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Cooling rate modifies the location of aquaporin 3 in spermatozoa of sheep and goat

Belén Pequeño[1], María Gemma Millán de la Blanca[1], Cristina Castaño[1], Adolfo Toledano-Díaz[1], Milagros Cristina Esteso[1], Esther Alba[1], Francisco A. Arrebola[2], Rodolfo Ungerfeld[3], Belén Martínez-Madrid[4], Manuel Alvarez-Rodriguez[1], Heriberto Rodriguez-Martinez[5], Julián Santiago-Moreno[1*]

[1]Dept. of Animal Reproduction, INIA-CSIC. Madrid, Spain,

[2]Instituto Andaluz de Investigación y Formación Agraria, Pesquera y Alimentaria (IFAPA) Hinojosa del Duque, Córdoba, Spain,

[3]Dept. Biociencias Veterinarias, Facultad de Veterinaria, Universidad de la República, Montevideo,Uruguay

[4] Dept. Medicina y Cirugía Animal, Facultad de Veterinaria, UCM, Spain,

[5]Dept. of Biomedical & Clinical Sciences, Obstetrics & Gynecology, Linköping University, Sweden.

* Corresponding author

E-mail: moreno@inia.csic.es

ABSTRACT

The freeze-thawing process induces osmotic changes that may affect the membrane domain location of aquaporins' (AQP) in spermatozoa. Recent studies suggest that changes in AQP3 localization allows better sperm osmo-adaptation, improving the cryoresistance. Ultra-rapid freezing is an alternative cryopreservation technique that requires less equipment than conventional freezing, and it is faster, simpler and can be used in the field. This study aimed to determine the influence of freezing-thawing rates (slow (control) vs. ultra-rapid) on AQP3 expression and location in the spermatozoa from small ruminants (sheep and goats) and its relationship with sperm cryo-damage. Spermatozoa were collected from 10 Merino rams and 10 Murciano-Granadina bucks. The presence and distribution of AQP3 were assessed by Western blotting and immunocytochemistry (ICC), employing a commercial rabbit polyclonal antibody. Sperm motility was CASA system-analyzed, and membrane and acrosome integrity assessed by fluorescence (PI/PNA-FITC). Western blotting did not detect a significant effect of freezing-thawing rate on the amount of AQP3 while ICC found freezing-thawing rate affecting AQP3 location ($P<0.05$). In both species, the percentages of spermatozoa showing AQP3 in the post-acrosome region, mid-piece, and principal piece of the tail were greater in samples cryopreserved by slow freezing-thawing (control) than ultra-rapid freezing-thawing rates ($P<0.05$). Spermatozoa cryopreserved using ultra-rapid freezing-thawing showed decrease motility, plasma membrane, and acrosome integrity ($P<0.05$), which might be related, at least in part, to a lower expression of AQP3. In conclusion, the cooling rate modifies the location of AQP3 in spermatozoa of sheep and goat, which might be associated with sperm cryosurvival.

Keywords: Aquaporins, domestic ruminants, sperm, cryoresistance.

1. Introduction

The small ruminant productive systems face many challenges, including emerging diseases, competition for natural resources, and climate change. The diminishing genetic diversity represents an obstacle to sustainable livestock production. Cryo-conservation of genetic resources through gene banking provides one of the most powerful tools to manage genetic diversity [1]. Trans-cervical artificial insemination (AI) using cryopreserved spermatozoa is commonly employed in goat does. However, the cervix of sheep ewes is a significant barrier limiting the application of artificial insemination that requires laparoscopic intrauterine insemination to bypass the anatomical challenges [2]. Moreover, in sheep, the fertility results obtained with cryopreserved semen are lower than those obtained with refrigerated semen [3,4], and, unfortunately, since initial studies by Colas in the 70' [5], the freezing procedures have not substantially improved [6,7]. Thus, a better knowledge of the causes that determine variations in sperm cryosurvival in small ruminants is a priority to provide new tools to identify new markers of sperm cryoresistance and thus improve sperm cryopreservation.

Aquaglyceroporins (AQP) are membrane proteins responsible for the transport of water and solutes such as glycerol [8]. These water channels are crucial to regulate sperm volume during freezing-thawing processes and, therefore, they are involved in the functional sperm response to cryopreservation [9]. Of all AQPs, AQP3 has a central role in osmoregulation [10], and thus, is crucial in post-ejaculatory events when spermatozoa interact with the female tract. AQP3 has been recently identified in the spermatozoa of rams [11], and sperm cryotolerance seems related to changes in AQP3 location. Specifically, the freeze-thawing

process increases the proportion of ram spermatozoa showing AQP3 in both the mid and the principal pieces in ejaculates depicting good freezability [11]. These findings suggested that changes in AQP3 location could be used as a biomarker for sperm freezability in rams. To the best of our knowledge, AQPs have not yet been identified in goat spermatozoa.

Freeze-thawing involves changes in the location of AQP3 in membrane domains of spermatozoa, but it is unknown if this relocation is related to the cooling rate. The use of cryopreservation procedures at high cooling rates (ultra-rapid freezing) has provided successfully results in some wild small ruminant species, but its application in domestic ruminant is scarce [12]. This is a simple and quick method to perform that does not require human expertise and sophisticated equipment. Certainly, ultra-rapid freezing is an alternative cryopreservation procedure widely used in some wild species [13], but leading to greater cryodamage, mainly at the mitochondrial level, compared to conventional freezing [14]. It is hypothesized that differences in the cryodamage among freezing methods could be related to a variation in the location of AQP3 after freezing.

