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RESEARCH ARTICLE

Relationship of Aquaporin-3 and Aquaporin-7 with Cryotolerance of Angus Bull Spermatozoa

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ABSTRACT

Semen of all bulls is not suitable for freezing; similarly, all ejaculates with excellent sperm vitality are not suitable for freezing. Based on sperm survival at post-thawing, bull ejaculates are categorized as good freezability ejaculates (GFE) and poor freezability ejaculates (PFE). In this study, sperm quality parameters of GFE and PFE, with the localization of aquaporin-3 (AQP-3) and aquaporin-7 (AQP-7) were investigated. Moreover, the relationship of AQP-3 and AQP-7 with cryotolerance of bull sperms was recorded. After fresh semen evaluation, 24 ejaculates from four Angus bulls were cryopreserved and their sperm quality parameters were analyzed by AndroVision before and after frozen-thawed. Expression of AQP-3 and AQP-7 was determined through Western blot, while their localization was detected by immunocytochemistry. The GFE and the PFE were well differentiated by sperm motility, progressive motility, viability, acrosome integrity, and mitochondrial activity, except for DNA integrity. AQP-3 was significantly higher in GFE than PFE in frozen-thawed sperm, while the AQP-7 in fresh sperm of GFE was significantly higher than that in fresh sperm of PFE and in frozen-thawed sperm of GFE ($P<0.05$). The location of AQP-3 was seen in the sperm mid-piece, while that of AQP-7 was recorded in the post-acrosome region, the mid and prime tailpiece. In conclusion, GFE and PGE can be well distinguished by the expression of AQP-7 in fresh semen, while AQP-7 could be a biomarker of sperm cryotolerance in bull sperm. This study is hoped to improve the efficiency of frozen sperm production and provide a reference for the improvement of frozen semen production technology in other male animals, as well as humans.

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INTRODUCTION

Since the birth of the world's first calf following artificial insemination by frozen semen in 1951 (Ombelet and Van Robays, 2015), semen freezing is currently the most widely used technology in different species, including dairy cattle. With the rapid development of artificial insemination technology, semen cryopreservation technology has also improved (Moore and Hasler, 2017). Preservation of bovine semen at ultra-low temperatures can effectively inhibit the metabolism of spermatozoa and prolong their survival time, achieving the purpose of long-term preservation and utilization of semen. Though the bovine semen cryopreservation technology has been developed and improved, there are still certain influencing factors in the process of cryopreservation and thawing which result in the reduced vitality, plasma membrane integrity and acrosome integrity of sperm after thawing compared with fresh sperm (Westfalewicz *et al.*, 2015; Arunkumar *et al.*, 2022) and there is still about 50% or even more loss of sperm vitality after freezing under the most developed cryopreservation technology (Moore and Hasler, 2017).

More importantly, the semen of all bulls is not suitable for freezing, and values of sperm quality parameters recorded in fresh semen are not necessarily guaranteed at post-thaw evaluation (Sawitri *et al*., 2021). Consequently, bull ejaculates are categorized based on their post-thaw sperm survival as good freezability ejaculates (GFE) and poor freezability ejaculates (PFE: Fujii *et al.*, 2018; Luo *et al.*, 2022). To date, sperm cryotolerance capability cannot be accurately predicted. The primary requirement for high-quality bulls is its ability to produce sperm of normal quality with low sperm quality loss after freezing, resulting in at least 35% sperm viability after freeze-thawing (Pardede *et al*., 2020; El-

Nagar *et al*., 2022). Therefore, scientists are trying to find reliable biomarkers to predict sperm cryotolerance of the bull before freezing, which could improve the efficiency in bull selection and reduce unnecessary wastage of resources.

