

—Original Article—

Expression and localization of aquaporins 3 and 7 in bull spermatozoa and their relevance to sperm motility after cryopreservation

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Abstract. Artificial insemination with cryopreserved semen is a well-developed technique commonly used for controlled reproduction in cattle. However, despite current technical advances, cryopreservation continues to damage bull spermatozoa, resulting in a loss of approximately 30 to 50% of viable spermatozoa post thawing. To further improve the efficiency of cryopreservation of bull spermatozoa, understanding the molecular mechanisms underlying the cryobiological properties that affect cryoinjuries during cryopreservation process of bull spermatozoa is required. In this study, we examined the expression and localization of aquaporin (AQP) 3 and AQP7 in fresh, cooled, and frozen-thawed bull spermatozoa. Furthermore, we investigated the relevance of AQP3 and AQP7 to motility and to membrane integrity in frozen-thawed bull spermatozoa. Western blotting against AQP3 and AQP7 in bull spermatozoa revealed bands with molecular weights of approximately 42 kDa and 53 kDa, respectively. In immunocytochemistry analyses, immunostaining of AQP3 was clearly observed in the principal piece of the sperm tail. Two immunostaining patterns were observed for AQP7 —pattern 1: diffuse staining in head and entire tail, and pattern 2: diffuse staining in head and clear staining in mid-piece. Cooling and freeze-thawing did not affect the localization pattern of AQP7 and the relative abundances of AQP3 and AQP7 evaluated by Western blotting. Furthermore, we demonstrated that the relative abundances of AQP3 and AQP7 varied among ejaculates, and they were positively related to sperm motility, particularly sperm velocity, post freeze-thawing. Our findings suggest that AQP3 and AQP7 are possibly involved in the tolerance to freeze-thawing in bull spermatozoa, particularly in the sperm's tail.

Key words: Aquaporin, Bull, Cryopreservation, Motility, Spermatozoa

(J. Reprod. Dev. 64: 327–335, 2018)

Artificial insemination (AI) is a well-developed technology commonly used for controlled reproduction in cattle. Cryopreservation is a useful method for storing bull spermatozoa and enables the AI of a large number of females using a single ejaculate. Many empirical studies, such as the examination of semen extender [1, 2], cryoprotectants [3], supplements [4, 5], and equilibration period [2], have been performed to improve the cryopreservation of bull spermatozoa, and various protocols optimized for different laboratory conditions have been developed. However, despite the currently optimized conditions, the cryopreservation process inflicts functional and structural damage that leads to the loss of approximately 30 to 50% of viable bull spermatozoa. Furthermore, individual variation in cryotolerance of spermatozoa among bulls [6] is of concern for semen producers. Thus, to further improve the

efficiency of cryopreservation of bull spermatozoa, it is required to understand the molecular mechanisms underlying the cryobiological properties that affect cryoinjuries during the cryopreservation process in bull spermatozoa.

The permeability of the plasma membrane to water and cryoprotectants is one of the most important cryobiological properties that determines the success of cryopreservation of mammalian cells because this property is closely related to major cryoinjuries such as intracellular ice formation, toxicity of cryoprotectants, and osmotically abnormal swelling and shrinking of the cells [7–9]. Aquaporins (AQPs) are a family of small integral membrane proteins that function as a channel for facilitating rapid water transport, and 13 isoforms of AQPs (AQP0–12) have been identified in mammals [10–12]. AQPs have been found in various mammalian organs, such as the kidney, lungs, digestive organs, and skin, as well as both male and female reproductive tissues and gamete [13–15], and they play crucial roles in the regulation of fluid homeostasis. Among AQPs, AQP3, AQP7, AQP9, and AQP10, the so-called aquaglyceroporins, facilitate the transport of not only water but also glycerol, which is commonly used as a cryoprotective agent (CPA) [10–12]. Thus, it is presumed that aquaglyceroporins also play crucial roles in the process of the cryopreservation of mammalian cells [8, 9]. Studies

Received: December 21, 2017

Accepted: April 20, 2018

Published online in J-STAGE: May 24, 2018

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of aquaglyceroporins will be useful for research aimed towards improving the efficiency of cryopreservation of bull spermatozoa.

Expression and function of AQP3 and AQP7 in rodent, human, and boar spermatozoa have been well investigated [16–25]. Chen *et al.* [16] clearly demonstrated that AQP3 is localized in the principal piece of the mouse and human sperm tail and that it plays a pivotal role in sperm volume regulation against osmotic changes *in utero* using experiments with *AQP3* knockout mice. For AQP7, a study of sperm from infertile men demonstrated its possible role in the maintenance of sperm motility [17]. Recently, Prieto-Martinez *et al.* reported that AQP3 and AQP7 are expressed in boar spermatozoa [21, 22] and that they are involved in sperm cryotolerance [25]. However, the information related to AQPs in bull spermatozoa remains considerably limited.

To the best of our knowledge, a few reports on AQPs focusing on their cryobiological roles in bull spermatozoa demonstrated that AQP3, AQP7, and AQP11 are expressed in bull spermatozoa and that AQP7 and AQP11 are possibly involved in cryotolerance of bull spermatozoa [26, 27]. On the other hand, in studies of boar spermatozoa, it has been reported that AQP7 relocates during the freeze-thawing process [23, 25]. Thus, to elucidate the cryobiological roles of AQPs in bull spermatozoa, changes in expression and localization of AQPs during cryopreservation process in bull spermatozoa should be investigated. In the present study, we investigated the expression and localization of AQP3 and AQP7 in fresh, cooled, and frozen-thawed bull spermatozoa using Western blotting and immunocytochemistry analysis (described as Experiment 1 and 2). Furthermore, in order to obtain insights into the roles of AQP3 and AQP7 in the cryopreservation of bull spermatozoa, we investigated the association of the relative abundance of AQP3 and AQP7 with sperm motility and integrity of plasma and acrosomal membranes in frozen-thawed bull spermatozoa (described as Experiment 3).

