27.4 ± 3.0	42.8 ± 3.0	6.2 ± 0.6	15.6 ± 1.0	-
	Frozen-thawed	T0	25.7 ± 5.3	185.9 ± 17.3	57.2 ± 8.6	77.9 ± 7.8	69.7 ± 5.4	33.9 ± 4.1	45.5 ± 3.3	5.9 ± 0.5	17.3 ± 1.4	36.1 ± 13.9
		T1	12.9 ± 4.3	165.7 ± 28.3	42.9 ± 8.4	65.9 ± 6.3	60.8 ± 10.6	28.2 ± 8.2	42.7 ± 6.4	5.3 ± 0.8	16.9 ± 2.1	-
C57BL/6N	Fresh	T0	54.9 ± 12.1	176.7 ± 18.2	48.8 ± 6.8	68.6 ± 6.8	66.6 ± 3.9	30.5 ± 3.2	41.9 ± 3.1	5.4 ± 0.5	18.3 ± 0.8	42.8 ± 17.6
		T1	39.3 ± 6.3	146.3 ± 9.9	36.2 ± 5.4	58.1 ± 6.9	58.6 ± 3.0	27.0 ± 4.2	42.1 ± 4.6	4.9 ± 0.4	16.2 ± 0.9	-
	Frozen-thawed	T0	21.8 ± 6.1	147.7 ± 16.7	43.2 ± 8.2	63.1 ± 8.2	64.6 ± 4.8	33.0 ± 6.3	47.1 ± 6.6	4.9 ± 0.4	17.0 ± 1.3	43.2 ± 15.6
		T1	9.8 ± 3.5	139.7 ± 32.3	37.3 ± 14.2	55.4 ± 14.1	64.1 ± 12.4	30.4 ± 11.4	43.3 ± 8.6	4.6 ± 1.0	16.5 ± 2.7	-
BALB/c	Fresh	T0	48.9 ± 6.9	164.8 ± 16.6	60.8 ± 11.9	76.2 ± 12.5	72.2 ± 4.7	36.7 ± 4.9	46.8 ± 4.0	5.0 ± 0.5	19.5 ± 0.7	39.1 ± 13.0
		T1	23.5 ± 4.7	147.4 ± 24.3	35.0 ± 10.5	54.4 ± 12.8	58.6 ± 5.8	24.5 ± 4.5	38.1 ± 5.2	4.7 ± 0.8	17.6 ± 1.4	-
	Frozen-thawed	T0	13.6 ± 4.4	127.7 ± 36.2	38.8 ± 11.1	53.6 ± 13.9	68.3 ± 6.8	35.2 ± 7.1	47.5 ± 5.9	4.2 ± 1.3	19.0 ± 3.4	38.1 ± 11.4
		T1	5.3 ± 1.2	106.5 ± 42.4	29.5 ± 9.6	41.6 ± 12.4	68.9 ± 10.7	31.9 ± 9.5	42.8 ± 9.0	3.4 ± 1.4	18.2 ± 2.8	-

Abbreviations: ALH, average lateral head displacement; BCF, beat-cross frequency; LIN, path linearity; MOT, sperm motility; STR, path straightness; VAP, sperm average path velocity; VCL, sperm curvilinear velocity; VSL, sperm straight line velocity; WOB, path wobble.