The plasma membrane of spermatozoa is the key structure most susceptible to damage under low-temperature environments, and the freezing process can affect the structural integrity and functions of the plasma membrane. Currently, sperm cryopreservation solutions mostly contain glycerol (Delgado-Bermudez *et al.,* 2022) as an osmotic protection agent, which can reduce the osmotic swelling of sperm cells and slow down the rate of cell dehydration. Aquaporins (AQP) are intrinsic membrane proteins that form "pore channels" in the cell membrane and control the flow of water in and out of the cell, serving as the "water pump of the cell" (Yeste *et al.*, 2017). There are 13 isoforms of aquaporins, AQP 0-12 (Ribeiro *et al.*, 2022; Zhang and Yang, 2023), of which AQP-3 (O'Brien *et al.*, 2022) and AQP-7 (Santiago-Moreno *et al.*, 2022) are known as water-glycerol channel proteins because of their involvement in cell membrane stability and glycerol transport in and out of the cell. Since glycerol plays an important role in sperm cryopreservation, we hypothesize that these aquaporins (AQP-3 and AQP-7) may be associated with spermatozoa cryopreservation. Previous studies by Prieto-Martinez *et al.* (2017a) and Fujii *et al.* (2018) suggested that aquaporins (AQP-3 and AQP-7) could be involved in bovine sperm cryo-resistance.

The objectives of this study were: i) to study the sperm quality parameters of GFE and PFE; ii) to determine the localization and comparative contents of bovine sperm AQP-3 and AQP-7 by Western Blot and immunocytochemistry and iii) to investigate the association of AQP-3 and AQP-7 with cryotolerance of bull sperm.

MATERIALS AND METHODS

Ethics: The present study was performed during the period from August 2022 to May 2023. The Animal Ethics Committee, Tarim University, Alar, Xinjiang 843300, China, approved the protocol and allowed the use of experimental bulls in the present study.

Animals and samples collection: Four Angus bulls, aged 2-5 years and having body weight of 810 to 900 kg were used in the present study. The semen of bull No. 65318272 and 65319672 was graded as PFE, while that of 65317948 and 65319272 was GFE, as had been identified previously by quality parameters of frozen-thawed sperm. These bulls were housed in the Xinjiang Dingxin Seed Industry Technology Co., Limited Aksu, Xinjiang, China, in open sheds having fresh water available around the clock. Seasonal fodder @ 1.2kg/100kg and concentrate 0.5kg/100kg (14-16% crude proteins) was offered per bull daily.

For the present study, semen from these bulls was collected weekly for six weeks, using the artificial vagina method. In total, 24 ejaculations (6 ejaculates per bull) were collected, with 12 ejaculates from GFE and the remaining 12 from PFE group. Sperm motility and morphology were assessed by using AndroVision (Minitube). Semen samples with at least 65% progressively motile spermatozoa were selected for further processing. Each ejaculate was split into four aliquots; one aliquot was utilized for the assessment of sperm quality, the 2nd aliquot was used for the extraction of proteins from the fresh semen, while 3rd and 4th aliquots were used for immunocytochemistry and cryopreservation, respectively.

Sperm quality analysis: The first aliquot of semen was used for the analysis of sperm quality parameters through AndroVision (Dini *et al*., 2019). Sperm total motility, progressive motility, viability, acrosome integrity, mitochondrial activity and DNA integrity were assessed according to the instructions of Minitube.

Extraction of proteins and Western blot: Method described by Westfalewicz *et al*. (2021) was followed for proteins extraction, while Western blotting method of Oberska and Michałek (2021) was followed. For the second semen aliquot, radioimmunoprecipitation assay (RIPA) lysis buffer (Epizyme Biomedical, China) was used to extract proteins from the spermatozoa (approximately 2.0×10^7 cells). Proteins were separated on 10% polyacrylamide gel (Epizyme Biomedical, China) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were gridlocked in TBST (tris-buffered saline + tween 20), mixed with 5% skim milk and nurtured with rabbit anti-AQP-3 polyclonal antibody (Ab) (bs-1253R; Bioss, China, 1:3000), rabbit anti-AQP-7 polyclonal Ab (bs-2506R; Bioss, China, 1:3000), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Ab (AF7021; Affinity, China, 1:3000). After washing, membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) HRP (S0001; Affinity, China, 1:5000). Immunoblots were established with enriched chemiluminescence (Mishubio, China). Images were taken by utilizing ChemiDoc Imaging System with Quantity One software (Bio-Rad, USA). Band sizes were determined utilizing a calibration curve set based on a molecular weight marker (Epizyme Biomedical, China). ImageJ software (National Institutes of Health, USA) was used to determine relative expression of proteins. GAPDH was used for standardization for the estimate of proportionate concentrations of AQP-3 and AQP-7, using replicates from each sample on another membrane and the values obtained were subjected to statistical analysis.

