Journal Pre-proof

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PII: S0093-691X(22)00256-4

DOI: https://doi.org/10.1016/j.theriogenology.2022.06.029

Reference: THE 16331

To appear in: *Theriogenology*

Received Date: 4 October 2021

Revised Date: 3 June 2022

Accepted Date: 25 June 2022

Please cite this article as: O'Brien E, Malo C, Castaño C, García-Casado P, Toledano-Díaz A, Martínez-Madrid Belé, Rodriguez-Martinez H, Álvarez-Rodríguez M, Santiago-Moreno Juliá, Sperm freezability is neither associated with the expression of aquaporin 3 nor sperm head dimensions in dromedary camel (*Camelus dromedarius*), *Theriogenology* (2022), doi: https://doi.org/10.1016/j.theriogenology.2022.06.029.

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Sperm freezability is neither associated with the expression of aquaporin 3 nor sperm head dimensions in dromedary camel (*Camelus dromedarius*)

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Abstract

The expression of aquaglyceroporin 3 (AQP-3) has been demonstrated in the spermatozoa of several mammalian species and its role has been associated with cryotolerance. Post-thaw sperm quality from individual dromedary males with different response to freezing-thawing process was evaluated through sperm head morphometry. In order to understand the cellular mechanisms affected by cryoinjury we have explored the presence and distribution of sperm AQP-3 using western blotting and immunocytochemistry. WB showed different intensity of the specific signal bands at 28 kDa. Immunofluorescence assessments allowed us to identify five different and clear AQP-3 distribution patterns of labelling in the sperm plasma membrane; acrosome, post-acrosome, mid-piece, and principal and final tail. Although expression of AQP-3 varied among male ejaculates, the individual sperm response to freeze-thawing was not associated with AQP-3 expression. Thus, AQP3 expressions do not seem like a reliable predictor of sperm response to freeze-thawing process in this species. This work is the first to describe the morphometric characteristics of the heads of dromedary spermatozoa. No correlation was found between sperm head dimensions and sperm quality variables after freeze-thawing suggesting that dromedary camel sperm head morphometry is also not a reliable predictor of cryosurvival.

Keywords: dromedary, cryopreservation, semen, aquaglyceroporin

1. Introduction

Reproductive technologies are used routinely in several domestic animals such as horse, cow, pig, and sheep, and more recently they have been successfully used for the genetic improvement of dromedary camel (*Camelus dromedarius*), focusing on milk production and racing [1]. Although methods for the collection and artificial insemination (AI) with fresh semen are now well established, there are no reliable methods for commercial application of AI using cryopreserved camelid semen.

The semen of dromedaries has characteristics that makes difficult its processing and preservation, including low volume, poor quality and high viscosity [2,3]. In addition, camelid spermatozoa present little tolerance to freezing and thawing [1,4] compared to other species. Perhaps this is not surprising, since most dromedary sperm freezing protocols have been extrapolated from other species. Nowadays, specific protocols have been tested, using different extenders [5], cryoprotectants [6,7], cooling rates and temperatures for thawing [8], and further, adding antioxidants to the extenders [3,9], with diverse, yet promising prospects.

Sperm cryoresistance, osmotic tolerance as well as its sensitivity to manipulation (i.e., centrifugation, pipetting) varies in a species-specific way [10]. Sperm cryoresistance has been related to membrane lipid composition [11, 12], despite this not been sufficient to account for the difference in susceptibility of the semen of various species to cold shock. As alternative, the large variation in the damage caused by a particular freezing technique has been proposed as related to the specific dimensions and shape of the sperm head, suggesting they could be a reliable indicator for cryosurvival. Evidence is, however, still controversial. On one hand, studies on individual bull ejaculates suggested that the variability in sperm head measurements follows sperm cryosurvivability [13]. On the other hand, follow-up studies carried out in 19 different species showed this relationship was less clear: no significant correlations were found

between cryoresistance and sperm head dimensions [14]. To the best of our knowledge, there are no parallel sperm morphometric studies carried out in dromedaries.

Another important cryobiological property that determines the success of sperm cryopreservation is the permeability of the plasma membrane to water and cryoprotective agents (CPAs) as glycerol. The capacity for spermatozoa to respond to cell volume adjustments is determined by several factors including active water channels as aquaporins (AQPs). These are small integral membrane proteins which regulate transcellular water flow across the plasma membrane. Thirteen types of AQPs have been identified in mammals (reviewed in 15), where AQP-3, AQP-7, AQP-9, and AQP-10, the so-called aquaglyceroporins, facilitate the transport of not only water but also glycerol [16,17]. All this makes aquaglyceroporins an interesting study target to find the mechanisms behind failures of sperm freezing protocols [18], aiming their improvement.

Particularly, the expression of AQP-3 in several mammalian spermatozoa [19] has been demonstrated and its role has been associated with the osmoadaptation, motility and mitochondrial membrane potential in human [19,20], sperm tolerance to freeze-thawing in bovine [21] and cryotolerance in porcine [22]. As far as we know, there are no previous studies on the determination and localization of AQP-3 in dromedary spermatozoa. Therefore, in order to understand the cellular mechanisms that affect cryoinjuries during the cryopreservation process in dromedary spermatozoa, we studied post-thaw sperm head morphometry in individual dromedary males with different responses to freezing-thawing. In addition, we explored the expression and membrane location of AQP-3 protein in frozen-thawed dromedary spermatozoa to determine whether its relative expression and/or distribution relates to sperm freezability.

