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Is the proAKAP4 a suitable biomarker of X-sorted sperm quality from Nelore and Gir bulls?

A proAKAP4 pode ser um biomarcador da qualidade de espermatozoides sexados-X de touros Nelore e Gir?

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Highlights

X-sorted sperm has higher concentrations of proAKAP4 than non-sorted sperm.
There was no correlation between sperm kinetics and proAKAP4 of X-sorted sperm.
ProAKAP4 correlated to sperm kinetics in the non-sorted sperm.
ProAKAP4 is not a suitable biomarker for the X-sorted sperm.

Abstract

ProAKAP4 is a protein precursor of AKAP4 present in the principal piece of the flagellum, associated with sperm motility. It has been described in several species, but its concentration has never been measured in sex-sorted sperm. Thus, the study aimed to test the proAKAP4 as a suitable sperm quality biomarker for *Bos indicus* X-sorted sperm. To achieve the study's goal, 14 semen straws from different bulls were evaluated, six X-sorted and eight non-sorted. Sperm kinetics and morpho-functional evaluations were carried out, in addition to the concentration of proAKAP4. The characteristics evaluated were compared by the t-test and correlated by the Pearson's correlation. A difference was found for total motility (P = 0.014), rapid sperm (P = 0.020), and sperm area (P = 0.013) between the non-sorted and X-sorted sperm. The proAKAP4 concentration was higher in the X-sorted sperm (X-sorted sperm: 67.54 ng/10^6 spermatozoa; non-sorted sperm: 29.76 ng/10^6 spermatozoa), but it was not correlated to the kinetics or morpho-functional characteristics evaluated. However, the proAKAP4 in non-sorted sperm showed

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a correlation to sperm total and progressive motility, rapid sperm, and sperm membrane integrity. The study concluded on the unreliability of the proAKAP4 as a biomarker to indicate sperm quality in the X-sorted sperm.
Key words: AKAP4 protein. Non-sorted sperm. Sex-sorted sperm. X chromosome.

Resumo

A proAKAP4 é uma precursora da proteína AKAP4, presente no flagelo espermático, a qual é associado à motilidade espermática. A concentração de proAKAP4 foi previamente avaliada em diversas espécies, mas sua concentração, nunca foi medida em espermatozoides sexados. Assim, o estudo teve por objetivo avaliar o potencial da proAKAP4 como um biomarcador de qualidade espermática de sêmen de touros *Bos indicus* sexados para fêmea. Foram avaliadas 14 palhetas de sêmen de diferentes touros, sendo seis sexadas para fêmea e oito contendo sêmen convencional. As doses inseminantes foram avaliadas quanto sua cinéticas e morfofuncionalidade, além da concentração de proAKAP4. Os parâmetros avaliados foram comparados pelo teste T de Student e correlacionados pela correlação de Pearson. Uma diferença significativa foi encontrada para motilidade total (P = 0,014), espermatozoides rápidos (P = 0,020) e área espermática (P = 0,013) entre os espermatozoides convencionais e as amostras sexadas. A concentração de proAKAP4 foi maior nos espermatozoides classificados sexados para fêmea (espermatozoides sexados: 67,54 ng/10^6 espermatozoides; espermatozoides convencionais: 29,76 ng/10^6 espermatozoides), entretanto a precursora proteica não se correlacionou com nenhum parâmetro cinético ou morfofuncional avaliado. Já em espermatozoides convencionais, a proAKAP4 mostrou uma relação com os parâmetros motilidade total e progressiva, *rapid sperm* e integridade da membrana espermática. O estudo concluiu que o proAKAP4 não é um biomarcador válido para indicar a qualidade do sêmen sexado para fêmeas.
Palavras-chave: Cromossomo X. Proteína AKAP4. Sêmen convencional. Sêmen sexado.