Immunocytochemistry: Procedure for immunocytochemistry was following as mentioned by Oberska and Michałek (2021). Briefly, the third aliquot of each ejaculate was fixed with 4% (w/v) paraformaldehyde, by placing three drops on poly-lysine-coated slides and air-dried. Air-died slides were immersed in ethylenediaminetetraacetic acid (EDTA) Antigen Retrieval Solution (Solarbio, China) and microwaved for antigen repair. Afterward, samples were permeabilized with 0.25% (v/v) Triton X-100 (Solarbio, China) and incubated with 5% skim milk. Then samples were nurtured with either rabbit anti-AQP-3 polyclonal Ab (bs-1253R; Bioss, China) or rabbit anti-AQP-7 polyclonal Ab (bs-2506R; Bioss, China) previously diluted to 1:120 in TBST containing 5% (w/v) skim milk overnight at 4ºC in a humid chamber. Afterward, slides were incubated with secondary antibodies (goat anti-rabbit IgG (H+L); Flour594-

conjugated-S0006 (Affinity, China) and diluted to 1:250 in TBST. After incubation with DAPI (4′,6-diamidino-2-phenylindole) solution (Solarbio, China), slides were mounted with a drop of antifading mounting medium (Solarbio, China), coated with nail varnish, shielded from direct light, and instantly examined under Nikon Ti2-U fluorescence microscope (Nikon, Japan). Assessment of AQP-3 and AQP-7 stain coloration was carried out at 595nm (Flour-594) and for nuclei examination, the wavelength was 415nm (DAPI).

Cryopreservation: The method described by Prieto-Martinez *et al*. (2017a) with slight modification was followed for cryopreservation of semen. Briefly, semen samples were diluted with Tris-egg yolk-glycerol extender (Table 1) to achieve final concentration of spermatozoa of $2x10^7$/mL and cooled to 4°C for 2h. After 2h of equilibration, the samples were loaded into 0.25mL straws. The straws were placed on a freezing rack filled with liquid nitrogen. The straws were placed about 10cm above liquid nitrogen and the fumigation height was approximately 12-13cm. After fumigation for 6min, the straws were immersed in liquid nitrogen for freezing.

Statistical analysis: All the experiments reported were replicated three times using independent samples. Analysis of variance, followed by Duncan's Multiple Range test, was applied to assess differences between means. All statistical analyses were performed by utilizing the GraphPad Prism 9 Statistical Software.

RESULTS

Quality parameters of fresh and frozen-thawed sperm of two groups: Table 2 shows mean±SEM values of sperm motility, viability, progressive motility, mitochondrial activity, acrosome integrity and DNA integrity before and after post-frozen-thaw stage in GFE and PFE groups. Before freezing, progressive motility, viability, acrosome integrity and mitochondrial activity were significantly lower in PEF than GEF group (P<0.05), while sperm motility and DNA integrity did not differ between the two groups. A similar trend was seen between the two groups after cryopreservation. When comparison between fresh and frozen-thawed samples was made, all parameters within a group showed significant decrease post-freezing compared to fresh semen (P<0.05), except DNA integrity which did not change in both groups before and after cryopreservation.

Western Blot analysis: Specific signal bands by Western blot of AQP-3 were identified at 54kDa both in fresh and frozen-thawed semen (Fig. 1a). Although variations were reflected, the proportionate abundances of AQP-3 (Fig. 1b) did not differ in sperm from GFE before and after freeze-thawing, whereas AQP-3 abundance in frozen-thawed sperm of GFE was significantly higher than in frozen-thawed spermatozoa of PFE group. Furthermore, the relatively large quantity of AQP-3 was not altered by cryopreservation.

In the case of AQP-7, Western Blot (Fig. 2) only recognized one specific signal at 52kDa in fresh semen, as well as in frozen-thawed semen (Fig. 2a). Proportionately AQP-7 in the sperm of fresh GFE group was significantly (P≤0.05) higher compared to fresh sperm of PFE and the frozen-thawed sperm of GFE group (Fig. 2b).