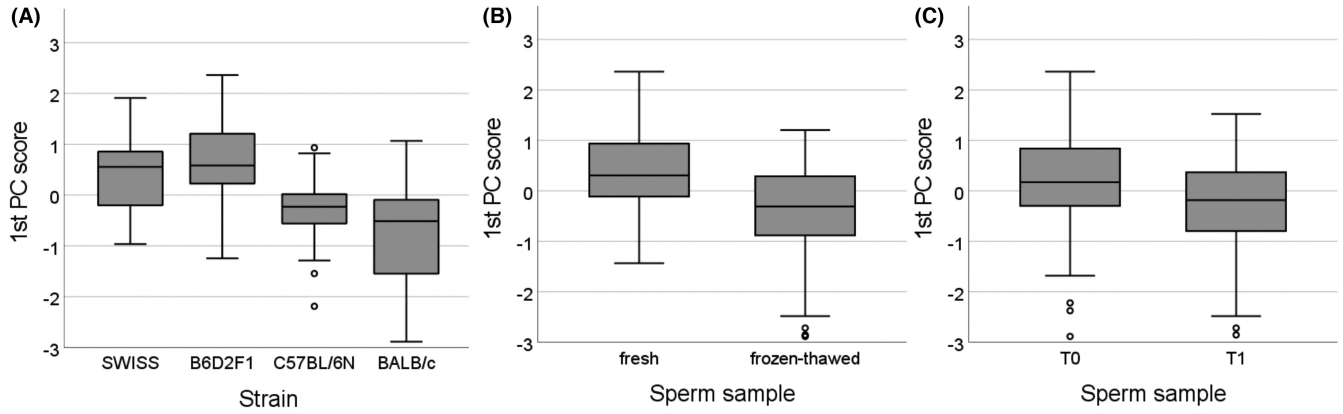

FIGURE 5 Influence of strain, cryopreservation, and incubation time on first PC scores comprising sperm motility, VCL, VSL, VAP, and ALH. (A) First PC scores of SWISS, C57BL/6N, B6D2F1, and BALB/c sperm samples. (B) First PC scores in fresh and frozen-thawed sperm samples. (C) First PC scores in sperm samples measured after swim out (T0) and after 90 min of incubation (T1). $N = 184$ data points in each figure, four measurements (fresh/frozen and T0/T1) from each of the 46 investigated mice across strains. Circles (○) refer to mild outliers (Q1/Q3 ± 1.5xIQR).

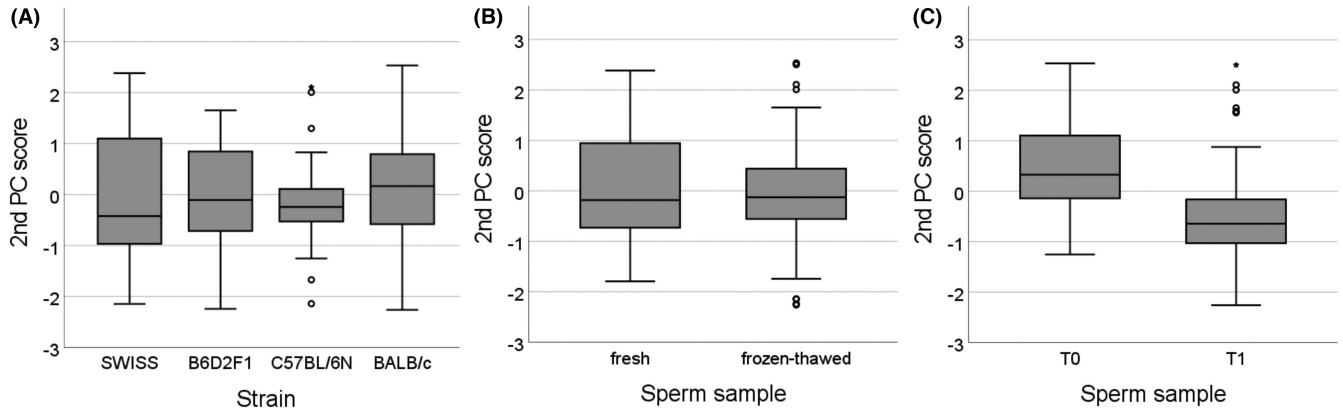

FIGURE 6 Influence of strain, cryopreservation, and incubation time on second PC scores comprising sperm STR, LIN, WOB, and BCF. (A) Second PC scores of SWISS, C57BL/6N, B6D2F1, and BALB/c sperm samples. (B) Second PC scores in fresh and frozen-thawed sperm samples. (C) Second PC scores in sperm sampes measured after swim out (T0) and after 90 min of incubation (T1). $N = 184$ data points in each figure, four measurements (fresh/frozen and T0/T1) from each of the 46 investigated mice across strains. Circles (○) refer to mild outliers (Q1/Q3 ± 1.5xIQR), and asterisks (*) refer to strong outliers (Q1/Q3 ± 3xIQR).