Materials and Methods

All experiments using animals were approved by the Animal Ethics Committee, Animal Research Center, Hokkaido Research Organization and Genetics Hokkaido Association.

Animals and semen samples

A total of 9 Holstein bulls (Bulls A to I, 13 to 15 months of age) housed in the Genetics Hokkaido association (Tokachi-shimizu, Hokkaido, Japan) were used for semen collection. One or two ejaculates were collected by artificial vagina from each bull mounted on the dummy cow on a single collection day, and combined for processing in a single batch. The semen volume and concentration of spermatozoa was assessed immediately after each collection, and partially sampled as fresh spermatozoa samples. Then, fresh semen was diluted (1:1) with an egg yolk citrate extender, and sperm motility and morphology was visually assessed under a light microscope. Only samples that have 65 to 75% of progressive motile spermatozoa at a high speed in visual assessment (+++, as described previously [28]) were used for the cryopreservation process. After assessing their motility and morphology, the semen sample was cooled to 4°C for 1 to 2 h, and partially sampled as cooled spermatozoa samples. Then, cooled semen was further diluted to a final volume by adding

an egg yolk citrate extender containing glycerol as a CPA. After 2 h of equilibration, the samples were loaded into 0.5-ml straws, frozen using a programmable freezer (Digitcool 5300; IMV technologies, France), and stored in liquid nitrogen until thawing. Frozen stored spermatozoa samples were thawed at 37°C for 1 min [29] immediately prior to use in experiments.

Motility analysis

The motility and kinematics of movement of frozen-thawed spermatozoa was evaluated with a computer-assisted sperm analysis (CASA) system (IVOS II; HTCasa version 1.7.1, Hamilton Thorne, Beverly, MA, USA) based on digitalized images recorded using a negative-phase contrast microscope. Five microliters of frozen-thawed spermatozoa sample was mixed with egg yolk citrate extender containing glycerol and incubated at 37°C for 5 min prior to any evaluations. Then, 3 μ l of the mixture was pipetted into counting chambers (Leja, Nieuw Vennep, Netherlands) warmed at 37°C. Evaluations were performed on any strip of 6 squares of the grid in counting chambers. The CASA recorded 60 frames per second. A total of nine motility parameters were assessed: percentage of motile spermatozoa (motile: defined as the spermatozoa that moved more than the distance of their head length, %), percentage of progressively motile spermatozoa (progressive: defined as spermatozoa with average path velocity and percentage of straightness being higher than 50 μ m/sec and 80%, respectively, %), average path velocity (VAP, μ m/sec), straight-line velocity (VSL, μ m/sec), curvilinear velocity (VCL, μ m/sec), percentage of straightness (STR = VSL/VAP, %), percentage of linearity (LIN = VSL/VCL, %), amplitude of lateral head displacement (ALH, μ m), and beat frequency (BCF, Hz). Three replicates per sample (at least 900 spermatozoa) were evaluated and the mean values were used in the statistical analyses.

Integrity analysis of plasma and acrosomal membranes

The integrity of the plasma and acrosomal membranes of frozen-thawed bull spermatozoa was evaluated by a fluorescent staining test using Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA), propidium iodide (PI; Sigma-Aldrich), and peanut agglutinin conjugated with fluorescein isothiocyanate (PNA-FITC; Vector Laboratories, Burlingame, CA, USA) according to the protocol reported by Celeghini *et al.* [30] with some modification. Two hundred microliters of frozen-thawed spermatozoa were incubated with Hoechst 33342 (final concentration, 18 μ M) at 37°C for 10 min in the dark, and then incubated with PI and PNA-FITC (final concentration, 30 μ M and 25 μ g ml⁻¹, respectively) at 37°C for 8 min in the dark. Stained spermatozoa samples were then fixed with 0.1% formaldehyde, placed on a slide glass, covered with a cover glass, and immediately inspected under a fluorescence microscope (Nikon E800; Nikon, Tokyo, Japan). PI-stained and PNA-FITC-stained spermatozoa were considered to have an injured plasma membrane and acrosomal membrane, respectively. Spermatozoa were classified into four groups, as follows: PI unstained and PNA-FITC unstained (PI–PNA–: normal spermatozoa), PI unstained and PNA-FITC stained (PI–PNA+: spermatozoa injured in acrosomal membrane), PI stained and PNA-FITC unstained (PI+PNA–: spermatozoa injured in plasma membrane), and PI stained and PNA-FITC stained (PI+PNA+: spermatozoa injured in both plasma and acrosomal membranes). Three

replicates per sample (at least 251 spermatozoa) were evaluated and the mean values were used in the statistical analyses.