Table 1: Chemical composition of tris-egg yolk-glycerol extender for bull semen

Chemical	Manufacturer	Quantity
Tris	Solarbio, China	24.22g
Citric acid	Solarbio, China	13.42g
Glucose	Solarbio, China	10g
Glycerol	Solarbio, China	64mL
Egg yolk	Locally procured	200mL
Distilled water	Lab product	736mL

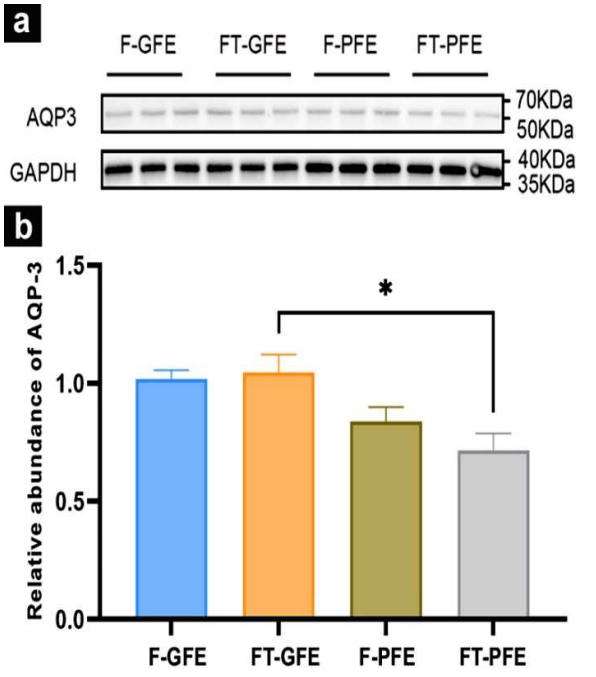

Fig. 1: a) Western blot of bull spermatozoa at fresh and frozen-thaw stage showing AQP-3, and b) comparative concentration (mean±SEM) of AQP-3 in GFE and PFE in fresh and frozen-thaw bull spermatozoa. There were non-significant differences among the groups, except the GFE and PFE in frozen thawed bull sperm (P≤0.05). F: Fresh; FT: Frozen-thaw.

Table 2: Quality parameters of spermatozoa of GFE and PFE (mean±SEM)

Parameters(%)	Fresh ejaculate		Frozen thawed ejaculate	
	GFE	PFE	GFE	PFE
Sperm motility	$77.07±1.37^{A,a}$	$71.58±2.49^{A,a}$	$52.56±0.94^{B,a}$	$29.76±0.74^{B,a}$
Progressive motility	$73.23±1.23^{A,a}$	$67.28±1.07^{A,b}$	$44.83±1.14^{B,a}$	$25.62±1.52^{B,b}$
Viability	$81.68±1.06^{A,a}$	$74.04±1.30^{A,b}$	$54.14±2.30^{B,a}$	$27.83±2.43^{B,b}$
Acrosome integrity	$80.20±1.65^{A,a}$	$78.94±1.02^{A,b}$	$58.71±2.00^{B,a}$	$33.18±3.19^{B,b}$
Mitochondrial activity	$83.37±1.71^{A,a}$	$76.41±1.51^{A,b}$	$54.18±1.82^{B,a}$	$31.20±1.75^{B,b}$
DNA integrity	$96.68±0.52^{A,a}$	$95.95±0.61^{A,a}$	$96.34±0.63^{A,a}$	$95.27±0.49^{A,a}$

Different uppercase letters indicate significant differences between fresh and frozen-thawed semen, while different lowercase letters specify significant differences among freezability groups (GFE vs PFE) either before freezing or after frozen-thawed (P≤0.05).