2. Materials and Methods

2.1 Reagents

All reagents were purchased from Merck KGaA and/or its affiliates (Darmstadt, Germany) and from Roche (Basel, Switzerland).

2.2 Experimental animals and semen collection

Six adult dromedary camel males, eight to ten years old, of proven fertility from the Camel Reproduction Centre, Dubai, UAE, were used in this study. Two ejaculates per male were obtained using an artificial vagina [23], between February and March, which is during the breeding season. Ejaculates were immediately taken to the laboratory and placed in a 37 ºC water bath. All animal procedures were approved by the Animal Care and Use Committee (ACUC) of the Camel Reproduction Centre, UAE.

2.3 Sperm cryopreservation

Semen was diluted (1:5) with Tris-Citrate-Fructose Buffer (TCF, 300 mM Tris, 94.7 mM citrate and 27.8 mM fructose) at 37 ºC and manually liquefied by gentle pipetting. Semen was then subjected to centrifugation at 300 x g for 20 min at 25 ºC. The supernatants were aspirated using a Pasteur pipet and discarded. Sperm concentrations were calculated and pellets were then re-suspended in the required volume of F1 (Green Buffer supplemented with 20% (v/v) egg yolk) to obtain a final concentration of 200 x 10^6 sperm/mL, and cooled to 5 ºC during a 2 hour period in a water jacket according to Malo et al. [9].

A second extension step (1:1) was performed with F2 [Green Buffer extender containing 20% egg yolk and 6% (v/v) glycerol] at 5 ºC, resulting in a final glycerol concentration of 3% and cell concentration of 100 x 10^6 sperm/mL [9]. Pre-cooled plastic straws (0.5 mL) were filled with cooled semen in a cold cabinet and, after 30 min of equilibration, were frozen in static liquid nitrogen vapour (1 cm above the liquid) for 15 min and then plunged into liquid nitrogen (-196 ºC) for storage.

2.4 Assessment of sperm variables

The evaluation of the fresh semen was carried out at the Camel Reproduction Centre, Dubai, United Arab Emirates. The pre-freeze sperm variables evaluated included total and progressive motility, kinematics (Computer Assisted Sperm Analysis, CASA) and acrosome and membrane integrity. Sperm motility was assessed by CASA as previously described [8] using the CEROS II® (Hamilton Thorne; MA; USA) at 10X on the heated stage of a phase contrast microscope (AX10 Zeiss, Gottingen, Germany). Plasma membrane integrity was evaluated using the fluorescent probes SYBR-14 (SY) and propidium iodide (PI: L-7011, Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, the Netherlands) and acrosome status was assessed by fluorescein isothiocyanate, conjugated with peanut agglutinin (FITC-PNA) staining, as previously described [9].

Thawing and analysis of the frozen-thawed dromedary semen was performed at the Department of Animal Reproduction, Spanish National Institute for Agricultural and Food Research and Technology (INIA-CSIC), Spain. Thawing was done at 37 ºC for 30 seconds and sperm motility (total, progressive and kinetic variables of the motility) and the membrane and acrosome integrity assessed. In addition, sperm samples were processed to morphometric analysis and inmuno-detection of AQP-3. When sperm extension was required, the Green Buffer (GB, IMV Technologies) extender + 3% glycerol were used.

Post-thaw sperm motility was examined using a computer-aided sperm analyses (CASA) system coupled to a phase contrast microscope (Nikon Eclipse model 50i; Nikon Instruments Europe B.V., Izasa S.A.; negative contrast) and employing a Sperm Class Analyzer (SCA®, Barcelona, Spain) v.6.5.0.5 software (Microptic S.L., Barcelona, Spain). Semen was extended to a concentration of approximately 40 million sperm/mL [24,25], and loaded into a warmed (37 ºC) 20 µm Leja® 8- chamber slides (Leja Products B.V., Nieuw-Vennep, The Netherlands). The percentage of total and progressive motility were recorded. A minimum of three fields and

500 sperm tracks were evaluated at a magnification of 100 x for each sample. The recorded motility kinetic variables included curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s) and amplitude of lateral head displacement (ALH, µm). The status of the plasma and acrosome membranes were assessed using a combination of propidium iodide (PI, Sigma P4170) and fluorescein isothiocyanate-conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC, Sigma L7381) as previously described [26]. A total of 200 cells per sample were evaluated using a Nikon Eclipse E200 epifluorescence microscope (Nikon Instruments Inc, NY). Also, post-thaw sperm viability were assessed using a seminal quality system SQS2® (ZoitechLab, S.L. – ARQUIMEA GROUP, Madrid, Spain) previously validated in our laboratory [27].

