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SHORT COMMUNICATION

Changes in aquaporins mRNA expression and liquid storage at 17°C: A potential biomarker of boar sperm quality?

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Abstract

Artificial insemination (AI) for pigs relies on liquid storage of extended semen at 17°C, which preserves sperm quality and ensures its fertilizing capacity. Routine quality controls include the evaluation of sperm motility, viability and capacitation status. The physiological functions of all these features depend on transmembrane aquaporins (AQPs), proteins playing key roles in osmoadaptation. In this study, we made a relative quantification, using RT-qPCR, of the mRNA of several sperm AQPs in AI-liquid semen doses before and after a 48-hr incubation period, aiming to determine possible quantitative compromising expression changes during the process that could serve as a diagnostic tool. Our results showed a decrease in classical sperm motility variables (total and progressive motility and velocity) and sperm viability after 48-hr storage, whereas capacitation status increased overtime. mRNA expression increased in the orthodox AQP4 and AQP6 after 48-hr incubation, relative to control (0 hr) and 24-hr time-points. Moreover, mRNA expression of aquaglyceroporins AQP3, AQP7 and AQP10 was higher after 48-hr incubation, confirmed by AQP7-protein validation using Western blot. Our results indicate that expression levels of AQPs-mRNA can change in ejaculated pig spermatozoa under conditions of ex-vivo incubation that could modify sperm homeostasis, suggesting it could eventually become a relevant molecular biomarker to assess the efficiency of liquid storage of pig semen.

KEYWORDS
AI doses, boar sperm, liquid storage, transcripts

1 | INTRODUCTION

In the swine industry, most artificial inseminations (AI) are carried out using extended liquid semen as sperm cryopreservation remains suboptimal, and with individual differences are becoming more marked as storage time increases (De Ambrogi et al., 2006). However, AI doses stored for more than 24 hr have been related to decreased fertility (Christensen et al., 2004). Unlike spermatozoa in seminal plasma, which retain motility for a few hours, AI doses pursue to control not only the reduction of the metabolic activity by

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lower temperatures (17°C) but also by providing the energy source and regulating pH, among other factors.

Aquaporins (AQPs), a family of ubiquitous transmembrane proteins, allow the transport of water and small molecules across the cell plasma membrane (Delgado-Bermúdez et al., 2021). In mammalian sperm, different AQPs have been identified being their main roles related to osmoadaptation and sperm motility activation after ejaculation (Delgado-Bermúdez et al., 2021). The group of orthodox AQPs, exclusively permeable to water, includes AQP2, AQP4, AQP5, AQP6 and AQP8. In contrast, aquaglyceroporins, including AQP3, AQP7, AQP9, AQP10 and AQP11, are also permeable to glycerol, urea and other small electrolytes. AQP3, AQP7 and AQP11 (Prieto-Martínez et al., 2016, 2017) and AQP9 (Vicente-Carrillo et al., 2016) have been identified in pig. AQP7 has been associated with sperm motility in humans (Saito et al., 2004) but not in mice (Sohara et al., 2007). Thus, considering the function of AQPs and their influence on pH through the regulation of water flow, this study aimed to track the changes in different AQPs during in vitro sperm liquid storage at 17°C for up to 48 hr, and their correlation with sperm quality parameters.

2 | MATERIALS AND METHODS

The chemicals used in the experiments were of analytical grade. Unless otherwise stated, all reagents were acquired from Sigma-Aldrich.

2.1 | Ejaculated spermatozoa

Boar ejaculates were supplied as commercial AI doses (10 batches of 3-boars per pool; Svenska Köttföretagen AB) extended in Hampshire Longlife (Durasperm; Kruuse) to 4.8×10^9 total spermatozoa/dose, each batch containing a pool of three different breeding, healthy, mature (1–2 years old) boars of proven fertility and semen quality (>80% motility, <15% total sperm abnormalities), and stored at 17°C, as recommended for AI purposes, for 0 hr (Control), 24 hr and 48 hr, from the time of arrival to the laboratory (24 hr after sample collection).

Each AI dose was evaluated for motility (velocity and total and forward progressive motility) using a light microscope (Zeiss) equipped with a thermal plate (38°C), positive phase contrast optics (10× objective), a Charge Coupled Device (CCD) camera (UI-1540LE-M-HQ, Ueye, IDS Imaging Development Systems GmbH) and the Qualisperm® Software (AKYmed). Two fluorescent probes in PBS were combined to evaluate cell physiology: viability (YO-PRO-1, 75 nM; stains the nuclei of apoptotic or dead sperm) and capacitation status (Merocyanine 540, 2 μM; binds to membranes with lower cholesterol:phospholipids ratio), after 15 min of incubation at 37°C in the dark. Analyses were performed using a fluorescence microscope at 40× magnification (Eclipse Ti; Nikon). We recorded the percentage of spermatozoa, in at least 4 random fields, identified as viable (negative YO-PRO-1 fluorescence) and viable and capacitated (bright M540 fluorescence and negative YO-PRO-1 fluorescence).