Protein extraction and Western blotting

Fresh, cooled, and frozen-thawed bull spermatozoa were washed three times in 5 ml of phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.02% polyvinyl alcohol (PVA; Sigma-Aldrich) by centrifugation at 600 g for 5 min per washing. The resulting spermatozoa pellets were subsequently used for protein extraction and Western blotting analyses. Proteins in the spermatozoa (approximately 5.0×10^7 cells), kidney (as a positive control) [31], and erythrocytes (as a negative control) [32] were extracted using ISOGEN reagent (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Protein pellets were resuspended with 100 μ l of sample buffer containing 2% SDS and 100 mM DTT (ATTO, Tokyo, Japan) and then boiled at 95°C for 6 min. For the detection of α -tubulin in spermatozoa samples, 10-fold dilutions were further performed using sample buffer. Ten microliters of sample were separated on 12% polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) by electrophoresis for 90 min at 60 mA. Following electrophoresis, gel proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) using the Trans-Blot system (AE-7500; ATTO) for 30 min. The membrane was blocked in PBS supplemented with 1% bovine serum albumin (BSA; essentially fatty acid-free, Sigma-Aldrich) and 5% normal goat serum (NGS; Wako Pure Chemical Industry, Osaka, Japan) for 30 min at room temperature, and then incubated with anti-human AQP3 rabbit polyclonal IgG (sc-20811; Santa Cruz Biotechnology, Dallas, TX, USA, 1:200), anti-human AQP7 rabbit polyclonal IgG (orb13253; biorbyt, San Francisco, CA, USA, 1:500), or anti- α tubulin mouse monoclonal IgG (MABT205; Millipore, 1:500) in PBS supplemented with 0.05% (v/v) Tween-20 (Bio-Rad laboratories, Hercules, CA, USA), 0.1% BSA, and 1% NGS overnight at 4°C. Control experiments were performed using normal rabbit serum (NRS; Invitrogen, 1:5000) instead of primary antibodies against AQP3 and AQP7. The membranes were washed five times for 5 min in PBS supplemented with 0.05% Tween-20 and then incubated with the horseradish peroxidase-conjugated goat anti-rabbit (sc-2004; Santa Cruz Biotechnology, 1:5000) or goat anti-mouse (sc-2005; Santa Cruz Biotechnology, 1:5000) immunoglobulin secondary antibody for 2 h at room temperature. Immunoblots were developed with enhanced chemiluminescence agents (Millipore) and images were acquired in a ChemiDoc CCD imager using Quantity One software (Bio-Rad laboratories). The sizes of obtained bands were estimated using a calibration curve prepared using a prestained molecular weight marker (LC5925; Invitrogen). The relative abundance was quantified by the band intensity using ImageJ software (National Institutes of Health, Bethesda, MD, USA). α -Tubulin was used for normalization for the calculation of relative abundances of AQP3 and AQP7. The relative abundances of AQP3 and AQP7 were determined in replicates from each sample on different membranes and the mean values were used in the statistical analyses.

Immunocytochemistry

Fresh, cooled, and frozen-thawed bull spermatozoa were washed three times in 5 ml of PBS supplemented with 0.02% PVA by cen-

trifugation at 600 g for 5 min per washing. The resulting spermatozoa pellets were subsequently used for immunocytochemistry analyses. Sperm samples were fixed in 4% paraformaldehyde (Wako Pure Chemical Industry) solution for 30 min at room temperature and washed three times in 5 ml of PBS supplemented with 0.02% PVA by centrifugation at 600 g for 5 min per washing. Then, sperm samples were smeared on aminosilane-coated slides (Matsunami Glass, Osaka, Japan), and air-dried for 60 min. The slides were washed in PBS, and then treated with 0.25% Triton X-100 (Sigma-Aldrich) in PBS containing 1% BSA for 10 min at room temperature. After washing in PBS, slides were blocked in PBS supplemented with 1% BSA and 5% NGS for 30 min at room temperature, and then incubated overnight in humid container at 4°C with anti-human AQP3 rabbit polyclonal IgG (sc-20811; 1:50) and anti-human AQP7 rabbit polyclonal IgG (orb13253; 1:100) in PBS supplemented with 0.1% BSA and 1% NGS. Control experiments were performed using NRS (1:5000) instead of primary antibodies against AQP3 and AQP7. The slides were washed five times for 5 min in PBS, and then the reactions were revealed by goat anti-rabbit FITC-conjugated antibody (sc-2012; Santa Cruz Biotechnology, 1:500) for 2 h at room temperature. The slides were washed five times for 5 min in PBS, mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories), and examined by fluorescence microscopy (Nikon E800; Nikon).

Experimental design

Experiment 1: Analysis of expression of AQP3 and AQP7 in frozen-thawed bull spermatozoa

To examine whether AQP3 and AQP7 are expressed in bull spermatozoa, Western blotting analyses were performed using frozen-thawed spermatozoa from a bull (Bull A), as well as kidney (positive control) and erythrocyte (negative control).

Experiment 2: Comparison of the localization and relative abundance of AQP3 and AQP7 among fresh, cooled, and frozen-thawed bull spermatozoa

To investigate the changes in the localization and relative abundance of AQP3 and AQP7 in bull spermatozoa during cryopreservation, immunocytochemistry and Western blotting analyses were performed using fresh, cooled, and frozen-thawed spermatozoa from 2 bulls (Bulls B and C), each with 4 ejaculates (total 8 ejaculates). In immunocytochemistry analyses of AQP7, at least 200 spermatozoa were examined to calculate the proportion of localization pattern 1 and 2.

Experiment 3: Relationship of relative abundances of AQP3 and AQP7 with sperm quality in frozen-thawed bull spermatozoa

The relationship of the relative abundances of AQP3 and AQP7 (evaluated by Western blotting analyses) with motility and integrity of plasma and acrosomal membranes in frozen-thawed spermatozoa from 6 bulls (Bulls D to I), each with 3 ejaculates (total 18 ejaculates), was investigated by multiple regression analysis.