2.5 Morphometric analysis of frozen-thawed dromedary samples

Sperm head morphometry was analyzed according to Esteso et al. [28]. Briefly, sperm smears were air-dried and stained using Hemacolor®. Sperm head length, width, area, perimeter and percentage of acrosome region data were acquired for 100 cells. Stained slides were examined for sperm head morphometry using the CASA-Morph module of the Sperm Class Analyzer® v.6.5.0.5 software package (Microptic S.L., Barcelona, Spain).

2.6. AQPs assay

Spermatozoa were examined for the presence and distribution of sperm membrane AQP-3 by western blotting (WB) and immunocytochemistry (ICC) using commercial rabbit polyclonal antibodies against AQP-3 (ab125219 – Abcam) according to Santiago Moreno et al, 2022 [29]. Before starting to carry out the immunodetections, a blast analysis was performed. It showed that the AQP-3 sequence is conserved and the antibodies are a reliable tool to recognize this protein in dromedary samples (**Supplementary material 1**). For WB, sperm proteins were extracted in lysis buffer (6% SDS, 125 mM Tris pH 7.5, 1 mM benzamide, 1 mM PMSF and

158 1% cocktail of proteases inhibitors) at 4 °C. Once extracted, the protein samples were
159 centrifuged at 11,000 x g for 5 min and the supernatants mixed with sample buffer containing
160 50 mM DTT, incubated at 94 °C for 4 min and subjected to 12% SDS–PAGE (Laemmli, 1970).
161 Electrophoresis was run at 150 V for 90 min and each lane was loaded with 35×10^6 cells.
162 Proteins were transferred to nitrocellulose membranes (Amersham™ Protran®, GE
163 Healthcare) at 300 mA (constant) at 4 °C for 90 min. Immunoblotting was performed using
164 1:1,000 dilution of anti-AQP-3 antibody (ab125219, Abcam) following the manufacturer's
165 directions, and a 1:5,000 dilution of a secondary mouse anti-rabbit IgG-HRP labelled antibody
166 (sc-2357-CM, Santa Cruz). The membranes were scanned using WesternSure® PREMIUM,
167 LI-COR® chemiluminiscent substrate (Lincoln, NE, USA), employing an Amersham™ ECL
168 Western Blotting ImageQuant™ 500 chemiluminiscent imaging system (Ge Healthcare).
169 Western Blotting of sheep small intestine tissue lysate (I_s) and mouse kidney tissue lysate (K_r)
170 (positive control) were performed to test the specificity of the antibodies used. The specificity
171 of the primary anti-AQP-3 antibody was assessed by incubation with the corresponding
172 synthetic AQP-3 peptide (ab195690, Abcam) concentrated 10-times in excess in relation to the
173 primary antibody, 1 h at room temperature, in gentle agitation. All membranes were stripped
174 and western blot for actin detection was performed as loading controls. The intensity of protein
175 bands was quantified by band densitometry analysis using ImageJ software (U. S. National
176 Institutes of Health, Bethesda, Maryland, USA). The signal from each extract was normalized
177 to housekeeping (actin).
178 For ICC, the spermatozoa were fixed in 4% paraformaldehyde, centrifuged (1,200 xg for 6
179 min) and the pellet resuspended in PBS to prepare smears on slides. The smears were allowed
180 to dry, washed with PBS-Tween and blocked with 5% BSA (Sigma- Aldrich, Sweden) in
181 PBS for 60 min. After washing, the slides were incubated with a 1:100 dilution of anti-AQP-
182 3 antibody at 4 °C, overnight. After incubation, the smears were washed before incubation

with the secondary antibody (polyclonal goat anti-rabbit Alexa Fluor 488, Molecular Probes, Invitrogen, Carlsbad, CA, USA, diluted 1:1,000 in PBS containing 1% BSA) in darkness for 180 min [30]. To visualize the sperm nucleus, Hoechst (33342) staining was performed. Washed slides were mounted with ProLong™Glass antifade medium (ThermoFisher).

The percentage of sperm showing AQP-3 in the different sperm membrane sub-domains (acrosome, post-acrosomal region, mid-piece, principal piece of the tail and final piece of the tail) was evaluated by one and the same observer counting 200 spermatozoa per sample using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc., New York, NY, USA). AQP3 presence and localization was determined by excitation of samples at 450-490 nm (emission 520 nm). Hoechst-counterstained nuclei were observed under 330-380-nm excitation (emission 420 nm). The image resulting from the capture of the different channels showed AQP3 and nuclei as green and blue, respectively. The negative control (NC) was performed without primary antibodies.

2.7 Statistical analysis

Data were expressed as means ± SEM (standard error of the mean). All statistical calculations were made using Statistica™ software for Windows v.13.3 (Tibco® Inc., Tulsa, OK, USA). The significant level was set at $p < 0.05$. Each ejaculate was treated as an independent statistical case. Data were first tested through Shapiro–Wilk and Levene tests for normality and homogeneity of variances and transformed when required. Quality sperm variables of fresh samples were analysed by t-test. Quality sperm variables and the proportion localization patterns of AQP-3 were analysed by one-way ANOVA, and the Tukey post hoc test was performed when significant differences were found ($P < 0.05$). Differences in the relative abundances of AQP3 bands in WB between animals with different sperm freezability were analysed by Mann-Whitney U test. Morphometric analysis of frozen-thawed sperm head was analysed by one-way ANOVA. For the k-means cluster analysis to identify four subpopulations

of sperm head area, Statistica specifically uses Lloyd's method to implement the k-means algorithm [31]. Spearman correlations were used between sperm variables, head area and expression of AQP-3 in frozen-thawed spermatozoa.