2.2 | RNA isolation and qPCR analysis

RNA was isolated using a commercial kit (RNeasy kit; Qiagen), following the manufacturer protocol, with slight modifications (Alvarez-Rodriguez et al., 2020). Total RNA content, from 50×10^6 spermatozoa, and quality were determined by NanoDrop® 1000 (Thermo Fisher Scientific). Only samples with high RNA quality (260/280 nm absorbance ratio of 1.8–2.2) were snap-frozen in liquid nitrogen and stored at −80°C for analysis. The first-strand cDNA synthesis was performed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems), which consisted of 4 μg RNA in a final volume of 20 μl. After the synthesis, the samples were stored at −20°C until further analyses. The Real-Time PCR Detection System (CFX96; Bio-Rad Laboratories, Inc) was used for the Quantitative Polymerase Chain Reaction (qPCR). The reactions consisted of 0.5 μl of synthesized cDNA, 0.5 μl commercial gene-specific primers for boar (PrimePCR SYBR® Green Assay: GAPDH, AQP2, AQP3, AQP4, AQP5, AQP6, AQP7, AQP9, AQP10 and AQP11, *Sus scrofa*; Bio-Rad Laboratories, Inc), 5 μl of PowerUp SYBR Green Master Mix (Applied Biosystems) and water to a final volume of 10 μl. One cycle of uracil-DNA glycosylase (UDG) activation at 50°C for 2 min; one cycle of denaturation at 95°C for 2 min; and 40 cycles of denaturation at 95°C for 5 s, annealing/extension at 60°C for 30 s, and a melting curve at 60–95°C (0.5°C increments) for 5 s/step were used. Two technical replicates were used for each sample. The gene relative expression levels were quantified using the Pfaffl method (Pfaffl, 2001). The housekeeping gene GAPDH was used for cDNA normalization.

2.3 | Western blotting for validation of AQP7 protein expression

Western blotting (WB) was performed following our previous protocol (Alvarez-Rodriguez et al., 2021). Briefly, sperm samples were homogenized by sonication in commercial lysis buffer (RIPA) at 4°C. Protein concentration was determined by the DC™ Protein Assay kit (Bio-Rad). Each sample (25 μg) was denatured in 4× sample buffer and DTT for 10 min at 70°C, loaded into 4%–20% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes. For protein identification, membranes were blocked at room temperature for 60 min and incubated overnight at 4°C with rabbit monoclonal anti-AQP7 antibody (ab32826, Abcam; and MBS540430; MyBiosource) at dilution 1/500. To standardize the results, a polyclonal IgG anti-GAPDH antibody (ab157156, Abcam) was used at a dilution 1/1,000 in the same membranes. To visualize immunoreactivity, membranes were incubated 60 min at room temperature with secondary antibody donkey anti-rabbit IgGs (IRDye 800 CW-labelled, 926-32213, LI-COR Biosciences, 238 Inc; Hamburg, The Netherlands) at dilution

1/10,000. The membranes were scanned using the Odyssey CLx (LICOR Biosciences).

2.4 | Statistical analysis

Normal distribution and homoscedasticity of the data were analysed using the Shapiro–Wilk normality test and Levene's test. Non-normal data distribution was restored using Log(x) transformation prior to analysis. R-version 3.6.1. (R Development Core Team, 2011) was used to conduct the statistical analyses, by linear mixed effects (LME) models and pairwise comparisons adjusted by Tukey's test. The threshold for significance was set at $p < .05$. Data are presented as mean ± SEM, unless otherwise stated. Our LME model included the incubation time as fixed effects and the pool of samples as the random part of the model. Spearman's correlation analysis was performed among AQPs expressions and sperm quality parameters.

3 | RESULTS

The mRNA expression of AQP3, 4, 6, 7 and 10 increased after 48 hr of storage (Figure 1A). In addition, mRNA from AQP4, 6, 7 and 10 increased after 24 hr of storage, relative to control (0 hr incubation) (Figure 1A). No differences were found in the rest of the AQPs measured (Figure S1). Moreover, AQP7 protein expression increases after 48-hr storage ($p < .05$), relative with control (1.00 ± 0.17), 24 hr (1.02 ± 0.55) and 48-hr storage (1.76 ± 0.37) (Figure S2).

Total and progressive sperm motility and sperm viability decreased after 48-hr storage and velocity dropped after 24-hr incubation (Figure 1B). Capacitated-viable spermatozoa increased in proportions after 24- and 48-hr incubation (Figure 1B).

All the AQPs had a significant negative correlation with total motility, progressive motility and sperm viability. All AQPs studied, except AQP6 and AQP10, presented a positive correlation with sperm capacitation (Figure S3).