Data analysis

In Experiment 2, the effects of bull and cryopreservation step (fresh, cooled, and frozen-thawed) on localization patterns of AQP7 and relative abundances of AQP3 and AQP7 were analyzed by a two-way ANOVA using R statistical package (R version 3.3.2). Data for the proportion of spermatozoa with localization pattern 1 of AQP7 were subjected to arcsine transformation. In Experiment

3, multiple regression analyses of the relative abundances of AQP3 and AQP7, sperm motility, and membrane integrity in frozen-thawed bull spermatozoa were performed by fitting the general linear model (assumed a normal distribution) for parameters VAP, VSL, VCL, ALH, and BCF or the generalized linear model (assumed a beta distribution, link function was logit) for parameters motile, progressive, STR, LIN, PI-PNA-, PI-PNA+, PI+PNA-, and PI+PNA+ using R statistical package. Each of the nine motility parameters and the four membrane integrity parameters were considered as individual dependent variables and relative abundances of AQP3 and AQP7 were considered as independent variables. The differences were considered statistically significant at $P < 0.05$.

Results

Experiment 1: Analysis of expression of AQP3 and AQP7 in frozen-thawed bull spermatozoa

The results of Western blotting for AQP3 and AQP7 in frozen-thawed bull spermatozoa, kidney, and erythrocyte are shown in Fig. 1. For AQP3, a clear band of approximately 42 kDa in spermatozoa and two bands of approximately 41–42 kDa and 52 kDa in the kidney were detected. For AQP7, a clear band of 53 kDa in spermatozoa and two bands of 40 kDa and 53 kDa in the kidney were detected. No clear band was detected in erythrocytes for either AQP3 or AQP7. Non-specific bands could be confirmed by the experiment using NRS instead of using primary antibodies against AQP3 and AQP7.

Experiment 2a: Comparison of the localization of AQP3 and AQP7 among fresh, cooled, and frozen-thawed bull spermatozoa

The results of immunocytochemistry analyses of AQP3 and AQP7 in fresh, cooled, and frozen-thawed bull spermatozoa are shown in Fig. 2 and 3, respectively. For AQP3, immunostaining was clearly found in the principal piece of the fresh, cooled, and frozen-thawed bull spermatozoa (Fig. 2). For AQP7, two localization patterns were observed in fresh, cooled, and frozen-thawed bull spermatozoa; pattern 1: diffuse staining in head and entire tail, pattern 2: diffuse staining in head and clear staining in mid-piece (Fig. 3). No staining was detected in the experiments using NRS (Fig. 2 and Fig. 3). Using a two-way ANOVA, there was no interaction in the proportion of spermatozoa with localization pattern 1 of AQP7 between bull and cryopreservation step. The cryopreservation step (cooling and freeze-thawing) did not affect the localization pattern of AQP7 in spermatozoa ($P = 0.092$), whereas the mean value of proportion of spermatozoa with localization pattern 1 of AQP7 differed between Bull B and C ($P = 0.016$, Fig. 4).

Experiment 2b: Comparison of the relative abundances of AQP3 and AQP7 among fresh, cooled, and frozen-thawed bull spermatozoa

Band patterns of AQP3 and AQP7 were not different among fresh, cooled, and frozen-thawed bull spermatozoa. Using a two-way ANOVA, there was no interaction in the relative abundances of AQP3 and AQP7 between bull and cryopreservation step. No significant difference was observed in relative abundances of AQP3 and AQP7 evaluated by Western blotting among fresh, cooled, and frozen-thawed

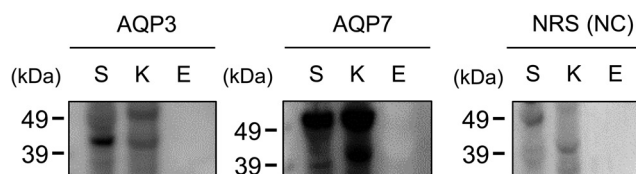


Fig. 1. Representative photographs of Western blotting for AQP3 and AQP7 in frozen-thawed bull (Bull A) spermatozoa, kidney, and erythrocytes. S: spermatozoa. K: kidney. E: erythrocytes. NRS: normal rabbit serum. NC: negative control.

bull spermatozoa (AQP3: $P = 0.889$, AQP7: $P = 0.726$, Fig. 5) or between Bull B and C (AQP3: $P = 0.504$, AQP7: $P = 0.166$, Fig. 5).

Experiment 3: Relationship of relative abundances of AQP3 and AQP7 with sperm quality in frozen-thawed bull spermatozoa

The relative abundances of AQP3 and AQP7, the motility, and the integrity of the plasma and acrosomal membranes varied among ejaculates (Fig. 6 and Supplementary Table 1: online only). The relationship of the relative abundances of AQP3 and AQP7 with motility and integrity of plasma and acrosomal membranes in frozen-thawed bull spermatozoa, analyzed by multiple regression analyses, is shown in Table 1. No significant interaction was detected in all the models. With linearly increasing AQP3, the parameters VAP, VSL, VCL, and STR significantly increased (VAP, VSL, and VCL: $P < 0.05$, STR: $P < 0.01$). With linearly increasing AQP7, the parameters progressive, VAP, VSL, VCL, and ALH significantly increased (progressive: $P < 0.05$, VAP, VSL, VCL, and ALH: $P < 0.001$). On the other hand, with linearly increasing AQP7, BCF was significantly decreased ($P < 0.01$). Neither AQP3 nor AQP7 had an effect on the integrity of the plasma and acrosomal membranes.

Discussion

In the present study, we examined the expression and localization of AQP3 and AQP7 in fresh, cooled, and frozen-thawed bull spermatozoa. In addition, the relationship of AQP3 and AQP7 with sperm motility and integrity of plasma and acrosomal membranes post freeze-thawing was investigated. We found that AQP3 is present in the principal piece of the tail, whereas AQP7 is present in the head and tail region with two localization patterns. Furthermore, we demonstrated that the amounts of AQP3 and AQP7 varied among ejaculates, and were positively related to sperm motility, particularly sperm velocity, post freeze-thawing. These findings suggest a novel insight that both AQP3 and AQP7 are possibly involved in the tolerance to freeze-thawing in bull spermatozoa, particularly in the sperm's tail.