3. Results

Among the fresh sperm quality parameters examined, only VCL and ALH differed significantly ($p < 0.05$) between males, mostly due to male 1 separating from the others (**Table 1**). All sperm variables declined after freezing-thawing (**Table 2**). Samples of male 1 showed best motility results (37.54 ± 1.38% total motility, 7.76 ± 1.14% progressive motility and 48.92 ± 2.77 μm/s VCL) while values of male 5 were the worst (18.92 ± 1.10% total motility, 1.63 ± 0.20 % progressive motility and 34.84 ± 1.11 μm/s VCL). Males 1, 2 and 3 showed higher values ($p < 0.05$) of viability than males 4, 5 and 6. In addition, males 1, 2, 4 and 6 showed higher values ($p < 0.05$) of acrosome integrity than males 3 and 5. Taking into account the viability of the six males, we identified three of them (males 1, 2, and 3) as "good freezers" (male 1 presented very good freezability) and three of them as "poor freezers" (males 4, 5 and 6).

The mean freezing-thawing sperm head dimensions (head length, width, perimeter, area and percentage of acrosome region) are shown in **Table 3**. The sperm heads area of male 3 was the smallest (18.94 ± 0.29 mm^2) and the sperm head area of male 4 was the largest (21.95 ± 0.57 mm^2). Four well-defined subpopulations (sp1 to sp4 of **Table 4**) were identified in the frozen-thawed sperm samples according to sperm head area. The proportion of the largest (sp1) and smallest (sp4) subpopulations were poorly represented. Yet, the sp3 subpopulation was most representative for all males: maximum for male 2 (19.62 ± 1.02, 42.82%) and minimum for male 6 (18.7 ± 1.00, 37.78%).

231 The WB confirmed the presence of AQP-3 in all frozen-thawed dromedary sperm samples
232 (**Figure 1**). Expression of AQP-3 varied among ejaculates, as shown by the different intensity
233 of the specific signal bands that appeared at 28 kDa. The specificity of the antibody was
234 confirmed by peptide competition assays and positive controls (Figure 1B). A clear band of 42
235 kDa in intestine tissue lysate (lane I_s, Figure 1A) and 34 kDa in kidney tissue lysate (lane K_r,
236 Figure 1B) were detected. There were no differences in the relative amount of AQP3 between
237 males with greater sperm viability (males 1, 2, 3) and males with lower viability (males 4, 5,
238 6) after freezing-thawing (Figure 1C).
239 Immunofluorescence established five localisation patterns of membrane AQP-3 (**Figure 2**) in
240 frozen-thawed dromedary spermatozoa. The patterns appeared in the different sub-domains of
241 the plasma membrane in the acrosome, post-acrosome region, mid-piece, principal piece of the
242 tail or final piece of the tail. Negative controls without primary antibodies (NC of Figure 2)
243 confirmed the specificity of all antibodies. The proportion of spermatozoa with localization
244 patterns of AQP-3 in frozen-thawed dromedary spermatozoa are shown in **Figure 3**. There
245 were no differences in AQP-3 distribution patterns between males except for acrosomal and
246 midpiece location. The proportion value for the acrosome pattern for male 3 was higher ($p <$
247 0.05) than male 5 and, for the midpiece pattern, the value for male 6 was higher ($p < 0.05$) than
248 those of male 2. There were only negative correlations ($r = -0.94$; $p < 0.05$) between the
249 expression of AQP-3 in sperm acrosome and the sperm acrosomal integrity.

250 **4. Discussion**

251 The presence of AQPs had not yet been previously demonstrated in dromedary spermatozoa.
252 The present findings revealed, for the first time, the presence of AQP-3 in frozen-thawed
253 dromedary sperm membranes. The WB assays revealed a single specific band of 28 kDa for
254 AQP-3 in frozen-thawed dromedary spermatozoa. Other studies showed similar results in
255 spermatozoa from other species: a single band of 25 kDa was identified in boar [32] while

bands were identified in bull either at 30 kDa [33] or 42 kDa [21]. Our findings showed that AQP-3 could be located in membrane domains at different distribution patterns in frozen-thawed spermatozoa: acrosome, post-acrosome region, mid-piece and principal or final piece of the tail. Similar patterns were found in other investigations carried out in boar [32]. Instead, AQP-3 was mainly found in bull sperm mid-piece samples [33]. Meanwhile, other studies showed that AQP-3 was clearly found in the principal piece of the tail of bull spermatozoa [21]. Interestingly, we found a negative correlation between sperm acrosomal integrity and the expression of AQP-3 in sperm acrosome. This would mean that the lower the percentage of acrosomal integrity after freeze-thaw the higher expression of AQP-3 in the sperm acrosome. This does not make any biological sense, since AQPs are supposedly present in the membrane, and, if the acrosome is damaged, the suprayacent plasma membrane must be gone. An alternative explanation is that the AQPs labelling remains in fragments of the damaged plasma membrane. Further analyses are thus required to determine the details behind this situation.