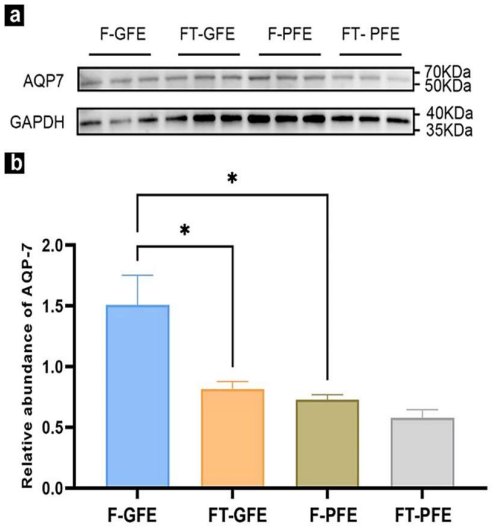

Fig. 2: a) Western blot of bull spermatozoa at fresh and frozen-thaw stage showing AQP-7, and b) comparative concentration (mean±SEM) of AQP-7 in GFE and PFE in fresh and frozen-thaw bull spermatozoa. The expression of AQP-7 of GFE in fresh semen is significantly higher than PFE in fresh and GFE in frozen-thawed bull spermatozoa (P≤0.05). F: Fresh; FT: Frozen-thaw.

Localization of AQP-3 and AQP-7 at the subcellular level: The subcellular localization of these aquaporins (AQP-3 & AQP-7) in bull sperm was observed under Nikon Ti2-U. All spermatozoa exhibited visible AQP-3 staining in the mid-piece, whereas in the head and principal piece of the tail AQP-3 showed a diffuse weak fluorescence distribution (Fig. 3). There was almost no fluorescence in the end piece of the tail.

As presented in Fig. 4, the AQP-7 was distributed in the post-acrosome region, mid and main piece of the tail. However, for deformed spermatozoa, such as those with broken tails, AQP-7 showed diffuse and weak fluorescence in the head, resultantly the acrosome could not be distinguished.

DISCUSSION

In the present study, GFE and the PFE were well differentiated by all the semen quality parameters except the DNA integrity, indicating that the freezing of sperm has little effect on the DNA integrity. Interestingly, the same conclusion was made in the study of frozen-thawed Italian Mediterranean buffalo semen (Serafini *et al.*, 2016). We discriminated the GFE and PFE of bulls mainly by sperm progressive motility and sperm viability, also known as plasma membrane integrity after thawing, which is in accordance with the studies on sperm of horses (Catalán *et al.*, 2022a), donkeys (Catalán *et al.*, 2022b) and boar (Delgado-Bermúdez *et al.*, 2019).