To avoid damage to the membranes and organelles that leads to reduced motility and fertility after cryopreservation, spermatozoa must control the flow of water and CPA via the plasma membrane and adapt to dramatic osmotic changes during cryopreservation process. Thus, the permeability of the plasma membrane to water and CPA is considered to be a crucial cryobiological property for determining the tolerance of spermatozoa to freeze-thawing [7–9]. AQP3 and AQP7

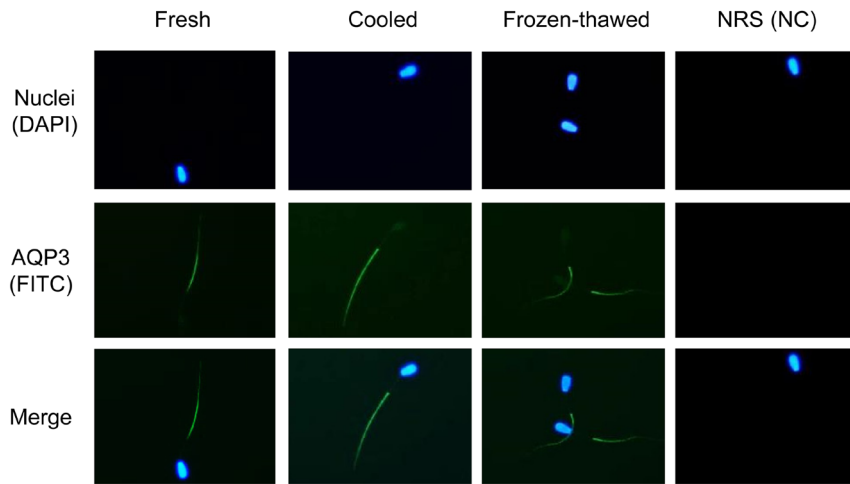


Fig. 2. Representative photographs of immunostaining for AQP3 in fresh, cooled, and frozen-thawed bull (Bull B or C) spermatozoa. NRS: normal rabbit serum. NC: negative control.

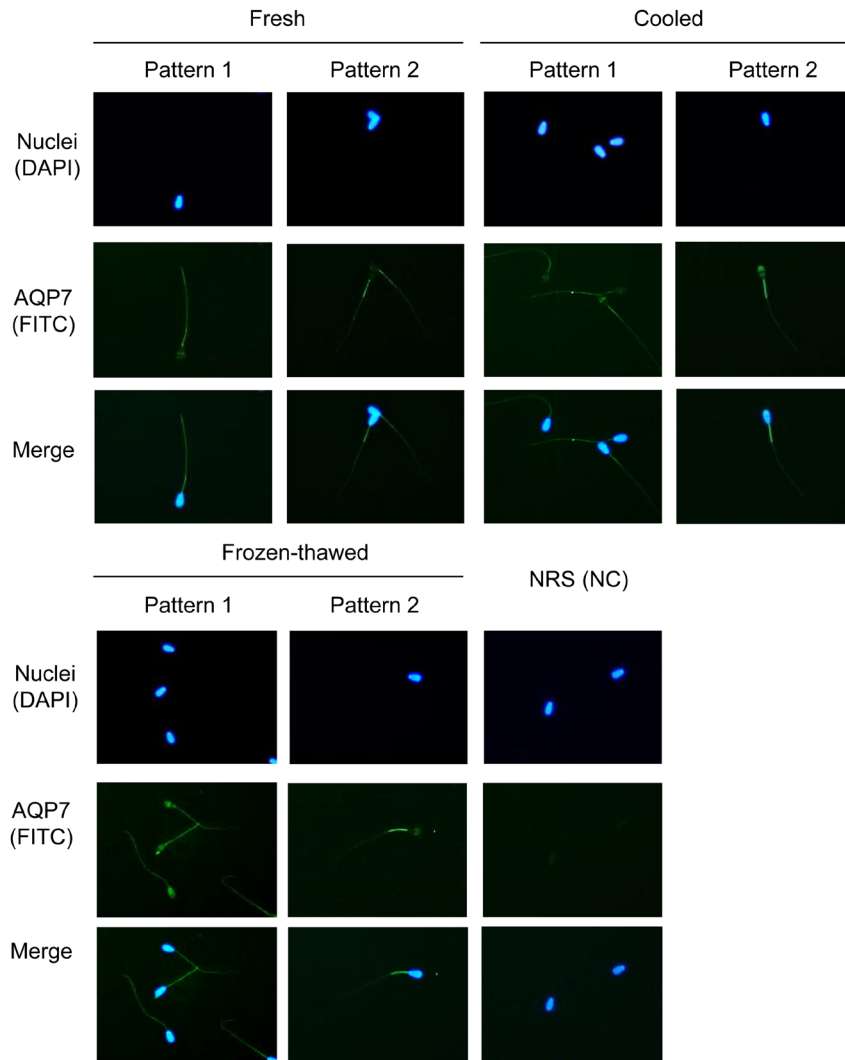


Fig. 3. Representative photographs of immunostaining for AQP7 in fresh, cooled, and frozen-thawed bull (Bull B or C) spermatozoa. Pattern 1: diffuse staining in head and entire tail. Pattern 2: diffuse staining in head and clear staining in mid-piece. NRS: normal rabbit serum. NC: negative control.