each strain. In fresh sperm samples, some individuals showed values more than 7-fold compared to others, and in frozen-thawed samples proAKAP4 levels differed up to 4.8-fold. This high inter-individual difference potentially qualifies proAKAP4 as a marker for sperm quality and fertilization ability in mice. Two studies in bulls have categorized frozen-thawed sperm samples based on their proAKAP4 levels and found that sperm from the high proAKAP4 group showed better motility[37] and fertility.[36] We also categorized sperm samples as having either a low (<25 ng/ml), medium (25–50 ng/ml) or high (>50 ng/ml) proAKAP4 concentration. However, in frozen-thawed samples we found no difference in sperm traits between concentration groups; and surprisingly, in fresh sperm samples we found that sperm from the low proAKAP4 concentration group showed the highest sperm direction parameters (i.e., LIN, WOB, and STR) and tended to have higher VSL and VAP compared to sperm from the high concentration group. The thresholds we used to classify sperm based on their proAKAP4 level were comparable to the thresholds used in bull sperm.[36] Across mouse strains, we found no difference in the proAKAP4 level of fresh and frozen-thawed sperm samples. We used sperm from the same individuals and measured proAKAP4 concentrations directly after sperm collection and after thawing. In ram, the proAKAP4 level in cryopreserved sperm was lower than in fresh sperm, and a decline in the amount of proAKAP4 was already observed in cooled samples, suggesting that the proAKAP4 protein might be sensitive to declining temperatures.[26] The freezing method used in our study is a method routinely applied for sperm cryobanking in reproduction laboratories[30,40,41] and large mouse repositories, as the European Mouse Mutant Archive.[42] Our results show that the proAKAP4 concentration in mouse sperm, other than in ruminant sperm, is insensitive to the freezing process and that the sperm samples from mouse mutant strains are relevant for biomedical research deposited in these repositories can be used for proAKAP4 analysis. Similar to our result, Dewulf et al.

were able to show that even multiple freeze-thaw cycles have no effect on proAKAP4 levels in boar sperm, pointing to the robustness of this parameter as a potentially time-independent marker for sperm motility and fertility.[27]

ProAKAP4 has been suggested as an indicator for sperm quality and even male fertility in a number of species such as humans,[43] rams,[26] bulls,[18,24,36] stallions,[21] and dogs.[23] Several of these studies reported a positive relationship between proAKAP4 levels and the specific sperm traits investigated. In contrast, we did not find any positive relationship between proAKAP4 and the sperm motility parameters identified by CASA, and even found significantly negative relationships between proAKAP4 and the parameters quantifying the velocity and direction of the sperm track, which were VSL, VAP, LIN, and WOB in fresh sperm, and VSL and STR in frozen-thawed sperm. Even though the respective correlation coefficients were small, this result is in line with our finding that fresh sperm in the low proAKAP4 concentration group shows the highest sperm direction parameters. In general, the parameters LIN, WOB, and STR will be higher in forward swimming sperm and lower in circling or wobbling sperm. Thus, our results might indicate that high proAKAP4 values are associated with spermatozoa swimming less forward and more in circles.

Mouse sperm have a peculiar swimming pattern and swimming in circles is not necessarily a sign of bad motility but can be, for example, an indicator of hyperactivity. In an in vivo situation, i.e., when caught in the pockets of the mucosa of the oviduct, the circling allows sperm to detach from the wall of the oviduct and to escape into the central lumen.[44,45] The movement of hyperactivated spermatozoa involves an increase in flagellar bend amplitude and beat asymmetry and as a result, hyperactivated spermatozoa tend to swim vigorously in circles.[44] Thus, one speculative but possible explanation for our result could be that sperm containing a high proAKAP4 concentration were more likely to be hyperactivated. Hyperactivation usually occurs at some point during capacitation and is a prerequisite for successful fertilization.

It has been hypothesized that proAKAP4 acts as a reservoir for mature AKAP4, and will be converted whenever a high demand for AKAP4 is required.[43] Accordingly, hyperactivation of sperm would be a stage where proAKAP4 has to be converted into AKAP4 in order to fuel sperm motility. If this is true, the time point for measuring proAKAP4 in sperm is crucial, as proAKAP4 concentrations would drop during capacitation and hyperactivation, and thus override or even reverse any relationships found between proAKAP4 concentrations and sperm motility or sperm swimming traits. We measured proAKAP4 concentrations in sperm after swim out from the epididymides, thus it could be possible that the spermatozoa were in the beginning of their capacitation and hyperactivation period. However, to test this hypothesis, several proAKAP4 measurements over the course of a longer sperm incubation time would be required, and we measured proAKAP4 concentrations only once in our study. Future studies are required that investigate the relationship between proAKAP4 concentration and mouse sperm hyperactivation, including the analysis of AKAP4 and proAKAP4 phosphorylation.

AKAP4 is among the most abundant proteins in the epididymis and increases in relative abundance as sperm traverses the epididymal duct from caput via corpus to the cauda epididymidis.[46] It is not only a major component of the sperm fibrous sheath but also among several proteins, which undergo post-translational modifications during epididymal maturation,[47,48] which are clearly linked to sperm function.[49] It would be worthwhile to examine the difference in proAKAP4 and AKAP4 concentration between epididymal and ejaculated spermatozoa, especially since other studies that found positive associations between proAKAP4 and sperm motility assessed ejaculated sperm.