AQP activity is modulated through regulation of gene expression, changing the localization of the already existing proteins in the cells and a direct regulation of their activity *in situ*. In the particular case of spermatozoa, where there is no *de novo* protein synthesis, the regulation mechanisms of AQPs could be by trafficking (reviewed in [34]) or re-localization and either by the switching-on of molecular mechanisms through post-translational modifications (reviewed in [35]). In addition, the cellular functions of AQPs are regulated by posttranslational modifications, e.g. phosphorylation [36], ubiquitination [37] glycosylation [38], among others. In boar spermatozoa, the redistribution of AQP-7 appears to be related to cell stress during thawing, through mechanisms involving osmotic variations in the extracellular media [32].

In mammals, AQPs have been associated with sperm osmoregulation. Chen et al. [20] demonstrated that AQP-3 was present in both mouse and human spermatozoa and was located in the plasma membrane of the principal piece of the sperm tail. In addition, AQP-3 knockout

mice show defects in sperm volume regulation and excessive cell swelling upon physiological hypotonic stress [20]. Alyasin et al.'s [19] results confirmed the expression of AQP-3 in human spermatozoa and the immunocytochemistry results showed an intense immunoreactivity in the entire sperm tail. The authors state that AQP-3 is involved in the motility of sperm and mitochondrial membrane potential. Such details ought to be examined for dromedary spermatozoa. The effect of individual males on post-thaw dromedary sperm quality was investigated in this study and we were able to identify variability between males with respect to their post-thaw quality. Taking into account the viability and motility of the six males, we identified one of them (male 1) as a 'good freezer' and three of them as a 'poor freezers' (males 4, 5 and 6). The rest of the males (males 2 and 3) presented an average freezability. These findings are consistent with previous studies in dromedary sperm where it concluded that there were individual variations in total motility, kinetic variables and viability of post- thaw dromedary spermatozoa [9]. The individual male response to the freeze-thawing process was not directly associated with neither the abundance nor the membrane localization of AQP-3. These observations coincide with that observed in boar, where cooling and freeze-thawing did not affect the relative abundance of AQP-3 evaluated by western blotting [21]. On the contrary, Prieto-Martínez et al. [32], reported that AQP-3 expression was significantly higher in 'good freezers' than in 'poor freezers' boars. Furthermore, Fujjii et al. [21] demonstrated that the amounts of AQP-3 varied among bull ejaculates, and they positively related to sperm motility, particularly sperm velocity post thawing. The model used in the present study (dromedaries) does not allow the use of a large number of animals in the experiments, to increase the statistical power. Although our findings provided valuable information about AQP-3 expression in spermatozoa of this species, more experiments should be designed to determine the mechanistic action of AQP-3, and other AQPs, on dromedaries' sperm freezability.

To the best of our knowledge, this work is the first to describe the morphometric characteristics of the heads of dromedary spermatozoa. The average value of the head area obtained (between 19 to 22 μm^2) was similar to other South American domestic camelids such as the llama [39] and alpaca [40]. We observed that the sperm area is relatively small compared to other domestic species such as pig (32 to 33 μm^2, [41]), bull (around 32 μm^2, [42]) or ram (around 35 μm^2, [43]). In addition, we found significant differences between males for all morphometric parameters. However, we did not detect any correlation between sperm head morphometry and response to freezing-thawing. The data confirm previous reports in other species showing that sperm head dimensions appear not to be useful predictors of how well spermatozoa might survive freezing [14].

5. Conclusions

The results revealed the presence of AQP-3 in dromedary spermatozoa. However, the individual sperm response to freeze-thawing was not associated with the expression of AQP-3. Thus, AQP3 expressions do not seem like a reliable predictor of sperm response to freeze-thawing process in this species. Interestingly, we found a negative correlation between sperm acrosomal integrity and the expression of AQP-3 in sperm acrosome. The absence of correlation between the sperm head dimensions and sperm quality variables after freeze-thawing suggest that sperm head morphometry is not predictive of cryosurvival in dromedary camel. Further studies are needed to determine the association, if any, of these parameters with fertility.

Acknowledgments

This work was supported by Zoitechlab S.L. (Arquimea Group) - INIA contract CON18-141, MCINN/AEI/FEDER and EU grant AGL2017-85753-R and PID2020-113288RB-100 / AEI / 10.13039/501100011033 grant.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

E. O´Brien: Methodology, Formal analysis, Investigation, Writing - Original Draft.

C. Malo: Methodology, Investigation, Writing - Original Draft.

C. Castaño: Methodology, Investigation, Writing - Original Draft.

P. García-Casado: Resources, Writing - Original Draft, Funding acquisition.

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Data Availability Statement

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

Figures legends

Figure 1. Immunoblot for AQP-3 in frozen–thawed dromedary spermatozoa. A) Each line contains 35 x 10^6 spermatozoa (Spz_d) analysed by 12% SDS/PAGE and Western Blot using anti-AQP-3. Sheep small intestine tissue lysate (Is) were included as a control of antibody specificity. B) Blots resulting from incubations with the AQP3-blocking peptide and controls for kidney mouse tissue lysate (K_r). The same blots were stripped and re-probed with anti-β-actin polyclonal antibody as a loading control. Molecular weight standards (MW) are indicated at the right of each blot. C) Relative abundances of AQP3 bands (as mean ± SD) in 'good freezers' and 'poor freezers' after freeze–thawing were calculated after quantification of 28 kDa bands and normalization using actin protein as an internal standard.