This study aimed to identify the AQP3 presence and location in spermatozoa from goat bucks and the influence of freezing-thawing method on AQP3 abundance and location in the spermatozoa from Merino rams and Murciano-Granadina bucks for its relationship with sperm cryo-damage.

2. Materials and methods

All diluents and media were prepared using reagent-grade chemicals purchased from Merck KGaA (Darmstadt, Germany).

2.1. Animals and semen collection

Ten Merino rams and ten Murciano-Granadina bucks were housed at the Department of Animal Reproduction of INIA-CSIC (Madrid-Spain, 40° 25′N latitude). The animals were fed a diet of barley straw, dry alfalfa, and grain. Water, vitamins, and mineral blocks were available ad libitum. All handling procedures were approved by the INIA Ethics Committee (reference regional government PROEX 046.0/21) and performed following the Spanish Policy for Animal Protection RD53/2013, which conforms to European Union Directive 2010/63 regarding the protection of animals used in scientific experiments.

Ejaculates were collected through an artificial vagina with non-estrous female teasers. Males were used to a twice weekly semen collection regimen. Six ejaculates per male, collected in August, October and December, were used in this study. Therefore, a total of 120 samples (60 from rams and 60 from bucks) were analyzed.

2.2. Cryopreservation

2.2.1. Conventional slow freezing-thawing (control method)

Ram semen samples were extended in TES-Tris-glucose-based medium (TTG-egg yolk-glycerol) containing TES (210.6 mM), Tris (95.8 mM), glucose (10.1 mM), streptomycin (0.54 mM), penicillin (2.14 mM), 6% egg yolk (vol/vol) and 5 % glycerol (pH adjusted to 6.8 -7.2, osmolarity 320-345 mOsm/kg). Buck semen was first washed by diluting (1:9, v:v) in a washing solution (TCG medium composed of Tris 313.7 mM, citric acid 104.7 mM, glucose 30.3 mM), centrifuging at 900 x *g* for 20 min, and the supernatant removed; the pellet was then extended in TCG-egg yolk-glycerol medium containing TCG plus streptomycin (0.54 mM), penicillin (2.14 mM), 6% egg yolk (vol/vol) and 5 % glycerol (pH 6.8 -7, osmolarity 320-345 mOsm/kg). All samples were extended to a concentration of 100×10^6 sperm/mL. The samples were cooled at 5 °C for a 3 h-equilibration period. Samples were

cryopreserved following conventional freezing in static liquid nitrogen vapor [15]. The samples were slowly frozen by placing the straws in a water bath at 27 ºC for 30 s.

2.2.2. Ultrarapid freezing-thawing (experimental method)

Ram semen samples were extended with a medium containing TTG–6% egg yolk +100 mM sucrose. Buck semen was washed as described above and diluted with a medium containing TCG–6% egg yolk + 100 mM sucrose. Afterwards, the extended samples were allowed to equilibrate at 5 °C for 30 min. Samples were then drawn into a pipette, and droplets of 50 μL were allowed to fall directly into liquid nitrogen [16]. The resulting pellets were quickly thawed by placing them on a DPP70 thermo-regulated conical hotplate (INIA, Madrid, Spain) set at 60–65 °C. Previous reports showed fast warming to be important [17], and work in our laboratory disclosed it prevented damage to sperm cryopreserved at high cooling rates [18].

2.3. Sperm analysis

Fresh and frozen-thawed samples were assessed for sperm viability, acrosome integrity, motility, and kinematic variables. Sperm membrane and acrosome integrity were assessed by fluorescence using propidium iodide (PI) (Sigma-Aldrich®, St. Louis, MO, USA) combined with fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC) (Sigma-Aldrich®, St. Louis, MO, USA). A total of 200 sperm cells were evaluated per sample using a Nikon Eclipse E200 epifluorescence microscope (Nikon Instruments Inc., New York, NY, USA). Sperm membrane integrity was calculated as the sum of all PI-negative cells whereas acrosome integrity was calculated as the sum of all PNA-negative cells [19].

Sperm motility and kinetic variables were evaluated using a computer-aided sperm analysis system (CASA) (Sperm Class Analyzer, SCA®, v.4.0 software, Microptic S.L., Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope with negative contrast capability. A minimum of three fields and 500 sperm cell tracks were examined. The following parameters were determined: total motility (%), curvilinear velocity (µm/s), straight-line velocity (µm/s), average path velocity (µm/s), and the amplitude of lateral head displacement (µm).

