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First results about ProAKAP4 concentration in stallion semen after cryopreservation in two different freezing media

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ABSTRACT

The quality of fresh or thawed sperm in stallions has been generally determined by the viability and total and progressive motility of the sperm. Today, the expression of ProAKAP4, a protein present in the flagellum of spermatozoa, appears to be an innovative and relevant functional marker to assess semen quality and male fertility. This study aims to compare the concentration of ProAKAP4 in the semen from 5 stallions frozen with two different extenders immediately after thawing (T0) and 4 h post-thawing (T4). Viability, total and progressive motility were measured in parallel. Significant differences for sperm viability and total motility were observed between the two extenders, as was the concentration of ProAKAP4 both at T0 and T4. At T4, all quality parameters and ProAKAP4 content significantly decreased compared to T0, but with a considerably slower decrease in one extender than the other. These preliminary results suggest that measuring the concentration of ProAKAP4 is a promising tool for the comparison of different extenders and the selection of the optimal freezing medium for each stallion ejaculate.

1. Introduction

Sperm quality in stallions is classically based on measurements of several sperm parameters. The primary parameters are volume, sperm, morphology and motility (total and progressive) before and after freezing process. Recent studies were conducted to identify protein markers associated with sperm quality and fertility. ProAKAP4 is one of the fertility associated protein markers that were identified more than 30 years ago and have been now developed as a robust marker in fresh/frozen sperm or isolated spermatozoa in various species including horses [6,10]. ProAKAP4 is the precursor of the AKAP4 protein. Both proteins are found in the principal part of the flagellum. Indeed ProAKAP4 appears as a storage form of the AKAP4 active molecule. High ProAKAP4 expression has been associated with high fertility and quantification of ProAKAP4 expression appears today as a pertinent and innovative approach to assess fertility. In bull, sperm from high fertility males showed a higher level of ProAKAP4 expression than sperm from less fertile males [8]. In men, ProAKAP4 expression was lower with non-fertilizing sperm even where other sperm parameters were normal [4,5]. ProAKAP4 was recently identified in stallion spermatozoa and its concentration was correlated with the mobility parameters in thawed semen [3] and also with the fertility of the stallions [6].

Our research group developed a new extender for stallion sperm freezing where egg yolk was replaced by a cyclodextrin cholesterol complex [1]. Such substitution allowed for a decrease in the concentration of glycerol in the medium and a significant improvement of the quality parameters of thawed spermatozoa, including viability and motility. We further improved this freezing medium by completely replacing glycerol, which is highly toxic for equine sperm, by mannitol, a non-toxic 6-C polyol [2] (WO/2020/201311A, Semen Cryopreservation).

This study aims to compare the concentration of ProAKAP4, the

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viability and the progressive and total motility of semen collected from 5 stallions and frozen with this new extender or with a commercial equivalent immediately after thawing and 4 h post-thawing.

2. Material and methods

Five stallions were housed at the Mont-le-Soie reproduction centre (Belgium) for the production of frozen sperm. They were aged from 4 to 25 years with an average weight of 580 ± 171 kg. Two frozen media were used: the INRA FREEZE (IMV Technologies, L'Aigle, France) and the SPERLIN medium (Mont-le-Soie, Belgium). The latter is a classical HBSS/HEPES based medium with glucose and lactose but having a casein salt and a cyclodextrin cholesterol complex as milk and egg yolk substitutes respectively and mannitol as the cryoprotectant agent instead of glycerol. After semen collection, each ejaculate was divided between the two extenders and diluted to reach a final concentration of 150 million spermatozoa/mL before being packaged and frozen in 0.5 mL straws. Each straw was thawed by immersion in a water bath at 37 °C for 1 min (T0). After sampling, the remaining semen was maintained at the same temperature for 4 h in 1.5 ml Eppendorf tube. A straw was dedicated for the determination of quality parameter just after thawing (T0) and 4h post-thawing (T4) while another straw was used for the determination of ProAKAP4 concentrations at T0 and T4.

Immediately following collection, the concentration of spermatozoa was determined using the NucleoCounter SP-100 with the Chemometec detergent (CHEMOMETEC, Allerod, Denmark). The concentration of dead spermatozoa was also determined using the same device, following the manufacturer's instructions. Total and progressive motility were determined on diluted samples (20×10^6 total spermatozoa/mL) using 20 μL Leja cells and a computer assisted semen analyser (CASA) (IVOS version 12.0, Hamilton Thorne Research, Beverly, MA, USA) according to the settings provided in Blommaert et al. [1].

The sample preparation (final dilution 20x including lysis and dilution buffer) and the measurement of ProAKAP4 concentrations were performed using a commercial ELISA kit according to the manufacturer's instructions (4BioDx, Horse 4MID® Kit, ref. 4VDX-18K3, France).

Statistical analyses were performed by MedCalc software (Belgium). Two-factor study with repeated measures on one factor was used. The first factor was the extender (IF versus SP), the second factor was the time just after thawing (T0) and 4h later (T4). A p value < 0.05 was considered as significant.