Figure 2. Localization of AQP-3 in frozen-thawed dromedary spermatozoa. Representative photographs of different patterns of immunostaining for AQP-3 (1: in the acrosome, 2: post-acrosomal region, 3: mid-piece, 4: principal piece of the tail and 5: final piece of the tail). Nucleus was stained by Hoechst. NC: negative control (400X).

Figure 3. Distribution pattern of AQP-3 expression. The proportion (mean ± SE) of spermatozoa with representative localization patterns of AQP-3 in membrane domains of frozen-thawed dromedary spermatozoa. Means with different superscript letters are significantly different ($p<0.05$) between males.

	MT (%)	MP (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	ALH (µm)	Viability (PI) (%)	Acrosomal integrity (%)
Male 1	71.85 ± 1.75	6.10 ± 3.40	238.06 ± 0.46^{a}	59.76 ± 5.60	101.81 ± 4.93	11.48 ± 0.25^{a}	61.75 ± 4.75	87.75 ± 6.75
Male 2	63.40 ± 4.60	12.65 ± 2.05	195.46 ± 1.96^{ab}	55.18 ± 4.11	81.82 ± 5.64	10.17 ± 0.07^{b}	61.00 ± 8.00	84.75 ± 1.75
Male 3	57.10 ± 10.80	8.95 ± 2.75	183.91 ± 22.80^{ab}	54.94 ± 9.51	83.30 ± 11.88	10.08 ± 0.89^{b}	61.00 ± 4.00	83.00 ± 5.00
Male 4	60.90 ± 11.50	8.65 ± 1.55	201.05 ± 6.82^{ab}	57.42 ± 1.97	90.14 ± 2.71	10.89 ± 0.42^{b}	45.00 ± 4.00	79.25 ± 6.75
Male 5	56.65 ± 4.35	16.65 ± 2.75	165.18 ± 5.21^{b}	57.56 ± 4.05	80.86 ± 3.53	9.17 ± 0.57^{b}	59.00 ± 4.00	80.00 ± 0.00
Male 6	41.50 ± 9.40	4.25 ± 2.15	209.04 ± 16.05^{ab}	56.30 ± 4.75	96.35 ± 6.89	11.14 ± 0.57^{b}	35.50 ± 7.50	88.50 ± 6.50

Table 1. Quality sperm variables of fresh dromedary samples (mean ± SEM). Sperm traits: total motility (MT), progressive motility (MP), motility kinetic variables; curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) and amplitude of lateral head displacement (ALH), viability (PI) and acrosomal integrity. Means with different superscript letters differ significantly ($p < 0.05$) between males.

	MT (%)	MP (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	ALH (µm)	Viability (SQS2) (%)	Viability (PI) (%)	Acrosomal integrity (%)
Male 1	37.54 ± 1.38^{a}	7.76 ± 1.14^{a}	48.92 ± 2.77^{a}	15.09 ± 1.12^{a}	24.17 ± 1.02^{a}	2.62 ± 0.11^{a}	26.50 ± 1.94^{a}	33.25 ± 2.17^{a}	78.25 ± 2.95^{a}
Male 2	23.78 ± 1.26^{a}	1.77 ± 0.23^{b}	37.29 ± 1.42^{b}	8.49 ± 0.47^{b}	16.55 ± 0.75^{b}	2.09 ± 0.06^{b}	21.75 ± 1.93^{a}	26.50 ± 2.72^{a}	77.75 ± 5.39^{a}
Male 3	26.68 ± 6.70^{a}	5.33 ± 1.64^{a}	45.67 ± 1.17^{a}	12.91 ± 0.63^{a}	22.22 ± 0.89^{a}	2.53 ± 0.05^{a}	19.25 ± 1.03^{a}	26.75 ± 1.88^{a}	64.00 ± 5.11^{b}
Male 4	23.99 ± 1.30^{a}	6.56 ± 1.68^{a}	49.77 ± 4.75^{a}	15.13 ± 1.01^{a}	24.22 ± 1.56^{a}	2.71 ± 0.20^{a}	16.00 ± 1.41^{b}	15.75 ± 1.10^{b}	80.75 ± 5.54^{a}
Male 5	18.92 ± 1.10^{b}	1.63 ± 0.20^{b}	34.84 ± 1.11^{b}	9.41 ± 0.33^{b}	16.71 ± 0.24^{b}	2.04 ± 0.07^{b}	18.25 ± 1.97^{b}	20.50 ± 3.77^{b}	65.50 ± 5.50^{b}
Male 6	24.4 ± 4.29^{a}	5.41 ± 1.70^{a}	45.18 ± 3.06^{a}	15.15 ± 0.97^{a}	24.37 ± 1.90^{a}	2.48 ± 0.12^{a}	14.25 ± 1.93^{b}	16.50 ± 1.55^{b}	87.50 ± 3.52^{a}