2.4. Determination and localization of AQPs

AQPs were assessed in frozen-thawed samples. Western blotting (WB) and immunocytochemistry (ICC) were used to detect the presence and distribution of AQP3 in frozen-thawed spermatozoa employing a commercial rabbit polyclonal antibody [AQP3 - ab125219 from Abcam (Netherlands) B.V]. The antibody specificity was assessed using the corresponding AQP3 blocking peptide (Supplementary Fig.1.). For WB-analysis, proteins were extracted from~35 million spermatozoa. After three rounds of sperm centrifugation at 8200 x *g* for 5 min, where the first centrifugation aimed to remove the seminal plasma and the following to wash the cells with phosphate-buffered saline (PBS 1X) solution, the pellet was subjected to crude mechanical disruption and incubated with lysis buffer at 4 ºC for 60 min in agitation. The lysis buffer contained 6% sodium dodecyl sulfate (SDS), 125 mM Tris, 1 mM benzamide, 1% protease inhibitor cocktail, and 1 mM phenylmethylsulfonyl fluoride. The samples were then centrifuged again at 8200 *g* for 5 min, the supernatant was collected, and Laemmli-sample buffer (DTT, SDS, Tris, glycerol, b-mercaptoethanol, and bromophenol blue) was added. These protein suspensions were then denatured by heating at

94 ºC for 4 min. Aliquots of 35 µL were subsequently loaded onto 12% SDS-PAGE gels. Electrophoresis was performed at 150 V for 90 min, then transferred the proteins to Amersham™ Protran® 0.45 µm nitrocellulose membranes (Global Life Sciences Solutions, Buckinghamshire, UK) at 300 mA for 90 min. These were then blocked with 5% BSA (Merck KGaA, Darmstadt, Germany) in PBS-Tween for 60 min and incubated at 4 °C overnight with the primary antibodies (AQP3 - ab125219) at a dilution 1/1000. The membranes were then washed three times in PBS-Tween, and incubated with a secondary antibody (mouse anti-rabbit IgG-HRP, sc-2357) (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at a dilution of 1/15000 at room temperature for 120 min, followed by extensive washing in PBS-Tween. Finally, the membranes were revealed using WesternSure® PREMIUM, LI-COR® chemiluminescent substrate (Lincoln, NE, USA), employing the C-DIGIT instrument (LI-COR Bio-sciences) and analyzed by the IMAGE STUDIO 4.0 software (LI-COR Bio-sciences). A Western blot of mouse kidney tissue lysate (K) was performed to evaluate the specificity of the antibodies.

For ICC, the spermatozoa were fixed in 4% paraformaldehyde, centrifuged (1200 *g*, 6 min), and the pellet resuspended in PBS to prepare smears on slides. These were allowed to dry, washed with PBS-Tween, and blocked with 5% BSA in PBS for 60 min. After washing, the slides were incubated with the primary antibody against AQP3, diluted 1/100 in PBS containing 0.1% Tween 20 and 1% BSA, at 4 °C overnight before washing them again, and incubating with the secondary antibody (polyclonal goat anti-rabbit Alexa Fluor 488) (Molecular Probes, Invitrogen, Carlsbad, CA, USA) diluted 1/500 in PBS containing 0.1% Tween 20 and 1% BSA, for 180 min in the dark [20]. Finally, the samples were incubated for 5 min at room temperature with 2.5 µg/mL Hoechst 33342 (Sigma). In addition, a complementary assay was made to evaluate simultaneously the acrosome integrity and the

presence of AQP3 in this membrane domain. For this, the samples were incubated for 5 min with 1 µg/ml lectin-PNA Alexa Fluor 568 (Invitrogen, L32458) and 2.5 µg/mL Hoechst 33342.

Controls for the specificity of AQP3 antibody were previously established in our lab, using the corresponding AQP3 blocking peptide, incubating AQP3 antibody together with AQP3 blocking peptide five times concentration; as well as omission of the AQP3 primary antibody (Supplementary Fig.1.). The sperm membrane location of the AQP3 was examined by optical sectioning in fluorescence imaging (Zeiss Apotome 3) using an inverted Zeiss Axio Observer microscope at x 630 magnification, connected to a camera Zeiss Axiocam Mono. In addition, the percentage of spermatozoa showing AQP3, in different cell regions was determined (examining 200 cells per sample) using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc, New York, NY, USA).

2.5. Statistical analysis

All statistical analyses were performed using the STATISTICA software for Windows v.13.3 (Tibco® Inc., Tulsa, OK, USA). The values for sperm variables that showed non-normal distributions, as determined by the Shapiro–Wilk test, were normalized with the arcsine-transformation before analysis. Differences in the expression of AQP3 between conventional and ultra-rapid freezing methods were compared by ANOVA. Differences in the relative abundances of AQP3 bands in WB between samples with different sperm freezability were analyzed by t-test. A linear correlation (Pearson) was used to assess the significance of correlations between the proportion of sperm with AQP3 in the different

membrane domains and the sperm variables in frozen-thawed samples. Data were expressed as mean ± SEM. Where applicable, significance was set at $p<0.05$.

3. Results

Sperm quality variables of Merino rams and Murciano-Granadina bucks are depicted in Fig 1. Cryopreservation affected all sperm variables studied in either species. In rams, motility and all kinetic variables except ALH after freezing were greater in samples conventionally frozen than ultra-rapid frozen ($p<0.05$). Also, sperm viability was greater in samples conventionally frozen than ultra-rapid frozen ($p<0.05$). However, there were no significant differences were found in the proportion of spermatozoa with acrosome integrity between slow (control) and ultra-rapid freezing-thawing. In bucks, all sperm variables except ALH after freezing were greater in samples slow frozen than in ultra-rapid frozen ($p<0.05$).