Introduction

The use of sex-sorted sperm gained ground in reproductive biotechnologies, by accelerating genetic progress and generating calves of the sex desired by the cattle breeder (Thomas et al., 2019). In recent years, due to sexing technique improvements, sex-sorted semen has become as viable as the non-sorted sperm in bovine reproduction (Scott et al., 2018; Thomas et al., 2019). However, to ensure the effectiveness of sex-sorted or non-sorted sperm, quality evaluation is essential for reproductive success.

Although the analysis of sperm quality is necessary for commercializing cryopreserved sperm, the methods are not sufficient to predict fertility rates in the field (Morrell et al., 2018; Sergeant et al., 2019). For this reason, researchers have been looking for protein biomarkers that can select fertile bulls with greater accuracy (Sergeant et al., 2019).

The AKAP4 (A-kinase anchor protein 4) and the proAKAP4 (AKAP4 precursor) has been studied as potential biomarker. The ProAKAP4 transcripts, encoded by an

X-chromosome gene, are observed in post-meiosis spermatids (Brown et al., 2003; Turner et al., 1999). As sperm are transcriptionally inactive cells, the proAKAP4 reserve is the source of the AKAP4, the active molecule. Additionally, the AKAP4 concentration is modulated according to the proAKAP4 stock (Sergeant et al., 2019).

The AKAP4 is the major sperm fibrous sheath protein (Sergeant et al., 2019). Due to its structural function, both the AKAP4 and proAKAP4 assist in the sperm motility, since the AKAP4 protein tether the sperm flagellum enzymes closer to their respective substrates, which induces the phosphorylation of cAMP-dependent proteins, resulting in sperm motility (Sergeant et al., 2019; Turner et al., 1999).

The proAKAP4 molecule is highly conserved in mammalian spermatozoa (Sergeant et al., 2019) and, the concentration of proAKAP4 has been considered as an indicator of sperm quality and fertility in men (Jumeau et al., 2018), stallion (Blommaert et al., 2019), ram (Riesco et al., 2020), and bull sperm (Almeida et al., 2022; Bastan & Akcay, 2021; Dordas-Perpiny et al., 2022); however, its level has not been determined in X-sorted sperm of any species.

As an X-chromosome gene encodes the proAKAP4 protein, we hypothesized that its level would be higher in X-sorted sperm than in non-sorted. Therefore, our objectives were to characterize the concentration of proAKAP4 in *Bos indicus* X-sorted sperm and its relationship with the sperm kinetics parameters, in an attempt to verify the proAKAP4 as a suitable biomarker in the sex-sorted sperm.

Materials and Methods

The study followed the ethical recommendations of the National Council for the Control of Animal Experimentation (CONCEA) and it was approved by the Institutional Ethics Committee for the Use of Animals (CEUA) under number 22460.2018.26.

Sperm collection and analysis

Fourteen commercial sperm frozen in 0.25 mL French straws (Nelore: 11 and Gir: 3) from *Bos indicus* bulls were used. Six straws contained $2x10^6$ X-sorted sperm (Nelore: 3 and Gir: 3) and eight, $35x10^6$ non-sorted sperm (Nelore).

The straws were thawed at 37ºC for 30 s, then the content was transferred to a microtube, and incubated at 37ºC for 3 min before analysis. For the sperm kinetics analysis, a 3 µL sample was placed in a preheated Cell-Vu® Sperm counting chamber (Hamilton Thorne, Beverly, MA, USA) and covered by a coverslip. The sperm kinetics evaluation was performed by the Computer Assisted Sperm Analysis (CASA-Hamilton-Thorne IVOS, Beverly, MA, USA, version 14.0), adjusted for the bovine sperm as follows: setup parameters-values: frames acquired: 30; frame rate: 60 Hz; minimum cell size: 5 pixels; minimum static contrast: 30; straightness (STR) threshold: 70%; VAP cut-off: 30 µm/s; VAP: 50 µm/s; VSL cut-off: 15 µm/s. The parameters evaluated included total motility (% TM); progressive motility (% PM); rapid sperm (% rapid); static sperm (% static), average path velocity (µm/s, VAP); linear speed (µm/s, VSL); curvilinear speed (µm/s, VCL); amplitude of lateral

head displacement (μm, ALH); straightness (%, STR); linearity (%, LIN); elongation (%, ELONG); area (μm^2, area).