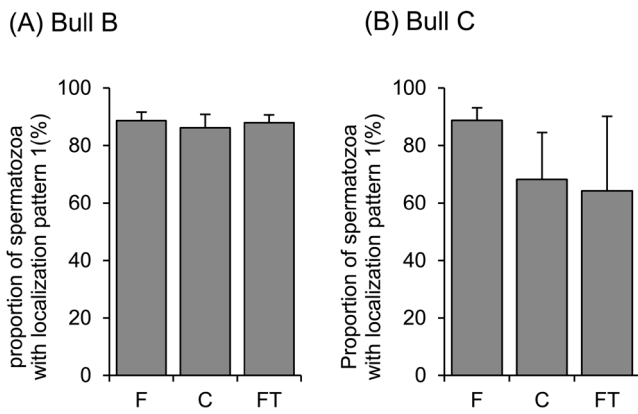


Fig. 4. The proportion of spermatozoa with localization pattern 1 of AQP7 in fresh, cooled, and, frozen-thawed steps evaluated by immunocytochemistry analysis (A: Bull B, B: Bull C). Columns and bars indicate mean value and standard deviation (SD), respectively. Using two-way ANOVA, there was no interaction in the proportion of spermatozoa with localization pattern 1 of AQP7 between bull and cryopreservation step. Proportion of spermatozoa with localization pattern 1 did not differ among fresh, cooled, and frozen-thawed spermatozoa (mean \pm SD in fresh, cooled, and frozen-thawed spermatozoa was 88.7 ± 3.5 , 77.2 ± 14.7 , and 76.1 ± 21.3 , respectively, $P = 0.092$), but there was difference between Bull B and C (mean \pm SD in Bull B and C was 87.6 ± 3.5 and 73.8 ± 19.7 , respectively, $P = 0.016$). F: fresh spermatozoa, C: cooled spermatozoa, FT: frozen-thawed spermatozoa.

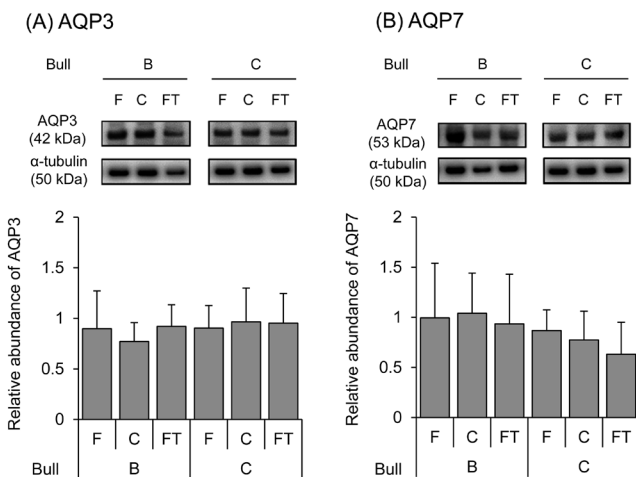


Fig. 5. The relative abundances of (A) AQP3 and (B) AQP7 in fresh, cooled, and, frozen-thawed bull spermatozoa evaluated by image analysis of the bands detected in Western blotting. Columns and bars indicate mean value and SD, respectively. Using two-way ANOVA, there was no interaction in the relative abundances of AQP3 and AQP7 between bull and cryopreservation step. No significant difference was observed in relative abundances of AQP3 and AQP7 evaluated by Western blotting among fresh, cooled, and frozen-thawed bull spermatozoa (AQP3: $P = 0.889$, AQP7: $P = 0.726$) or between Bull B and C (AQP3: $P = 0.504$, AQP7: $P = 0.166$). F: fresh spermatozoa, C: cooled spermatozoa, FT: frozen-thawed spermatozoa.

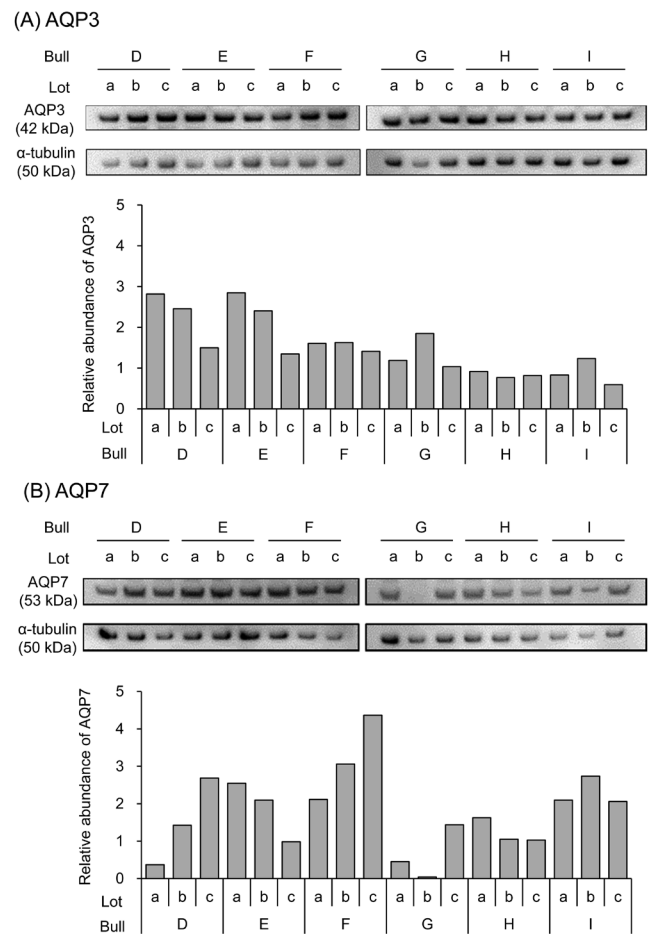


Fig. 6. The relative abundances of (A) AQP3 and (B) AQP7 evaluated by image analyses of the bands detected by Western blotting of frozen-thawed bull (Bulls D to I) spermatozoa.