AQP3 immunolabeling in all frozen-thawed sperm samples revealed AQP3 location in acrosome, post-acrosomal region, mid-piece, principal piece, and end-piece in both, ram (Fig 2) and buck samples (Fig 3). The proportion of spermatozoa showing immunolabeling of AQP3 in post-acrosome, mid-piece, and principal piece were greater after slow freezing-thawing (control) than after ultra-rapid freezing-thawing in both ram and buck samples (Fig 4). Simultaneous evaluation of acrosome integrity and presence of AQP3 in this membrane domain revealed that immunolabeling of AQP3 was always present in intact acrosomes, but was not seen when acrosome was damaged (Fig 5).

In overall frozen-thawed ram samples, the proportion of sperm with AQP3 located in the mid-piece was correlated with sperm motility ($R=0.44$, $p<0.01$), VCL ($R=0.45$, $p<0.01$), VSL

(R=0.47, p<0.01) and VAP (R=0.45, p<0.01). The proportion of sperm with AQP3 located in the principal piece was only correlated with sperm motility (R=0.35, p<0.05) and VSL (R=0.43, p<0.05) (Supplementary Fig. 2). In overall frozen-thawed buck samples, the proportion of sperm with AQP3 located in the mid-piece was correlated with sperm motility (R=0.46, p<0.01), VCL (R=0.55, p<0.01); VSL (R=0.65, p<0.001), VAP (R=0.62, p<0.001), sperm viability (R=0.54, p<0.01) (Supplementary Fig. 3). The proportion of sperm with AQP3 located in the principal piece was only correlated with sperm motility (R=0.46, p<0.01) and VAP (R=0.36, p<0.05). No significant correlation was seen between the proportion of sperm with AQP3 located in the post-acrosome and any of the sperm variables in both bucks and rams.

WB confirmed the presence of AQP3 in the spermatozoa of the studied species. WB identified AQP3 as a band of about 32 kDa in either slow or ultrarapid freezing samples (Fig 6.A), coinciding with mouse kidney tissue lysate. Relative abundances of AQP3 32 kDa band did not show significant differences between control (slow freezing-thawing) and ultra-rapid freezing-thawing samples (Fig 6.B).

4. Discussion

This is the first study identifying AQP3 in spermatozoa of goat. Even though no significant differences were found in the amount of AQP3 according to the freezing-thawing methods used, the results revealed that freezing-thawing method affected the domain location of AQP3 in both species. The domain localization of AQP3 in both sheep and goat spermatozoa was observed in the acrosome, post-acrosome, mid-piece, principal-piece, and

end-piece. A similar location has been found in other small ruminant species, such as ibex (the wild ancestor of goats), and mouflon (the wild ancestor of sheep) [21,22]. In rams, the location was similar to that described in a previous report [11]. The WB-assay revealed a band of about 32 kDa, similar to previous findings in other small ruminant species: mouflon, ibex [21], and chamois [22]. All these data suggest that AQP location remained constant in phylogenetically related species. Unlike small ruminants, AQP3 is only located in the mid-piece of bull sperm [23], and the band is observed at 42 kDa [24]. A band of 28 kDa has been observed in dromedary camel [25]. This high heterogeneity might be related to different factors, such as dimerization capacity with other proteins or different isoforms. Moreover, many bands are visible in the immunoblots, which disappear after incubations with the AQP3-blocking peptide suggesting the existence of different isoforms of AQP3 in membrane sperm possibly due to glycosylation and multimer formation [26] of the protein.

The fact that AQP3 was observed located in different areas of small ruminant spermatozoa (e.g. acrosome, mid-piece, principal piece) suggests a putative role in many physiological functions [27], i.e.in acrosome reaction and the metabolism (oxidative phosphorylation in the mid-piece and anaerobic glycolysis in the principal piece) supporting kinetic activity of spermatozoa. This metabolic function of AQP3 and other aquaglyceroporins may be related to the transmembrane passage of solutes such as glycerol and lactate [28]. The velocity or global capacity for the transmembrane passage of glycerol might be involved in sperm cryoresistance and cryodamage exerted by the cryopreservation method. AQP3 allows better sperm osmo-adaptation, but an adequate flux of water and glycerol is needed to avoid osmotic stress related to their expression in the different areas of sperm [21]. The fact that the proportion of sperm with AQP3 located in the mid-piece, which is packed with mitochondria

performing oxidative phosphorylation, was correlated with the sperm motility and the kinematic variables in both bucks and rams, highlights the role of AQP3 in sperm bioenergetic metabolism. AQP3 re-localization appears linked to an increase in the osmo-adaptative capacity of ejaculates with a better capacity to withstand freeze-thawing. Consistently, the expression of AQP3 in the mid-piece and principal piece was greater in frozen-thawed samples than in fresh samples of ejaculates classified as of good freezability [11]. The greater harmful effect of ultra-rapid freezing-thawing seems associated with a lower proportion of sperm showing AQP3 in the post-acrosome, mid-piece, and principal-piece. Although AQP3 was not analyzed in fresh samples in the present study, data suggest that after ultra-rapid freezing-thawing, AQP3 had a lower relocation capacity in these sperm areas and, thus, a smaller osmo-adaptative capacity, providing new exciting information about the factors that affect AQPs relocation. Along with the known influence of freeze-thawing [9], the results suggest that AQP3 also adapt their membrane location to osmotic changes depending on the cooling rate. Changes in subcellular localization of AQPs appear to be a fast way to regulate dynamic fluid homeostasis. AQPs are relocated through internalization by endocytosis into intracellular vesicles (endosomes), which transfer them to other membrane domains [29]. Other alternative way of AQPs relocation is by exocytosis of storage vesicles, which can also lead to changes in the amount of AQPs in the plasma membrane [29]. In neoplastic prostatic cells, a cAMP-PKA (protein kinase A) dependent pathway promoted AQP3 relocation [30]. In colonic epithelial cells, intracellular Ca2+ signaling resulted in the activation of PKC which promoted AQP3 translocation [31]. Cryopreservation process might redistribute AQP3 relocation in sperm cells through similar ways by signaling pathways including PKA and/or PKC as those described above, but molecular details are yet to be investigated.