For the morpho-functional analysis, 100 μL of sample was diluted in TALP medium to achieve the 5 x 10^6 spermatozoa/mL; then, the volume was divided into two microtubes to add fluorescent probes and incubated at 37ºC. In the first microtube, 20 μmol of the MitoTracker Red CMXRos (M-7512; Molecular Probes, Eugene, OR, USA) were added to evaluate sperm mitochondrial activity (MITO %), following the manufacturer's instructions. In the second microtube, 15 μmol of propidium iodide (0.5 mg/mL, L0770, Sigma-Aldrich Co., Saint Louis, MO, USA) were added to measure the sperm membrane integrity (PI %). Both analyses were conducted in the BD Accuri C6™ flow cytometer (Beckton-Dickeson, San Jose, CA, USA), equipped with an argon laser (488 nm pass) and a red laser (640 nm range), which assured the fluorescence emission of 10,000 events.

ProAKAP4 sperm concentration

A commercial ELISA kit was used to quantify the proAKAP4 sperm concentration (4BioDx, 4VDX-18K3, France) following the manufacturer's instructions. Briefly, 50 μL of frozen-thawed sperm was lysed and added to each well of the microplate containing the proAKAP4 antibody. Next, the microplate was incubated for 2 h at room temperature (24oC). Then, the wells were washed to remove the lysis buffer, the antibody detection was added, and the microplate was again incubated at room temperature for 1 h. The wells were rewashed, the substrate solution was added, and the plate incubated for 10 min. Finally, the stop solution was added to each well, and the optical density was measured at 450 nm in an ELISA microplate reader. The proAKAP4 concentration was expressed as ng/10^6 spermatozoa (Bastan & Akcay, 2021; Dordas-Perpiny et al., 2022).

Statistical analysis

The variables were submitted to normality test by Shapiro-Wilk test, and homogeneity distribution by Levene test. A general linear model was conducted to verify the influence of bull breeds and the type of sperm (X-sorted or non-sorted) over the proAKAP4 concentrations.

To evaluate the influence of bulls' breed and sperm type over the proAKAP4 concentrations, a two-way ANOVA was performed.

The differences in sperm endpoints between non-sorted and X-sorted sperm were evaluated by t-test or Mann-Whitney test, according to test normality distribution of variables. Since the variables presented normal distribution (P>0.05), the Pearson correlation analyses were performed to determine the correlation between the sperm proAKAP4 concentration and sperm kinetics. Statistical analyses were conducted in the SigmaPlot 14.0 software, and data were presented as mean ± SEM. Differences were considered significant when P < 0.05.

Results and Discussion

The X-sorted sperm had lower TM and percentage of rapid cells (P < 0.05) but greater area compared to the non-sorted sperm (Table 1).

Table 1
Mean (± SEM) values of CASA kinetics parameters of bovine X-sorted *vs.* non-sorted sperm

Sperm characteristic	X-sorted sperm (Mean ± SEM)	Non-sorted sperm (Mean ± SEM)	P value
Total motility (%)	26.33 ± 4.15	49.62 ± 6.19	0.014
Progressive motility (%)	19.50 ± 2.99	34.37 ± 6.81	0.100
Rapid sperm (%)	22.17 ± 3.59	43.63 ± 6.32	0.020
Static sperm (%)	55.17 ± 9.43	33.25 ± 5.79	0.055
VAP (µm/s)	89.00 ± 4.60	92.22 ± 5.76	0.686
VSL (µm/s)	75.53 ± 4.19	72.00 ± 3.03	0.496
VCL (µm/s)	160.11 ± 8.13	157.37 ± 11.55	0.860
ALH (µm)	7.20 ± 0.26	6.63 ± 0.41	0.264
STR (%)	83.67 ± 1.40	80.00 ± 3.33	0.755
LIN (%)	48.83 ± 1.66	49.87 ± 2.81	0.776
ELONG (%)	43.50 ± 1.87	41.50 ± 0.73	0.292
AREA (µm²)	7.28 ± 0.14	6.58 ± 0.13	0.013