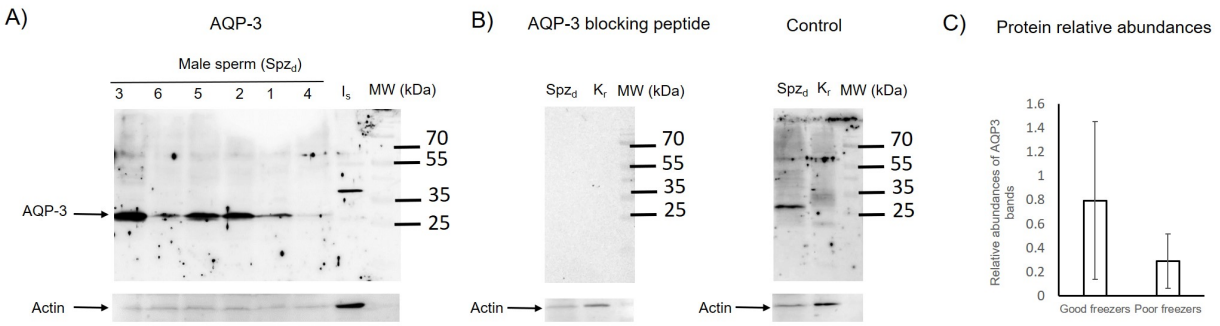
Table 2. Quality sperm variables of frozen-thawed dromedary samples (mean ± SEM). Sperm traits: total motility (MT), progressive motility (MP), motility kinetic variables; curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) and amplitude of lateral head displacement (ALH), viability (SQS2 and PI) and acrosomal integrity. Means with different superscript letters between males are significantly different ($p < 0.05$).

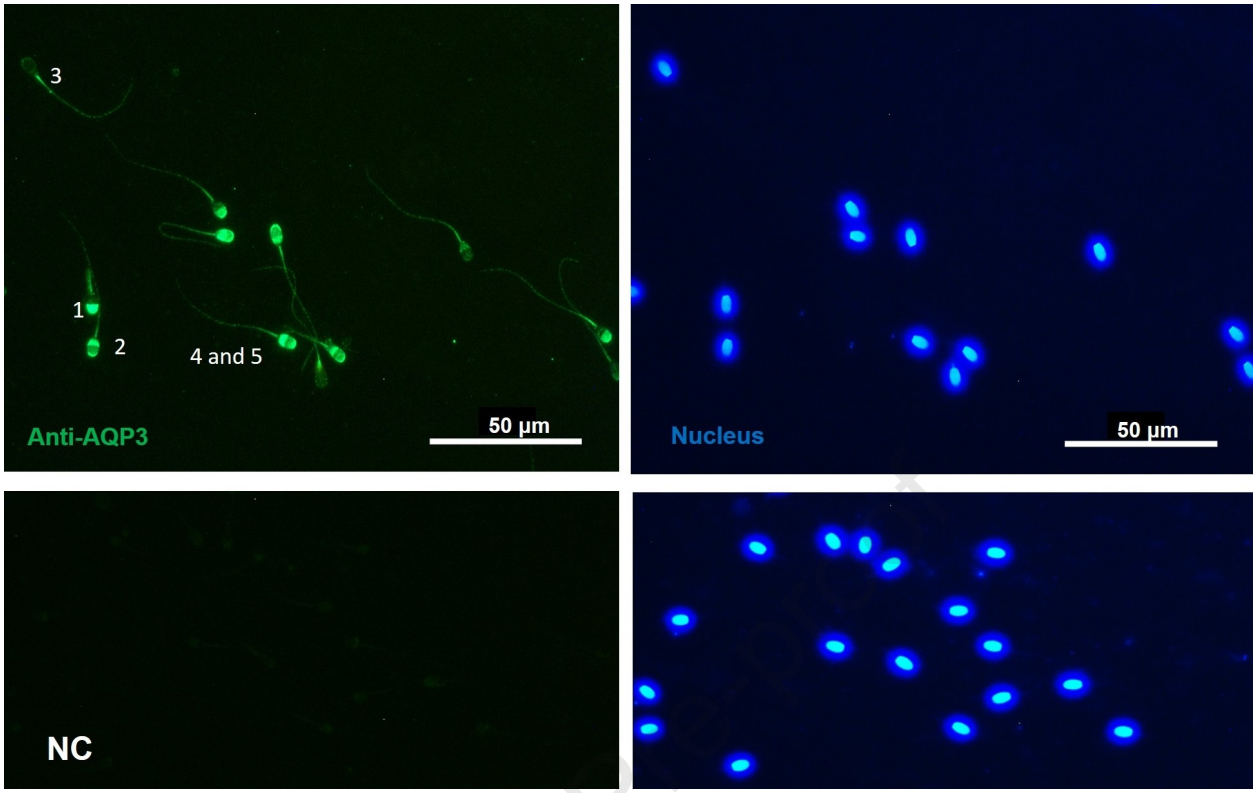
	Length (μm)	Width (μm)	Area (μm²)	Perimeter (μm)	Acrosomal region (%)
Male 1	5.95 ± 0.09^{c}	3.15 ± 0.04^{b}	20.23 ± 0.45^{c}	13.19 ± 0.22^{c}	53.99 ± 0.52^{ab}
Male 2	5.94 ± 0.07^{c}	3.07 ± 0.04^{c}	20.16 ± 0.41^{c}	13.11 ± 0.17^{c}	54.29 ± 0.34^{ab}
Male 3	5.66 ± 0.05^{d}	3.02 ± 0.03^{d}	18.94 ± 0.29^{d}	12.54 ± 0.10^{e}	53.28 ± 0.26^{b}
Male 4	6.09 ± 0.06^{b}	3.21 ± 0.03^{b}	21.95 ± 0.57^{a}	13.48 ± 0.09^{b}	54.04 ± 0.69^{ab}
Male 5	6.27 ± 0.03^{a}	3.07 ± 0.02^{c}	21.39 ± 0.11^{b}	13.71 ± 0.07^{a}	54.87 ± 0.21^{a}
Male 6	5.63 ± 0.07^{d}	3.34 ± 0.02^{a}	19.76 ± 0.52^{c}	12.87 ± 0.14^{d}	53.21 ± 0.40^{b}