Despite there were no differences in the relative amount of AQP3 between samples cryopreserved by both methods, a significant increase in the proportion of spermatozoa labelled for AQP3 was found after conventional freezing. This might be explained by a more efficient AQP3 translocation from the perinuclear cytoplasm to the plasma membrane [32] in slow freezing-thawing samples. We can hypothesize that a high cooling rate during ultra-rapid freezing (the ultra-rapid freezing by spheres procedure allows a cooling rate of about 600 ºC/min; [33]) does not allow sufficient time for adequate relocation of AQP3, and therefore, can be a partial explanation for the different results with both techniques. Alternatively, these effects could also result from thawing, as differences were seen between the control slow freezing-thawing hereby used with the experimental method that included ultra-rapid freezing but also ultra-rapid thawing; considering that fast warming is relevant to prevent sperm membrane damage [17,18].

As reported in other species (i.e. pig; [34]), the expression of AQP3 improves sperm cryotolerance. A decreased proportion of spermatozoa showing AQP3 in the mid-piece and principal-piece when frozen using the ultra-rapid method could indicate a decrease in the trans-membrane flux of glycerol and other solutes. Considering these play a relevant intermediary role in energy production during the glycolysis pathway, providing energy as the ATP form for tail movement and sperm motility [35]; such changes could lead to a metabolic imbalance [28, 36] that would explain the lower kinetic variable values as observed in the present study. Indeed, the inhibition of AQP3 with phloretin in mouflon sperm negatively affects sperm motility variables [37]. In human spermatozoa, where AQP3 is also located in the tail, a low expression of AQP3 is associated with low sperm motility [38]. AQP3 is also considered a peroxiporin, which allows the transport and removal of reactive

oxygen species (ROS) such as H_2O_2, thus, modulating oxidative stress [39]. A decreased proportion of spermatozoa showing AQP3 in the mid- and principal-piece after ultra-rapid freezing-thawing might contribute to greater oxidative damage and decreased sperm viability

In conclusion, the cooling rate modifies the location of AQP3 in spermatozoa of sheep and goat. Further studies are needed to confirm that changes in AQP3 localization, related to the freezing-thawing method, underlie sperm cryosurvival.

Supporting information

Supplementary Fig.1. (A) Negative control: sample only incubated with secondary antibody, omitting the primary antibody step (optical sectioning in fluorescence imaging; Zeiss Apotome 3). (B) Immunofluorescence of the peptide competition assay for the anti-AQP3 antibody (AQP3 + blocking peptide) (epifluorescence microscopy; Nikon Eclipse E200). (C) Western blot resulting from incubations with the AQP3-blocking peptide. K: kidney mouse tissue lysate. R10, R6, R13, R17, R14, R16, R19, R8: Individual ram identification. B68, B72, B70, B48: Individual buck identification.

Supplementary Fig. 2: Correlation between the proportions of sperm with AQP3 located in different membrane domains and sperm motility variables in ram frozen-thawed samples (samples cryopreserved using slow and ultra-rapid methods).

Supplementary Fig. 3: Correlation between the proportions of sperm with AQP3 located in different membrane domains and sperm motility variables in buck frozen-thawed samples (samples cryopreserved using slow and ultra-rapid methods).

Conflict of interest

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

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Figure captions

Fig 1. Sperm quality variables (mean±SEM) of Murciano-Granadina buck and Merino ram semen samples. Motility and kinematics variables in rams (A) and bucks (C). Percentage of viable spermatozoa and spermatozoa with acrosome integrity in rams (B) and bucks (D). Different letters (a, b, b) indicate significant differences ($p < 0.01$) between cryopreservation methods for each sperm variable. Curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head (ALH). Results are expressed as mean ± SEM.