VAP: travel speed; VSL: progressive speed; VCL: curvilinear speed; ALH: lateral head amplitude; STR: straightness; LIN: linearity; ELONG: elongation. Statistical significance set to P<0.05.

There was no difference (P <0.05) in mitochondrial function, but the X-sorted sperm had a higher plasma membrane integrity percentage (Table 2).

There was an effect of breed on the proAKAP4 concentrations (P =0.002) for non-sorted and sex-sorted sperm. In Nelore and Gir bulls, the concentrations of proAKAP4 were 48.81±11.39 ng/10^6 spermatozoa and 35.51±6.45 ng/10^6 spermatozoa, respectively. An influence of sperm type on the proAKAP4 results (P < 0.001) was also observed. In the X-sorted, the proAKAP4 concentrations ranged from 22.62 to 135.53 ng/10^6 sperm, while in non-sorted sperm it ranged from 12.22 to 48.29 ng/106 sperm. The X-sorted sperm (67.54 ± 17.40) presented higher concentrations of proAKAP4 compared to the non-sorted one (29.76 ± 4.16) (P=0.033).

Table 2
Mean (± SEM) values of mitochondrial activity and plasmatic membrane integrity of bovine X-sorted vs. non-sorted sperm

Parameters	X-sorted sperm	Non-sorted sperm	P value
MITO (%)	81.28 ± 7.05	87.91 ± 3.05	0.362
PI (%)	95.77 ± 1.02	85.12 ± 3.37	0.022

MITO: Mitochondrial activity; PI: Plasmatic membrane integrity. Statistical significance set to P<0.05.

For X-sorted sperm, there was no significant correlation between proAKAP4 concentration and TM (P = 0.83), PM (P = 0.82), rapid sperm (P = 0.88), VAP (P = 0.20), VSL (P = 0.13) and VCL (P = 0.56), mitochondrial activity (P = 0.34) or plasma membrane integrity (P = 0.280).

In contrast, in the non-sorted sperm, positive correlations were detected between proAKAP4 and TM, PM, rapid sperm, and sperm membrane integrity. Furthermore, there was a negative correlation between proAKAP4 concentrations and the percentage of statical sperm (Figure 1).

The authors are unaware of reports describing the proAKAP4 concentration in sex-sorted sperm from any species. Therefore, our study brings unprecedented results regarding the concentration of this protein precursor for sex-sorted sperm of bulls.

Since the breed impacted proAKAP4 concentrations, this allows us to hypothesize that the polypeptide may be expressed in different concentrations in different breeds and/or subspecies. A previous analysis from our research group identified lower proAKAP4 in Nellore bulls' sperm (25.75 ± 4.15 ng/10^6 sperm) (Almeida et al., 2022), when compared to others studies from taurine bulls' sperm (38.67 ±8.55 ng/10^6 sperm) (Dordas-Perpiny et al., 2022).

Recent proteomics analysis of sex-sorted sperm described that the cytoskeleton proteins, such as the AKAPs, are in greater amounts in the X-sorted sperm (De Canio et al., 2014; Scott et al., 2018). These facts corroborate our hypothesis and results, in which a higher concentration of proAKAP4 was found in the X-sorted sperm compared to the non-sorted sperm.