3. Results

Mean values, standard deviation and statistical analysis were reported in Table 1 and Fig. 1. A significant difference was observed between media ($p < 0.05$) for ProAKAP4 concentration, viability and total motility. Just after thawing (T0), the spermatozoa in the SPERLIN medium showed higher mean ProAKAP4 concentrations (+8.6 ng/10 M spermatozoa for SP vs IF, $p < 0.05$), viability (+15% for SP vs IF, $p < 0.05$) and total motility (+8.2% for SP vs IF, $p < 0.05$) in comparison to the IF medium.

Table 1
Viability, total and progressive motility (%) and ProAKAP4 concentrations (ng/10 million spermatozoa) measured just after thawing (T0 h) and 4 h later (T4h) in equine spermatozoa frozen with INRA Freeze and SPERLIN media (Mean ± SD, n = 5 horses, 1 ejaculate per horse).

	INRA Freeze (IF)		SPERLIN (SP)	
	T0	T4	T0	T4
ProAKAP4 (ng/10 M SPZ)	60.9 ± 2.1	15.9 ± 3.2	69.5 ± 2.8	22.6 ± 4.0
Viability (%)	65.4 ± 4.4	60.2 ± 5.1	79.2 ± 5.0	76.0 ± 5.1
Total motility (%)	34.0 ± 4.2	29.4 ± 4.6	42.2 ± 5.9	39.4 ± 6.5
Progressive motility (%)	22.4 ± 8.6	17.8 ± 9.3	30.6 ± 9.0	28.2 ± 9.5

After 4h post-thawing (T4), the spermatozoa in the SPERLIN medium showed a higher mean ProAKAP4 concentration (+6.7 ng/10 M spermatozoa for SP vs IF, $p < 0.05$), viability (+15.8% for SP vs IF, $p < 0.05$), total motility (+10% for SP vs IF, $p < 0.05$) and progressive motility (+10.4% for SP vs IF, $p < 0.05$) in comparison to the IF medium.

A significant difference was observed between T0 and T4 for the mean ProAKAP4 concentration, viability, total motility and progressive motility.

Moreover, a significant interaction ($p < 0.05$) was observed for the progressive motility indicating that the decrease in the progressive motility between T0 and T4 was less severe in the SPERLIN medium by comparison with IF.

4. Discussion

These first results obtained with semen collected from 5 horses showed concordance between the classical quality parameters of spermatozoa and the ProAKAP4 concentrations measured after thawing. Blommaert et al. [3] showed that the concentrations of ProAKAP4 varied between stallions but correlated to both total and progressive motility in post-thawed stallion semen samples. Furthermore, high- and low-quality semen from stallions were also shown to contain respectively higher and lower concentrations of ProAKAP4 [6]. Environmental factors such as oxidative stress factors are known to promote ProAKAP4 degradation and a subsequent decrease in the quantity of ProAKAP4 available for conversion to active AKAP4 protein [7]. Thus, modification of the composition of freezing media may have a positive or negative impact on semen functionality following thawing. In this study, we demonstrated higher viability, motility and ProAKAP4 concentrations in semen samples conditioned with the SPERLIN as compared with the IF medium, suggesting that SPERLIN is a more favourable medium for freezing stallion semen and could better preserve spermatozoa functionality in post-thawed semen. The replacement of egg yolk and glycerol respectively by a cyclodextrin-cholesterol complex and mannitol could explain the higher post-thaw quality of the semen [1,2].

Both quality parameters and ProAKAP4 content significantly decreased at T4 compared to T0 but with a slower decrease for the progressive motility in SPERLIN as compared with IF. An important decrease of ProAKAP4 concentration after thawing was already observed [9]. In our study, the drastic decrease of ProAKAP4 was not correlated with a similar decrease of the quality parameters (viability or motility). After thawing, the loss of the storage form of the AKAP4 active molecule is rapidly solicited not only for the maintenance of motility but also for other spermatozoa function necessary for spermatozoa fertility such as sperm capacitation and fertility [4]. The reduction of ProAKAP4 levels occurs through a conversion of ProAKAP4 into the active AKAP4 protein in order to maintain the structure of the fibrous sheath of the principal piece of the flagellum and a functional signalling system to transduce and control spermatozoa motility over time. This reduction could therefore provide a valid indicator of the maintenance of a functional molecular system in post-thawed spermatozoa in the selected media. It also suggests that this process will remain functional in the female genital tract following insemination. However, degradation of ProAKAP4 cannot be excluded and further investigations are needed to test this hypothesis. Whether the different rates of ProAKAP4 reduction related to the extender used would be similar inside the female genital tract also remains unknown.