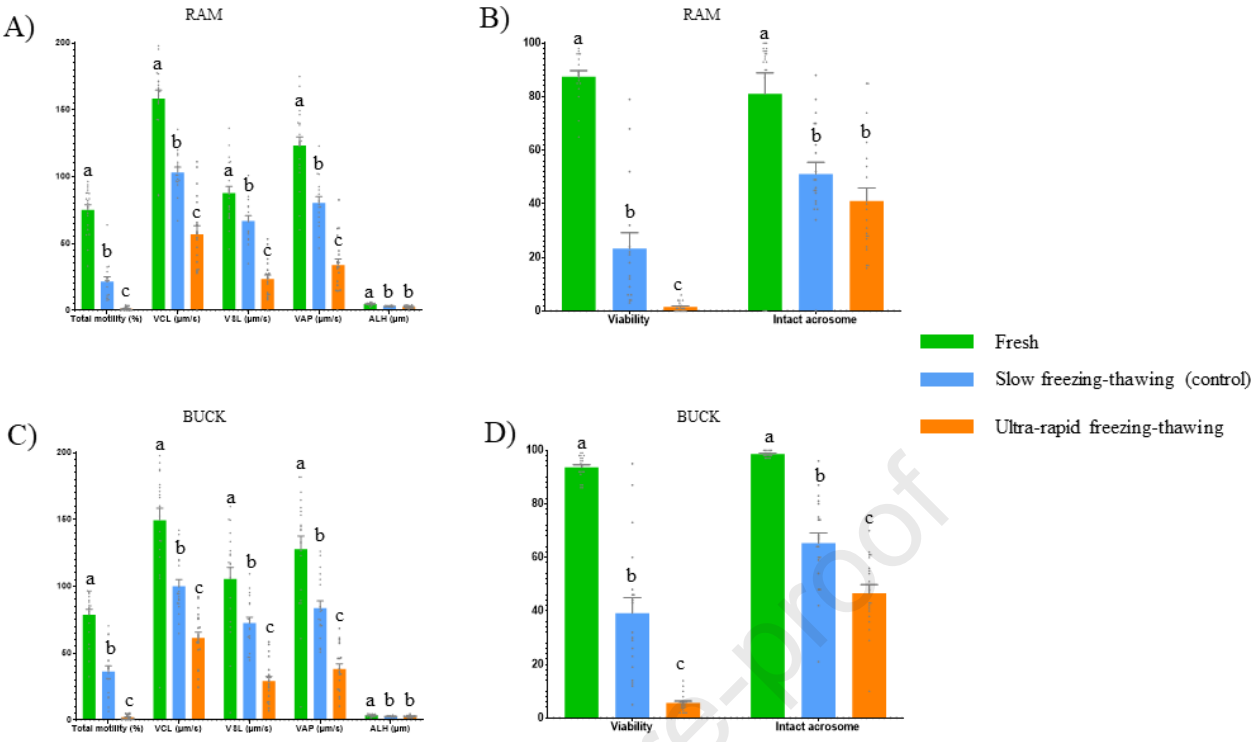
Fig 2. Immunolabeling of AQP3 in conventional (A) and ultra-rapid (B) frozen-thawed. Immunolabeling of AQP3 located in the acrosome, post-acrosomal region, mid-piece, principal piece, and end piece in ram spermatozoa (shown with arrows).

Fig 3. Immunolabeling of AQP3 in conventional and ultra-rapid freezing-thawing. Immunolabeling of AQP3 located in the acrosome, post-acrosomal region, mid-piece, principal piece, and end piece in buck spermatozoa (shown with arrows).

Fig 4. Proportion (mean±SEM) of fresh and frozen-thawed spermatozoa showing AQP3 in different membrane domains of spermatozoa. Different letters indicate significant differences (A-B $p<0.01$, a-b $p<0.05$) between cryopreservation methods.

Fig 5. Immunolabeling of AQP3 in slow (A) and ultra-rapid (B) frozen-thawed spermatozoa. Sperm nuclei were stained with Hoechst (blue) and acrosomes by PNA staining (red). Immunolabeling of AQP3 (green) located in intact acrosome, but was not seen when acrosome was damaged (arrows) in either ram or buck.

Fig 6: Identification of AQP3 by Western blotting. A) Immunoblots for AQP3 in Merino ram and Murciano-Granadina buck spermatozoa of ejaculates frozen-thawed by slow (S) and ultra-rapid (R) methods; K: mouse kidney tissue lysate. Arrow indicates AQP3 band of about 32 kDa in either slow or ultrarapid freezing samples, and K. B) Relative abundances of AQP3 bands (as mean ± SEM) from the samples cryopreserved by slow (control) and ultra-rapid cryopreservation method. No significant differences were found between cryopreservation methods after quantification of 32 kDa bands and normalization using tubulin protein as an internal standard.