4 | DISCUSSION AND CONCLUSIONS

To our knowledge, this is the first study to describe the differential expression of AQPs mRNA during liquid storage of boar AI doses at 17°C up to 48 hr. Our results showed a decrease in sperm total motility at 48 hr (72 hr after sample collection), in agreement with previous studies describing a decrease in the total motile spermatozoa after 72-hr storage (De Ambrogi et al., 2006; Khoi et al., 2021). Although these authors did not find any differences in sperm membrane integrity, our results showed a decrease after 48-hr incubation, agreeing with a previous study (Dubé et al., 2004). Moreover, the capacitation status in our study increases after only 24-hr incubation in a time-dependent manner as has been previously suggested (Dubé et al., 2004).

AQP7, but not AQP9, has been previously described as a relevant marker for non-empirical studies on boar sperm handling (Vicente-Carrillo et al., 2016). According to that statement, we validated AQP7 mRNA results at the protein level. Our results confirm the increase in AQP7 mRNA and protein during storage, but not AQP9, the latter being positively related to sperm velocity

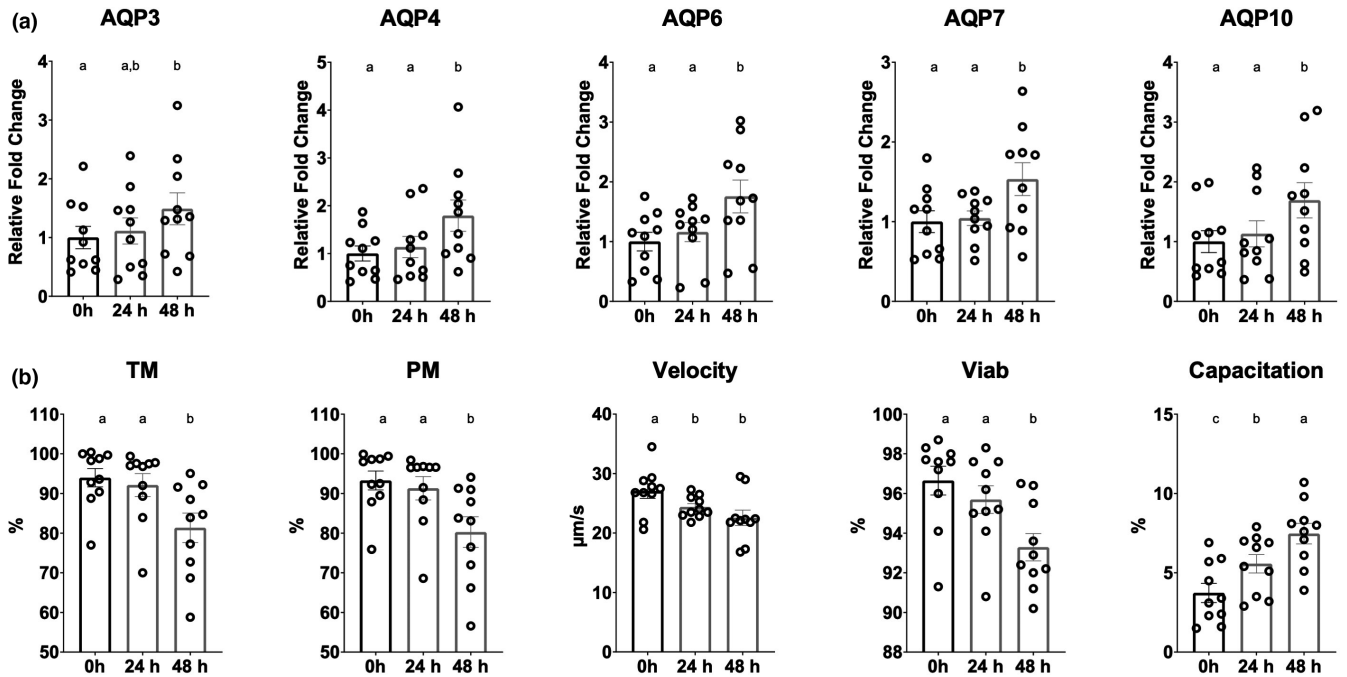

FIGURE 1 Relative AQPs mRNA expression changes (relative fold change) (A) and sperm quality parameters (B): total motility (TM), progressive motility (PM), velocity (Velocity), viability (Viab) and capacitation status (Capacitation), ($n = 10$) after 24- and 48-hr liquid storage at 17°C. Results are expressed in mean ± SEM. Different letters indicate differences among storage times

and capacitation status. Also, as previously defined, AQP3 and AQP7 could be used as freezability biomarkers (Prieto-Martínez et al., 2016, 2017), which would be of high relevance in the porcine industry. Therefore, the increase in AQP3 and AQP7 mRNA in our results might suggest a role to cope with the long-time sperm liquid storage. Our results confront the main idea of spermatozoa being considered a transcriptionally silent cell. However, this concept has been criticized recently, arguing that they could not be completely quiescent cells (Santiago et al., 2021).

Overall, the differential cargo on several AQPs and, particularly, the AQP7 results at mRNA and protein level should lead to further localization and functional level analysis for elucidating the exact mechanism behind this AQPs expression changes.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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