Recently, it has been shown in frozen-thawed bull sperm that proAKAP4 levels were positively associated with sperm motility and swimming velocity after 3 h of incubation.[37] We did not find any relationship between proAKAP4 concentration and sperm traits measured after 90 min of incubation, neither in fresh nor in frozen-thawed sperm samples, suggesting that proAPA4 is not a good predictor for sperm longevity in epididymal mouse spermatozoa.

We found differences in sperm traits between the high and low proAKAP4 groups in fresh but not in frozen-thawed sperm samples. Similarly, we found that only VSL, but none of the other parameters that were correlated with proAKAP4 in fresh sperm samples, was correlated with proAKAP4 in frozen-thawed sperm samples. This partial discrepancy in results can be explained by the negative impact of sperm freezing on sperm swimming traits. All frozen-thawed values were below prefreeze values, and our result indicates that some parameters might be less sensitive to the freezing process than others.

Since several of our measured sperm parameters were interrelated, we performed a PCA to reduce the dimensionality of the data,[50] before assessing the effects of our experimental manipulations. We retained two principal components (PC1 and PC2) and found that PC1, where sperm motility, the three velocity parameters VCL, VSL, VAP, and ALH were loading, significantly declined with cryopreservation. Thus, our results confirm previous findings that cryopreservation—which is an indispensable tool for the conservation of genetically valuable animals—has detrimental effects on various sperm parameters. Cryopreservation causes oxidative and osmotic stress, and the structural damages to the membranes and mitochondria lead to a decline in the motility of spermatozoa.[51–53] Interestingly, PC2, where the three direction parameters STR, LIN, WOB, and BCF were loading, was not affected by the cryopreservation procedure, indicating that cryopreservation affects sperm velocity parameters more than sperm direction parameters. There are known differences in the cryopreservation ability of different mouse strains, with C57BL/6 substrains being more difficult to freeze.[54,55] To reduce sperm damage related to the freezing process, we applied a cryopreservation method, that overcomes the detrimental freezing effects by adding the amino acid L-glutamine to the CPA.[33,56]

We also observed significant strain differences in sperm traits. The PC1 score increased from BALB/c to C57BL/6N, and further to SWISS and B6D2F1, which did not differ from each other. This result is not surprising as hybrid and outbred strains are known for

their hybrid vigor and fertility compared to inbred strains.[57] Among the two investigated inbred strains, BALB/c mice—especially the substrain ByJ used in our study—display a lower in vivo and in vitro fertility,[57] which can be attributed to lower sperm motility[58] and a higher rate of morphologically abnormal sperm compared to other inbred strains, so that they have been used as a model for human infertility.[59] Given that we did not find significant differences in the PC2 score between strains, we can show that strain differences in sperm traits are more pronounced in sperm velocity parameters than in sperm direction parameters, and conclude that strain differences in fertility are more likely caused by velocity parameters than direction parameters.

Finally, we found that the 90 min incubation period negatively affected both sperm motility and direction parameters. Given that cryopreservation had no significant effect on PC2 scores, our results indicate that incubation over a prolonged period of time has a more severe effect on sperm direction parameters than sperm freezing.

Only a few studies provide CASA data on inbred and/or outbred mouse strains in the literature, which would allow a comparison with our results, and none performed a PCA on the different sperm parameters.[35] Studies investigating sperm motility traits usually select only one CASA parameter, e.g., VSL,[60] and sperm is then classified as having either high, medium, or low VSL.[61] Similarly, others classify mouse sperm motility patterns according to their progressivity and hyperactivity.[62] As there are no generally accepted threshold values for these subpopulations in mouse sperm—as there are in human sperm defined by the WHO[15]—we did not define subpopulations within the motile sperm population in our study.

In conclusion, we were able to analyze for the first time proAKAP4 concentration together with sperm motility parameters in four laboratory mouse strains. We found high individual variation but no significant effect of the freezing and thawing procedure on proAKAP4 levels. This result opens up the possibility to analyze proAKAP4 levels in thousands of mouse lines deposited in mouse mutant repositories. Surprisingly, we found a negative relationship between proAKAP4 levels and sperm motility parameters. To elucidate the significance and the postulated indicative value of proAKAP4 for the estimation of male fertility in mice, future studies using in vitro fertilization assays are required.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The institutional ethics and welfare committee (ETK) of the University of Veterinary Medicine, Vienna, discussed and approved this study in accordance with GSP guidelines and national legislation (file number ETK 199/12/2019).

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