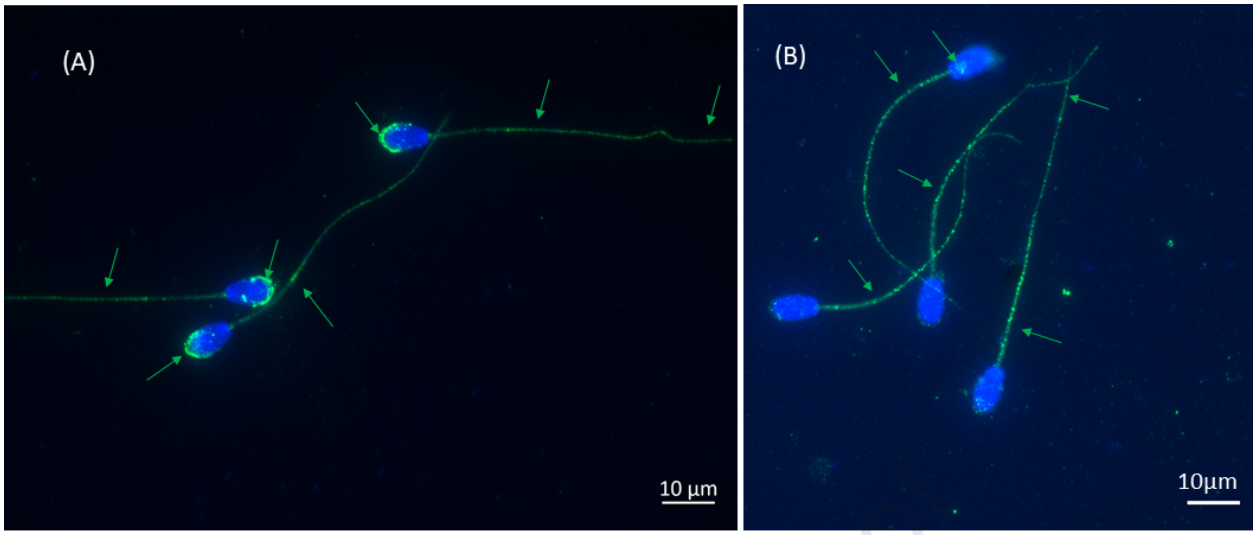
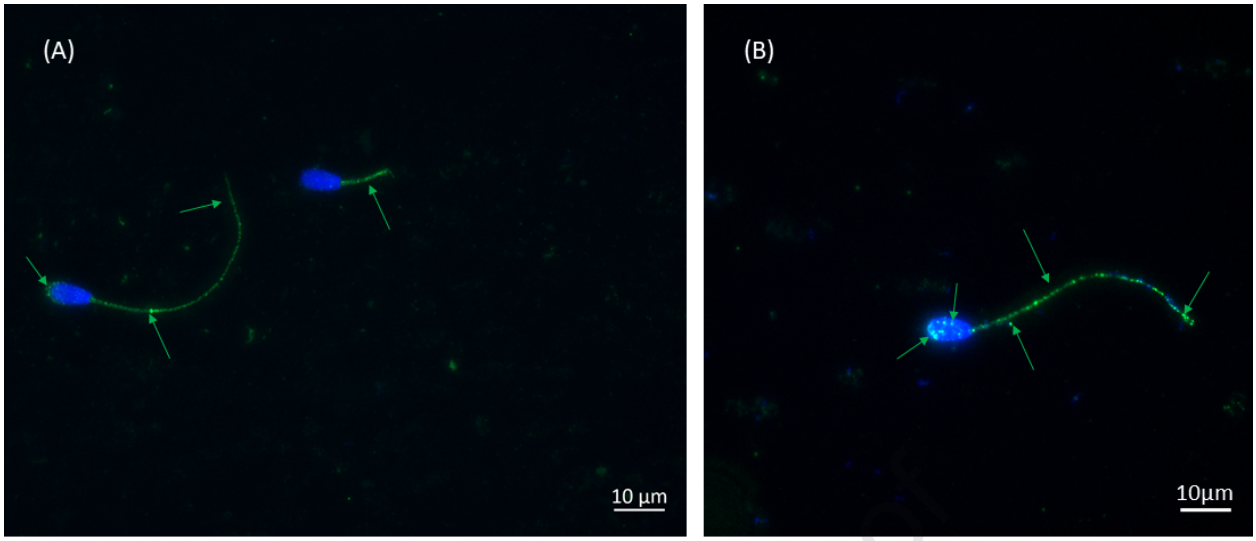


Fig 2. Immunolabeling of AQP3 in conventional (A) and ultra-rapid (B) frozen-thawed. Immunolabeling of AQP3 located in the acrosome, post-acrosomal region, mid-piece, principal piece, and end piece in ram spermatozoa (shown with arrows) .



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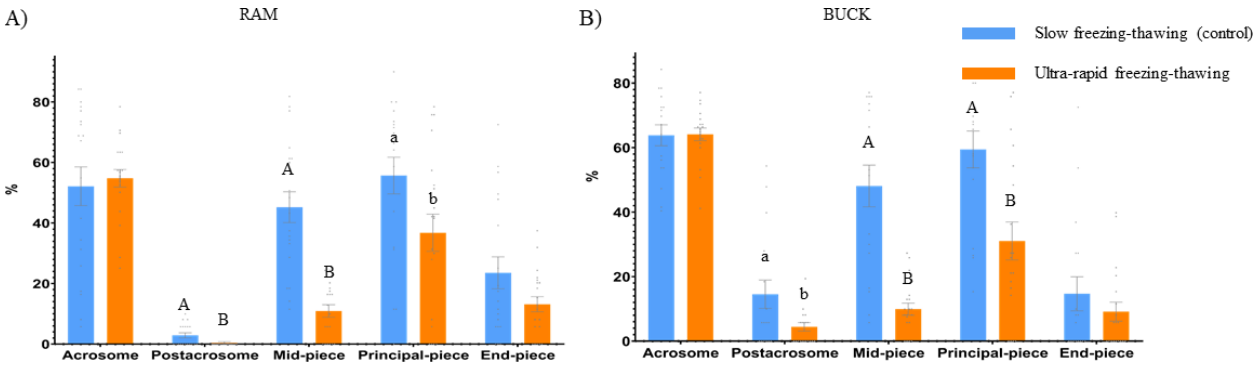