ProAKAP4 concentrations are undoubtedly a functional indicator of sperm functionality and motility as spermatozoa without ProAKAP4/AKAP4 are immature, not motile and infertile (reviewed in Delehedde et al. [4]). We observed in our study that when ProAKAP4 concentrations remained higher at T4 with SPERLIN medium (22.6 ± 4.0 ng/10 M of spz), total and progressive motility parameters also remained higher at T4 (39.4 ± 6.5% and 29.2 ± 9.5% respectively), almost 10 points above the total motility observed in IF media (29.4 ± 4.6% and 17.8 ± 9.3% respectively). Freezing conditions favouring high concentrations

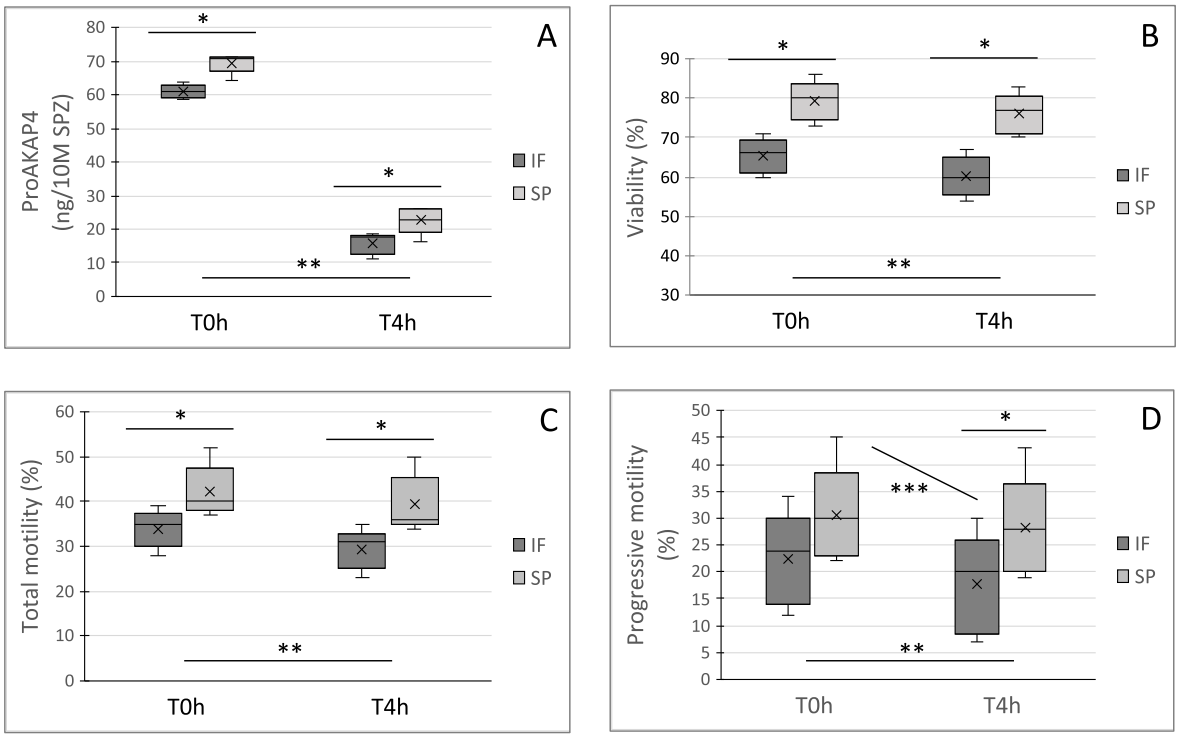

Fig. 1. Effect of medium [INRA freeze (IF) and SPERLIN (SP)] and time (T0 h and T4h) after thawing on ProAKAP4 concentrations, viability, and total and progressive motilities (Mean ± SD, n = 5 horses). *p < 0.05 between groups (SP and IF); **P < 0.05 between T0 and T4; ***p < 0.05 interaction (groups x T0-T4).

of ProAKAP4 post thawing could contribute to the maintenance of high sperm motility and consequently to higher fertility rates. Long-lasting motility and functionality will also increase the chances of fertilization if insemination and ovulation are not synchronized.

ProAKAP4 expression has been associated with fertility and appears today as a pertinent and innovative approach to assess fertility in several species. In boars, it was shown that the processing of dose semen presenting a ProAKAP4 concentration above 45 ng/10 million spermatozoa exhibited a clearly increased fertility index [10]. Just after thawing, we observed ProAKAP4 concentration about 60 ng/10 million spermatozoa. Based on our results, it would be useful to define the threshold concentration for equine semen in a larger field study.

In conclusion, the results of the present study showed a parallelism between ProAKAP4 concentrations and the quality parameters for the selection of the optimal medium for semen cryopreservation. The determination of ProAKAP4 concentration even appear less variable between horses than progressive motility, considered as one of the most important parameters when evaluating male fertility. These preliminary results suggest that the concentration of ProAKAP4 as a reflect of long-lasting motility and functionality in post-thaw conditions, could be a promising tool for the comparison of different extenders, and the selection of the best one for each stallion.

Declaration of competing interest

Maryse Delehedde and Nicolas Sergeant are both co-founders of the SPQI company (Lille, France).

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