belong to the aquaglyceroporin family, which participates in the transport of not only water but also glycerol [10–12]. Therefore, it is presumed that AQP3 and AQP7 play important roles in the process of cryopreservation [8, 9]. The physiological roles of AQP3 and AQP7 in the male reproductive system, including spermatogenesis, sperm motility, and sperm volume regulation, has been well investigated in several species, including rodents, humans and boars [33, 34]. However, few studies have investigated the expression of AQPs in bull spermatozoa and their role in the process of cryopreservation, although it was recently reported that AQP3, AQP7, and AQP11 are expressed in bull spermatozoa and that the amount of AQP7 and AQP11 in fresh spermatozoa may be useful as a marker to predict tolerance to freeze-thawing in bull spermatozoa [26, 27]. In addition, changes in the expression and localization of AQP3 and AQP7 during the cryopreservation process (cooling and freeze-thawing) in bull spermatozoa has not been investigated. Therefore, we first investigated the expression and localization of AQP3 and AQP7 in fresh, cooled, and frozen-thawed bull spermatozoa (Experiment 1 and 2).

In Western blotting analysis, we detected clear bands of AQP3

Table 1. Effect of the relative abundances of AQP3 and AQP7 on motility and membrane integrity parameters in frozen-thawed bull spermatozoa

Dependent variables		Independent variables					
		AQP3			AQP7		
		Estimate	SEM	P value	Estimate	SEM	P value
Motility	motile	0.039	0.119	0.741	0.060	0.078	0.436
	progressive	0.144	0.106	0.177	0.152	0.069	0.027 *
	VAP	5.968	2.471	0.029*	7.182	1.607	< 0.001 ***
	VSL	6.375	2.330	0.015*	6.782	1.515	< 0.001 ***
	VCL	12.271	4.659	0.019*	13.852	3.031	< 0.001 ***
	STR	0.140	0.053	0.008**	0.033	0.034	0.332
	LIN	-0.014	0.033	0.666	-0.027	0.021	0.199
	ALH	0.420	0.212	0.067	0.636	0.138	< 0.001 ***
	BCF	0.375	0.542	0.499	-1.061	0.353	0.009 **
Membrane integrity	PI-PNA-	0.083	0.120	0.489	-0.015	0.078	0.850
	PI-PNA+	-0.034	0.254	0.893	-0.221	0.172	0.199
	PI+PNA-	-0.003	0.103	0.979	0.001	0.067	0.983
	PI+PNA+	-0.120	0.099	0.226	0.022	0.063	0.730

Asterisk indicates statistically significant (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). motile: percentage of motile spermatozoa. progressive: percentage of progressively motile spermatozoa. VAP: average path velocity. VSL: straight-line velocity. VCL: curvilinear velocity. STR: percentage of straightness (VSL/VAP). LIN: percentage of linearity (VSL/VCL). ALH: amplitude of lateral head displacement. BCF: beat frequency. PI-PNA-: PI unstained and PNA-FITC unstained. PI-PNA+: PI unstained and PNA-FITC stained. PI+PNA-: PI stained and PNA-FITC unstained. PI+PNA+: PI stained and PNA-FITC stained.

and AQP7 with the molecular weights of approximately 42 kDa and 53 kDa, respectively, in frozen-thawed bull spermatozoa. In contrast, Prieto-Martinez *et al.* [26] reported that specific bands were detected at 30 kDa and 60 kDa for AQP3 and at 25 kDa for AQP7 in bull spermatozoa. Although the reason for this difference is not fully understood, they could be attributed to differences in antibodies used and protocols of protein extraction and Western blotting utilized. However, in this study, Western blotting of the kidney as a positive control also showed the bands of AQP3 and AQP7 with sizes of approximately 41–42 kDa and 53 kDa, respectively, while no clear bands were detected in erythrocytes as a negative control. Furthermore, we also detected the bands of 42 kDa for AQP3 and 53 kDa for AQP7 after using alternative antibodies against AQP3 and AQP7, which react with different epitopes from AQP3 antibody sc-20811 and AQP7 antibody orb13253, respectively (Supplementary Fig. 1: online only). Taken together, the 42-kDa and 53-kDa bands in bull spermatozoa detected in Western blotting analyses of this study are likely to be specific bands for AQP3 and AQP7, respectively. On the other hand, the molecular weights of bovine AQP3 and AQP7 calculated from reported amino acid sequences are approximately 32 kDa and 36 kDa, respectively. It is well known that AQPs have some nitrogen-linked glycosylation sites in extracellular loops. Therefore, we speculated that glycosylation may be one of the factors related to the different molecular weights of AQP3 and AQP7 expected in bull spermatozoa.