The absence of correlation between the proAKAP4 and CASA kinetics characteristics for the X-sorted sperm was unexpected. We hypothesize that, when compared to Y-sorted sperm (Boro et al., 2016; Scott et al., 2018), the greater size and weight of X-sorted sperm is the main reason for the absence of correlation. Additionally, the injuries inherent to the sex-sorting process may also impair sperm kinetics (Scott et al., 2018; Steele et al., 2020).For that, further researches are needed to enlighten this matter.

Furthermore, the proAKAP4 and AKAP4 genes are only transcripted during spermatogenesis, so their concentrations are not modified after ejaculation (Sergeant et al., 2019). Since the AKAP4 is a hemizygote gene (Fang et al., 2019), the sex sorting process accumulates this protein precursor. For this reason, we suppose that the proAKAP4 may not be a reliable biomarker for the X-sorted sperm quality.

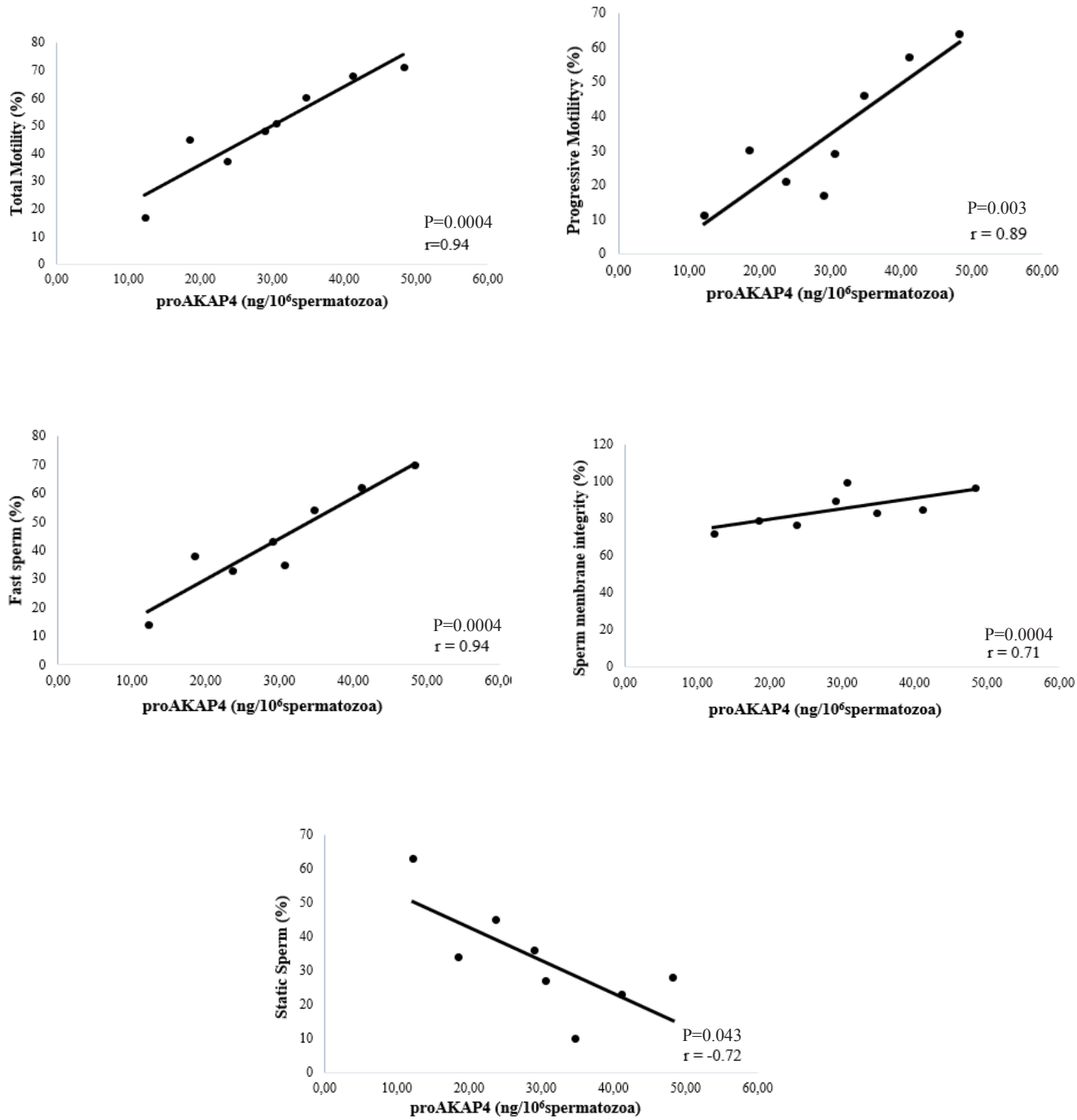

Figure 1. Correlation between proAKAP4 concentration and total motility, progressive motility, percentage of fast sperm, sperm membrane integrity, and percentage of statical sperm in conventional semen.

The lower kinetics parameters in the X-sorted sperm observed in our study corroborate with a previous study findings, that showed a decrease of progressive motility in sex-sorted sperm (60.8% for non-sorted and 25.6% for sex-sorted sperm) (Steele et al., 2020). Besides, differences in total motility between non-sorted and sex-sorted sperm (respectively, 58.1 and 35.2%) were also observed (Carvalho et al., 2018).

In a previous study, sex-sorted sperm showed lower values of LIN and VAP but higher results for ALH and STR, which may characterize sperm hyperactivation induced by the sorting process (Steele et al., 2020). In our investigation, hyperactive behavior was not detected since there was no difference in the VAP, VSL, VCL, ALH, STR, or LIN parameters between the types of sperm.

Regarding mitochondrial functionality, the results from a previous study demonstrated higher mitochondria potential in the non-sorted sperm (Carvalho et al., 2018), in contrast to our findings, in which there was no difference for this parameter between the types of semen evaluated. Additionally, we detected a higher percentage of sperm membrane integrity for the X-sorted sperm. It opposes to results from a previous research in which no differences were detected in plasma membrane integrity between the sex-sorted and non-sorted sperm (González-Marín et al., 2018). However, we emphasize how current methods for sperm sexing effectively maintain sperm membrane integrity.