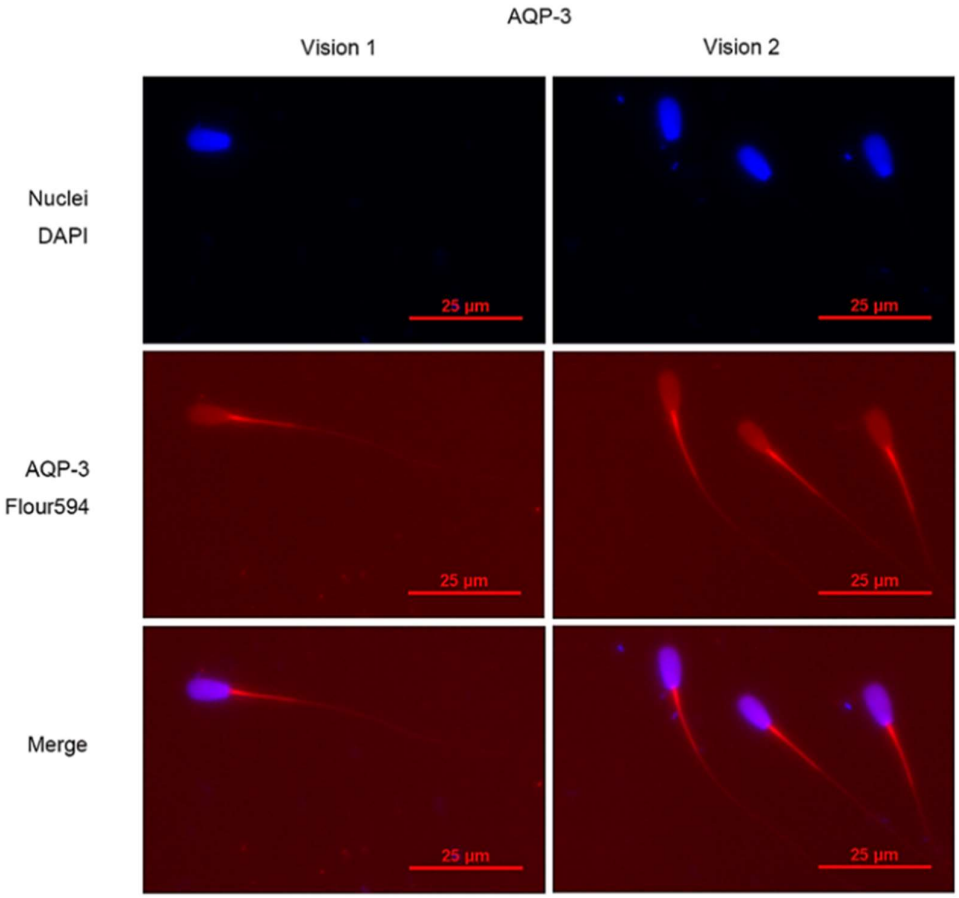

Fig. 3: The representative subcellular localization of AQP-3 in bull sperm using a fluorescence microscope under different visions. Stained spermatozoa with primary anti-AQP-3 antibodies coupled with Flour594 (red) and nuclei observed (blue) with DAPI counterstain. Stained coloration is obvious in the sperm mid-piece, while in the head and principal piece of the sperm tail AQP-3 showed a diffuse weak fluorescence distribution and there was almost no fluorescence in the final piece of the tail.

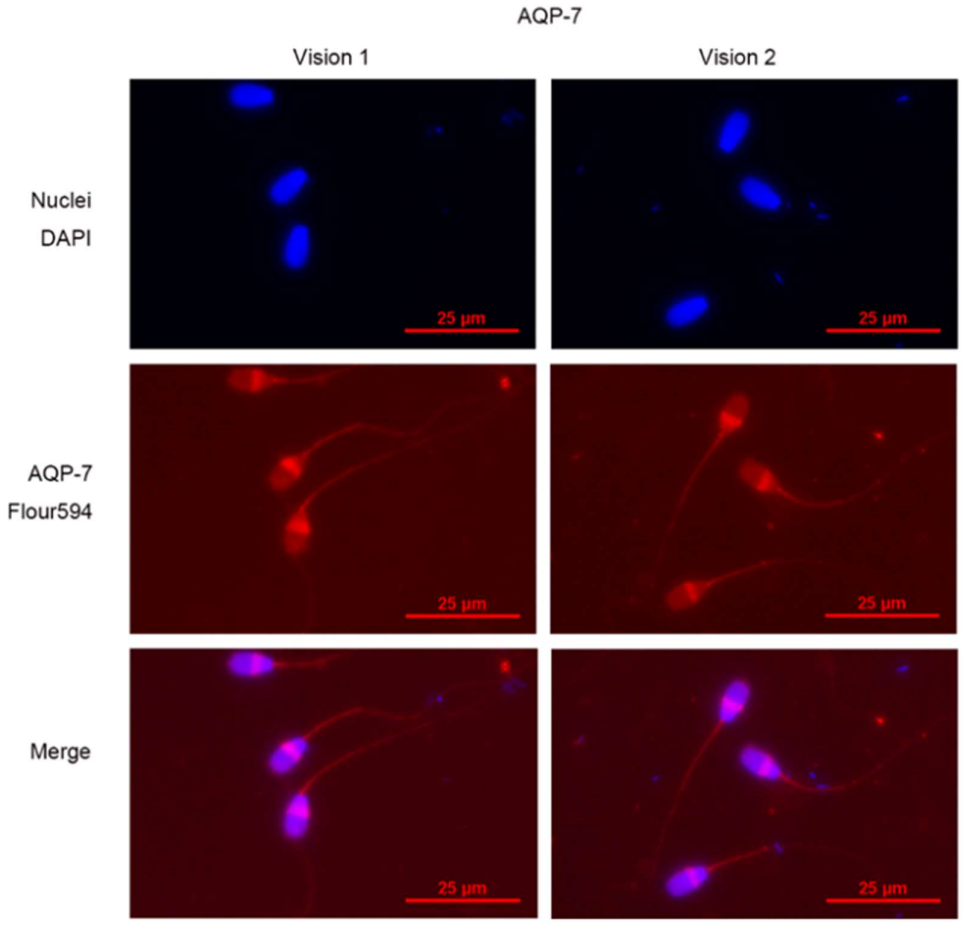

Fig. 4: The representative subcellular localization of AQP-7 in bull spermatozoa using fluorescence microscope under different visions. Stained spermatozoa with a primary anti-AQP-7 antibody coupled with Flour594 (red) and nuclei observed (blue) with DAPI counterstain. Clear staining of AQP-7 was seen in the post-acrosome region, midpiece and principal piece of the tail.