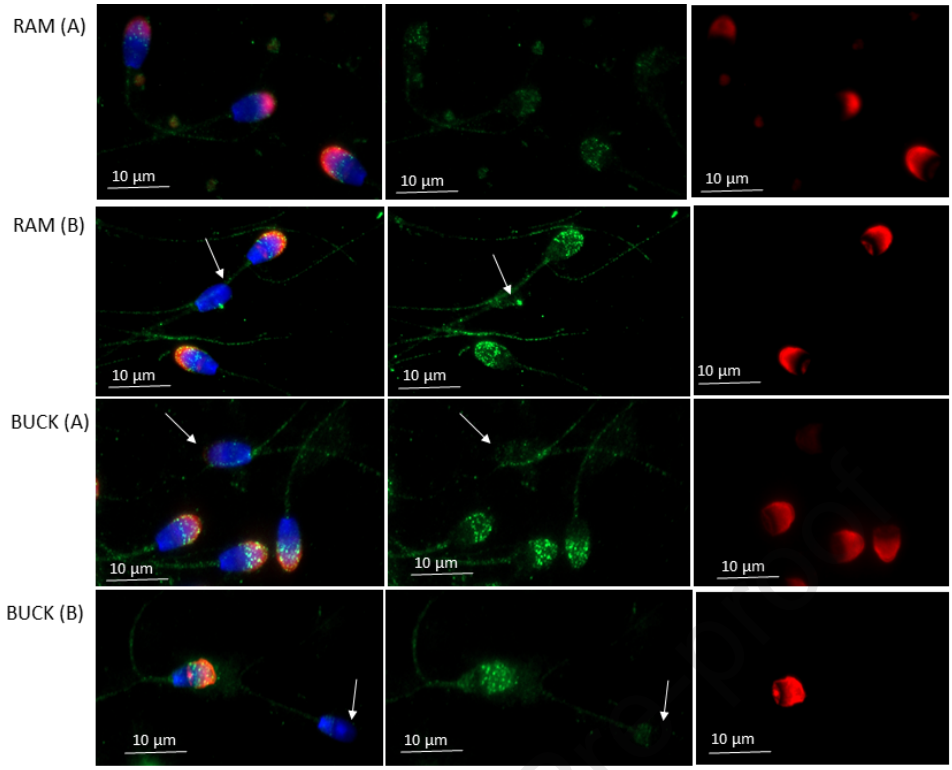

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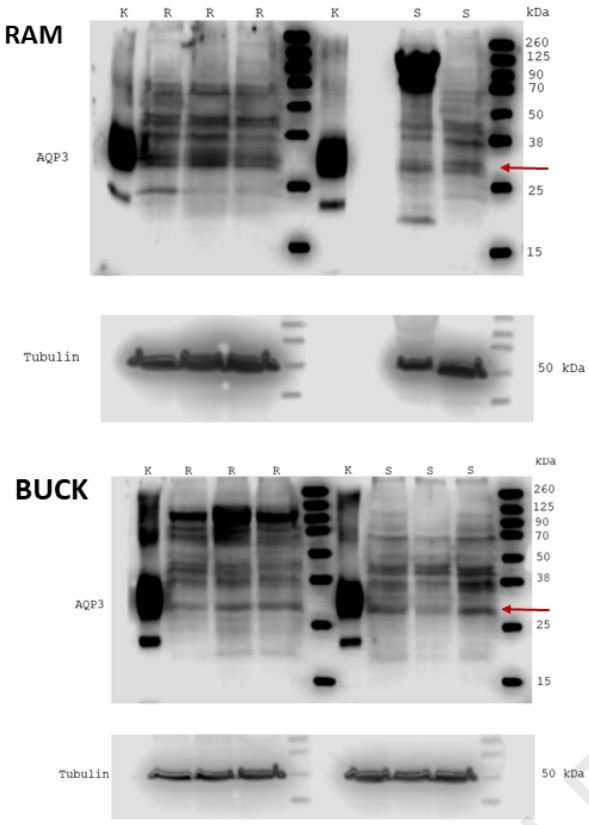
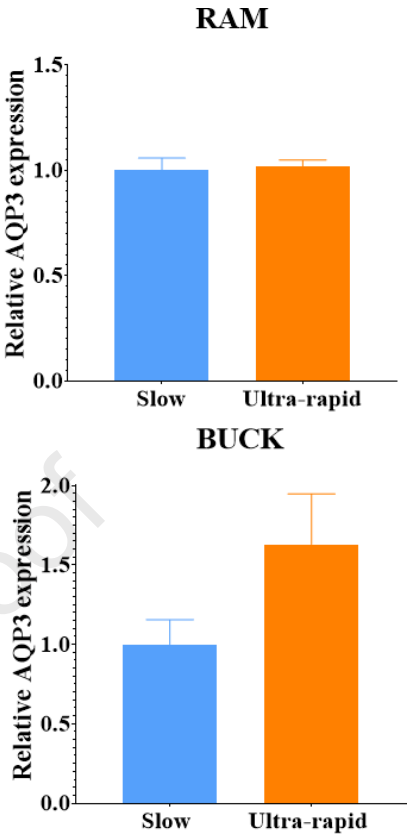


B)



Highlights

- This is the first study identifying AQP3 in spermatozoa of goat.
- Cooling rate modifies the location of AQP3 in spermatozoa of sheep and goat.
- Changes in the location of AQP3 appears to be related to sperm cryosurvival.