The correlation between proAKAP4 concentrations and kinetics characteristics in the non-sorted sperm corroborates previous reports, confirming how the proAKAP4 impacts sperm motility, and even its fertility (Almeida et al., 2022; Bastan & Akcay, 2021; Dordas-Perpiny et al., 2022). In light of this analysis results, it is expected that in a near future the use of biomarkers, such as the AKAP4 precursor, may become a great tool for selecting breeding bulls, since it shows the ability to predict the sperm quality of non-sorted sperm doses.

Although, in the X-sorted the proAKAP4 concentrations did not correlate to the kinetics characteristics, this correlation could occur in the Y-sorted sperm. In addition, the first main limitation of the present study was the incapability of searching proAKAP4 concentrations in the Y-sorted sperm. However, it is worthy emphasizing how our report was made possible only through the support of great commercial partners who provided us the sperm straws for analysis. Unfortunately, there were no Y-sorted straws available for donation. The second limitation was the lack of fertility results (as pregnancy rate in FTAI or blastocysts rates in PIVE production), especially from the X-sorted sperm, so we cannot assure if the proAKAP4 can predict sperm fertility in sex-sorted sperm. In view of that, future studies could benefit from addressing both our limitations as well as some of our study gaps

Conclusions

This is the first report of proAKAP4 concentrations in sex-sorted sperm. Although the proAKAP4 showed greater concentrations for the X-sorted sperm, we concluded that this protein precursor is an unreliable biomarker of X-sorted sperm quality. It occurs since there are no

positive correlations between the sperm kinetics parameters and the proAKAP4 concentrations in X-sorted sperm.

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Conflicts of Interest

The authors declare no conflict of interest.

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