In the present study, the AQP-3 was detected in 54kDa and there were no bands in 27kDa, as the instruction of the manufacturer, which is different from the 30 and 60kDa (Prieto-Martinez *et al.*, 2017a) or 42kDa in bulls (Fujii *et al.*, 2018). The AQP-3 was also detected at 25kDa in boar (Prieto-Martinez *et al.*, 2017b) and 28Kda in dromedary camel (O'Brien *et al.*, 2022). However, for AQP-7, bands were only detected in 52kDa and there were no bands in 37kDa, as the instruction of the manufacturer, which differs from the 25kDa in study of AQP-7 in bull (Prieto-Martinez *et al.*, 2017a) and boar (Prieto-Martinez *et al.*, 2017b) and 53 kDa in another report in bulls (Fujii *et al.*, 2018). According to Yeung *et al.* (2010), there are four additional AQP-7 isoforms in human sperm (27, 29, 30 and 40kDa). AQP-3 and AQP-7 in bovine spermatozoa are based on amino acid sequences that have 32 and 36kDa molecular weights, respectively (Fujii *et al.*, 2018). However, different molecular weights emerge from different studies, and the explanations for these differences are not yet clear. There could be several reasons for this, the first being the use of different antibodies in different studies, which detect different molecular weights. The other explanation could be the fact that AQP may be present in a dimeric structure (Prieto-Martinez *et al.*, 2017b; Sultan, 2023) and in the present study AQP-3 was probably detected in dimer. Thirdly, different molecular weights may be due to post-transcriptional modifications of AQP, such as glycosylation of the protein nitrogen chain (Prieto-Martinez *et al.*, 2017b).

Immunocellular fluorescence of AQP-3 was distinctly demonstrated in the mid-piece of spermatozoa in the present study, which is supported by the study of Prieto-Martinez *et al.* (2017a). The staining of AQP-3 in our study was spread more gently along the mid-piece of the sperm, while in the study of Prieto-Martinez *et al.* (2017a) position was relatively short and ended directly. AQP-3 in the study of Fujii *et al.* (2018) was found in the principal piece, without any staining in mid-piece. Previous studies have shown that AQP-3 is expressed and localized in the head of the sperm in boar (Prieto-Martinez *et al.*, 2017b), in the mid-piece of boar sperm (Prieto-Martinez *et al.*, 2017b) and mouse (Chen *et al.*, 2011; Ribeiro *et al.*, 2022) and in the tail of the sperm in human (Laforenza *et al.*, 2016; Michalek *et al.*, 2021; Pellavio *et al.*, 2020), boar (Prieto-Martinez *et al.*, 2017b) and mouse (Chen *et al.*, 2011). Regarding AQP-7, the clear staining of post acrosome and mid-piece of sperm was observed in the present study, and there was a diffuse weak staining in the head of abnormal spermatozoa, which is similar to the previous report of Prieto-Martinez *et al.* (2017a).

According to Fujii *et al.* (2018), two localization outlines were noted for AQP-7 in bull sperm i.e., diffuse