In the present study, immunostaining of AQP3 was clearly found in the principal piece of the tail of bull spermatozoa. For AQP7, two localization patterns were observed —pattern 1: diffuse staining in head and entire tail, pattern 2: diffuse staining in head and clear staining

in mid-piece. Localization patterns of both AQP3 and AQP7 were not different among fresh, cooled, and frozen-thawed bull spermatozoa. Similar to the Western blotting analyses, our immunostaining results for AQP3 and AQP7 in bull spermatozoa were different from those of Prieto-Martinez *et al.* [26], who showed that immunostaining of AQP3 and AQP7 was mainly found in the midpiece of the tail in fresh and frozen-thawed bull spermatozoa. Although the reason for differences in AQP3 and AQP7 localization between our results and those of Prieto-Martinez *et al.* [26] is not fully understood, we infer that difference in the antibody used might be one of the contributing factors. This has been reported in boar spermatozoa, whereby immunocytochemistry results of AQP7 varied depending on the antibodies used [23]. In mouse and human spermatozoa, localization of AQP3 in the principal piece of the sperm tail was clearly shown by immunofluorescence or an immunogold-labeled electron microscopic test, and AQP3-deficient sperm showed increased vulnerability to hypotonic cell swelling, characterized by increased tail bending after entering the uterus [16]. In addition, Saito *et al.* [17] reported that AQP7 immunostaining was found in the tail region of human spermatozoa, and sperm motility was low in an infertile man who lacked AQP7 compared to that in AQP7-positive sperm. Considering the localization of AQP3 and AQP7 and their physiological functions demonstrated in mouse and human spermatozoa, we believe that our results of immunocytochemistry analyses are reasonable. In addition, our results are likely plausible because the epitopes of anti-human AQP3 and AQP7 antibodies used in this study are more than 99% of homology to bovine antigens. Nevertheless, it is noted that the specificity of antibodies is an ongoing issue of concern for research in sperm AQPs across several species, as previously reported [35].

In Experiment 3, we used frozen-thawed bull spermatozoa for evaluation of relative abundances of AQP3 and AQP7. However, we have recognized that the freeze-thawing procedure affects some of the sperm protein amounts detected by Western blotting [36–38]. Therefore, we conducted a preliminary examination of the difference in the relative abundances of AQP3 and AQP7 in bull spermatozoa before and after cryopreservation (Experiment 2). We found that cooling and freeze-thawing did not affect the relative abundances of AQP3 and AQP7, which is consistent with previous results reported by Prieto-Martinez *et al.* [26]. Thus, we decided that it was permissible to use frozen-thawed samples rather than fresh samples to evaluate the relative abundances of AQP3 and AQP7 in Experiment 3.

Interestingly, relative abundances of AQP3 and AQP7 evaluated by Western blotting varied among ejaculates. During spermatogenesis, RNAs are transcribed at the pre-meiotic and early haploid stages, and thereafter RNA transcription does not occur [39]. In addition, it is generally accepted that protein translation does not occur in matured spermatozoa. Therefore, variation in the level of AQP proteins in each ejaculate was considered to have been determined during spermatogenesis. Individual differences in mRNA and in amounts of protein expressed by some genes have been reported in the testis and spermatozoa [40, 41]. Some conditions during spermatogenesis in each bull may influence the variation in the amount of AQPs among ejaculates, but further studies are required.

Our multiple regression analyses demonstrated that the relative abundances of both AQP3 and AQP7 are positively related to the frozen-thawed sperm motility, particularly sperm velocity, but bear no relation with the integrity of plasma and acrosomal membranes. In this study, we did not perform CASA analysis of sperm motility or fluorescent staining test of membrane integrity before freezing. We have recognized that the objective evaluation of sperm motility and membrane integrity should be performed before freezing to elucidate the association of AQP with sperm cryotolerance, similar to that in previous reports [25–27]. However, only samples visually assessed with 65 to 75% of progressively motile spermatozoa at high speed before freezing (meeting the criteria of the Genetics Hokkaido Association) were used in Experiment 3. Thus, in Experiment 3, the difference in motility and membrane integrity after freeze-thawing could be considered as a result of differences in the cryotolerance of each ejaculate. Recently, Prieto-Martinez *et al.* [25] reported that AQP3 are localized in acrosomal and entire tail region and AQP7 localized in connecting piece in fresh boar spermatozoa. Furthermore, they reported that relative AQP3 and AQP7 abundances in fresh boar spermatozoa are positively correlated with sperm motility and viability assessed at 30 min post-thawing, which is in agreement with the results of the present study using bull spermatozoa. On the other hand, they also reported that ejaculates with good freezing ability have higher amounts of AQP7 but not AQP3 compared to ejaculates of poor freezing ability in bulls [26]. Our findings in this study suggest a new insight that not only AQP7, but also AQP3, are possibly related to cryotolerance of bull spermatozoa. Combined with the results of our immunocytochemistry analysis that AQP3 and AQP7 are mainly localized in the tail region through the cryopreservation process and the positive relationships between the relative abundance of AQP3 or AQP7 and sperm motility, we infer that the ejaculates with higher amounts of AQP3 and AQP7 could adapt to dramatic

osmotic changes during the cryopreservation process by efficient transportation of water and CPA particularly in the sperm's tail, resulting in high motility post freeze-thawing.

In studies of oocytes and embryos, several researchers have demonstrated the function of AQP3 and AQP7 in the molecular pathways for transport of water and CPAs, and their crucial roles for cryotolerance in mouse [42–44], bovine [45], and porcine [46, 47]. In order to elucidate the association of AQP3 and AQP7 with cryotolerance in bull spermatozoa in more detail, further investigations, such as studies using experimental systems permitting the specific inhibition of the channel function of AQP3 and AQP7 in bull spermatozoa, are necessary. In addition, further research is required to elucidate whether the localization pattern of AQP7 is different among individual bulls or lots and whether it is related to sperm quality before and after cryopreservation.

In conclusion, we have demonstrated the possible involvement of AQP3 and AQP7 in the tolerance to freeze-thawing in bull spermatozoa, particularly in the sperm's tail. Although further studies, such as elucidating the regulatory mechanism of AQP3 and AQP7 expression during spermatogenesis, are needed, our findings have advanced the research on AQPs in bull spermatozoa and provided valuable information for improving the efficiency of cryopreservation in bull spermatozoa.

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