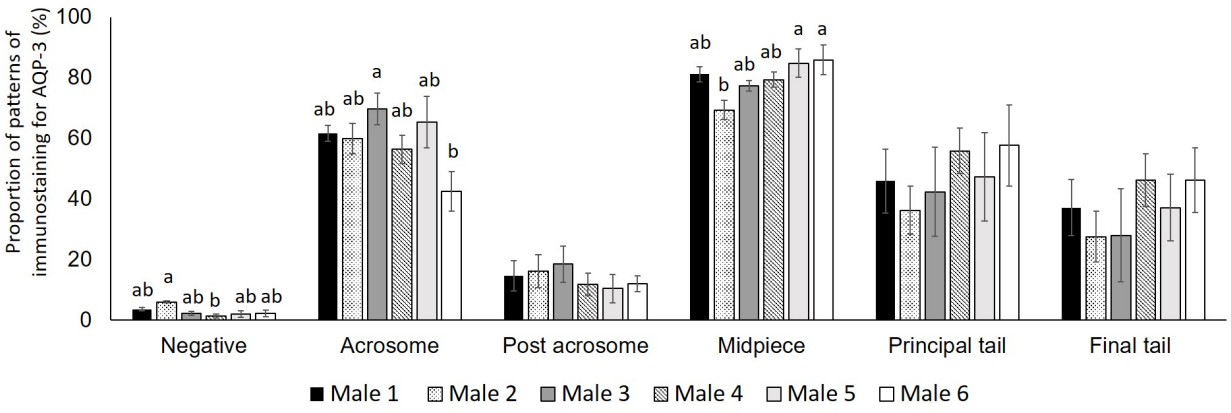
Table 3. Sperm head morphometrics of frozen-thawed dromedary samples (mean ± SEM). Length, width, area, perimeter and percentage of acrosome region of the sperm head as measured in 100 spermatozoa of each male. Means with different superscript letters between males are significantly different ($p < 0.05$).

	Male 1	Male 2	Male 3	Male 4	Male 5	Male 6
sp1	26.23 ± 1.42^{e} (12.50%)	28.26 ± 1.02^{c} (5.83%)	33.31 ± 4.37^{a} (0.98%)	31.37 ± 2.14^{b} (8.88%)	27.54 ± 1.87^{d} (13.45%)	26.15 ± 1.63^{f} (10.86%)
sp2	22.65 ± 0.86^{d} (24.40%)	23.32 ± 1.20^{c} (27.02%)	23.45 ± 1.71^{c} (29.42%)	25.08 ± 1.55^{a} (37.82%)	23.60 ± 1.03^{b} (29.19%)	22.18 ± 1.00^{e} (30.37%)
sp3	19.86 ± 0.93^{b} (38.40%)	19.62 ± 1.02^{c} (42.82%)	18.63 ± 1.34^{e} (41.50%)	20.70 ± 1.29^{a} (31.25%)	20.21 ± 1.13^{b} (38.58%)	18.79 ± 1.00^{d} (37.78%)
sp4	15.87 ± 1.47^{a} (24.70%)	15.70 ± 1.45^{d} (24.33%)	13.99 ± 1.52^{f} (28.10%)	15.83 ± 1.48^{b} (22.05%)	15.81 ± 1.60^{c} (18.78%)	14.78 ± 1.32^{e} (20.99%)

Table 4. Sperm clusters or subpopulations (sp) of frozen-thawed dromedary sperm samples according to head area (mean ± SD): sp1 showed the largest head size and sp4 the smallest values. The proportion of sperm in each subpopulation is showed in parenthesis. Means with different superscript letters between males for each subpopulation are significantly different ($p < 0.05$).







Highlights

- The dromedary sperm response to freeze-thawing was not associated with aquaporin 3 expression
- The sperm head morphometry is not predictive of cryosurvival in dromedary camel