coloration in the head and the tail or in the head with fine staining in mid-piece. AQP-7 has been reported in the mid-piece, the pericentriolar area, the entire principal piece, and the equatorial segment of the boar (Prieto-Martinez *et al.*, 2016), human (Saito *et al.*, 2004; Chen *et al.*, 2011; Ribeiro *et al.*, 2022) and mouse sperm (Sohara *et al*., 2007). The AQP-7 was predominantly observed in the connecting piece of fresh spermatozoa and in the acrosomal region and mid-piece of frozen-thawed spermatozoa in boar (Prieto-Martinez *et al.*, 2017b; Oberska and Michałek, 2021), which is similar to the findings of Fujii *et al.* (2018) in bulls, showing that freezing alters the localization pattern of AQP-7 in boars and bulls. Similar to our results, human sperm with usual progressive motility showed AQP-7 staining in the equatorial segment, pericentriolar area, midpiece, and tail, while frail and diffuse AQP-7 immunoreactivity was noted in sperm with cytoplasmic deposits, cytoplasmic droplets and coiled tails (Moretti *et al.*, 2012). Thus, the results of the present and previous studies regarding Western blot analysis and immunocytochemistry detection of AQP-3 and AQP-7 suggest that molecular masses and the dissemination of these aquaporins could be species-specific.

The present study revealed that AQP-7 expression of GFE was significantly higher than that of PFE in fresh semen, suggesting that AQP-7 may be a biomarker of sperm cryotolerance. Similar findings were recorded by Prieto-Martinez *et al.* (2017a) and Fujii *et al.* (2018). The most critical aspect of the sperm cryopreservation is resistance to damage caused by freezing (Ďuračka *et al.*, 2023; Hungerford *et al.*, 2023). It is well known that the AQPs are the chief channels linked to fluid transfer among biotic membranes (Agre, 2004), while the aquaglyceroporins (AQP-3, AQP-7, AQP-9, AQP-10) also transfer a series of small uncharged solutes, specifically glycerol (Sales *et al.*, 2013). Glycerol prolongs the freezing process, leading to a more even distribution of the hydrostatic pressure of water and making it easier for cells to survive (El-Sheshtawy *et al*., 2017; Beșchea *et al*., 2021; Paul *et al*., 2021). Another popular theory is that glycerol prevents the formation of ice crystals (Gao and Critser, 2000), as glycerol alters the hydrogen bond structure and intermolecular cohesiveness of the global solvent (Schrader *et al.*, 2016). According to Fu *et al.* (2000), there may be **s**tructure of a glycerol-conducting channel of the lipid bilayer and thus protect the membrane structure of cells. Upregulation of AQP-7 has been reported due to the presence of cryoprotectants (Tan *et al.*, 2015). The outcome of such upregulation is better in the transportation of water as well as cryoprotectants, which improves the cell resistance to hyperosmotic urgency and its endurance during vitrification and thawing (Tan *et al.*, 2015). It has been reported that high concentrations of AQP-3 and AQP-7 in the bull sperm could enable them to acclimatize to histrionic osmotic variations during cryopreservation by effectual transport of water and cryoprotective agent, resulting in high motility in frozen-thawed sperm (Fujii *et al.*, 2018). All these facts highlight that AQP-7 has a critical function in the cryopreservation survival of spermatozoa through minimizing the sperm damage. The AQP-7 makes the cell membrane feasible to endure the osmotic shock post cryopreservation and also during thawing. After isotonic medium intensity, AQP-7 preserves not only sperm membranes integrity but also mitochondrial membrane (Ribeiro *et al.*, 2022), which is also consistent with our results of sperm quality parameters.

As AQP-3 and AQP-7 are species-specific, the association among AQPs and sperm cryotolerance differs across species. The AQP-3 and AQP-7 but not AQP-11, are relevant in the boar; AQP-3, AQP-7 and AQP-11 are associated with sperm cryotolerance in the bull; while AQP-3 and AQP-11, but not AQP-7, are significant in stallion (Ribeiro *et al.*, 2021).

Conclusions: Results of the present study confirmed the GFE and PFE nature of semen samples by quality parameters of frozen-thawed bull sperm, and by the localization and quantification of AQP-3 and AQP-7. It was also found that AQP-7 may be a biomarker of sperm cryotolerance in the bull. Our future research may focus on the molecular mechanisms of AQP-7 affecting sperm cryotolerance, and we expect to develop a rapid and efficient method to detect cryotolerance of the bull sperm by using AQP-7.

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Authors contribution: QG and JS conceived the idea, designed the study and conducted experimental work. JW analyzed the data. AK prepared and reviewed the manuscript. All authors interpreted the data, critically reviewed the manuscript for important intellectual contents and approved the final version.

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