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Evaluation of boar and bull sperm capacitation and the acrosome reaction using flow cytometry

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ABSTRACT

Flow cytometry can be used to evaluate many sperm attributes and Dr. Duane Garner was influential in developing assays to understand sperm physiology and function. We review some of Dr. Garner's work and describe experiments that evaluate sperm capacitation using Dr. Garner's philosophy. In exploratory experiments, boar sperm were cryopreserved in lactose egg yolk (LEY) or Beltsville Freezing Extender 5 (BF5) and incubated in one capacitating medium. In another experiment, frozen-thawed bull sperm were incubated in TALP-Ca or CFDM1 capacitating media. In both experiments, sperm viability and capacitation were evaluated using multiple probes. Boar sperm frozen in LEY had greater survival rates (38%) than sperm frozen in BF5 (22%; $P < 0.05$) but did not capacitate as effectively as sperm in BF5 ($P < 0.05$). In Experiment 2, bull sperm survived to a greater extent when incubated in TALP-Ca than in CFDM1 ($P < 0.05$) and had greater capacitation for most parameters ($P < 0.05$). Of particular interest, 77% of sperm incubated in TALP-Ca had activated second messenger systems involved in capacitation, compared with < 5% of sperm incubated in CFDM1. The results indicate different freezing and capacitating media induce different responses to sperm capacitation and functions. If only sperm viability and acrosomal integrity were evaluated, these results would be interpreted very differently. Dr. Garner's philosophy of evaluating multiple sperm parameters was an impetus to determine unique treatment differences which help in understanding sperm capacitation, and design further experiments to determine how media content causes sperm physiology differences.

1. Introduction

Flow cytometry is a practical technique for assessing cell physiology and function with applications across many cell types. Dr. Duane Garner was one of the first to recognize and utilize the power of flow cytometry to evaluate many attributes of sperm cells in an attempt to understand how these attributes contributed to sperm function and fertility. In this paper, there is delineation among some of the seminal contributions Dr. Garner made in evaluating sperm using flow cytometry and then, in Dr. Garner's spirit of constantly testing new flow cytometric assays in attempting to understand sperm physiology, there will be presentation of data from assays that may increase the understanding of sperm capacitation.

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One of the foundational components of flow cytometry is the use of fluorophores to assess a particular aspect of cell physiology or morphology. Early work from the Garner laboratory focused on investigation of the use of fluorescent stains to determine the proportions of viable (CFDA or CDMFDA) and non-viable/damaged sperm (HED or PI) using fluorescent microscopy and flow cytometry procedures (Garner et al., 1988; Ericsson et al., 1989). Several of those stains are difficult to use, however, because these stains tend to leak out of the cells during incubation (Ericsson et al., 1989). An understanding of how fluorescent stains and flow cytometry itself affected the physiology and fertilizing capacity was always paramount in the Garner laboratory, and he understood that sperm are fragile by nature, lack the organelles that enable self-repair, and consequently had to determine such basic things as the toxicity concentrations of fluorescent stains for various sperm attributes (Downing et al., 1991; Garner, 2009). Improvements to those analyses are documented in publications developing the use of SYBR-14 combined with propidium iodide (PI, Garner and Johnson, 1995) and comparing the use of rhodamine 123, JC-1, and MitoTracker Green FM (Garner et al., 1997a, 1997b). The content of these often referenced publications did not just introduce and validate new fluorescent stains for assessing the viability (SYBR-14/PI) and mitochondrial potential (JC-1) of bull sperm, but the publications were a basis that encouraged a broader use of flow cytometry and creation of a diverse array of fluorophores that could be more easily used with cells, because of excitation at 488 nm rather than UV excitation, which can be detrimental to some cells and cell systems (Garner and Johnson, 1995; Garner, 2009). Perhaps more importantly, the contents of these publications were part of a research continuum of results into fluorescent staining and applications (Garner et al., 1996a, 1996b; Garner and Thomas, 1999; Thomas et al., 1997) that have become a basis for sperm analysis in andrology.

Flow cytometry can be used to understand effects of processes and validate unproven hypotheses. For example, the toxicity effects of glycerol as part of a cryopreservation diluent (Garner and Thomas 1999; Garner et al., 1999), seminal plasma (Garner et al., 2001), and cryopreservation (Garner et al., 1997a, 1997b; Thomas et al., 1998), all of which are components of the artificial insemination process have been studied, resulting in means to ameliorate deleterious effects of these factors on fertility. Furthermore, when the technology was used for conducting multiparametric analysis procedures including evaluations of plasma membrane and acrosome integrity, and mitochondrial function, this enabled identification of bulls with the potential for greater fertility (Ericsson et al., 1993). This same perspective has since been utilized to investigate a significant number of sperm structural and physiological characteristics to enhance our knowledge of gamete biology in vitro and in vivo.

One prominent application of flow cytometry technologies is for “sexing” mammalian sperm based on sperm DNA content. Again, the focus of the research by Dr. Garner was not merely if mammalian sperm could be separated into its X and Y chromosome populations, but also on validating the results to ensure that the two cell populations were in fact separated because of DNA content. Garner et al. (1983) accomplished this using a variety of heterogametic species, which contain both X- and Y-bearing sperm, and roosters which are homogametic and produce only a single population of sperm. The results from these experiments validated the accuracy of the flow cytometric analyses and the efficacy of the technology for detection of subtle differences in DNA content within, and by, species (Garner et al., 1983). In addition, once those characteristics were confirmed further validation of the technology was performed to ensure that sperm damage was minimal or avoidable (Garner et al., 1984) and then the technique was used to assess the success of other purported sperm sexing techniques (Pinkel et al., 1985). As the flow cytometric techniques for sex-sorting have improved over the years, these procedures are now widely used by the dairy industry (Steele et al., 2020).

Flow cytometry has proven to be very useful for analyzing many cellular attributes, therefore, the quantity of available fluorophores has expanded greatly. Furthermore, the capability to mix and match probes (e.g. lectins or antibodies) with fluorescent conjugates has led to a greater ability to multiplex fluorescent stains and simultaneously analyze multiple physiological or structural aspects of a cell simultaneously. These techniques, coupled with the development of affordable bench top flow cytometers, has resulted in an increase in the number of experiments and publications by andrologists, that have enhanced the knowledge of sperm biology. Furthermore, that ease of access to a variety of probes and fluorophores, coupled with the findings from the early studies in the Garner laboratory on sperm, has provided for an impetus to evaluate sperm functions that are based on, but well beyond that which could be accomplished using the original SYBR-14/PI analyses.

By embracing Dr Garner’s perspectives, namely that flow cytometry can be used to understand a sperm process, there was application of this technology to discern how different media affect mammalian sperm capacitation and the acrosome reaction. Sperm capacitation is modulated by many factors including: pH, temperature and the ingredients of the incubation medium used. Experiments conducted to compare the effectiveness of different media on sperm capacitation, frequently have led to elucidation of differences in gross cellular characteristics such as motility or membrane integrity, but often there is omission of an analysis of physiologic functions (e.g., second messenger signaling). Consequently, discerning the effects of a medium or an incubation condition on sperm physiology is severely limited. The goal of this exploratory research, therefore, was to develop methods to quantitatively evaluate sperm capacitation and the acrosome reaction which incorporate 1) the evaluation of second messengers, such as PKA (Pommer et al., 2003) and PKC (via PDK1, Kumaresan et al., 2011), and 2) enable monitoring of plasma membrane reorganization using Quant-IT (Purdy et al., 2016) and Merocyanine 540 (Guthrie and Welch, 2005), for protein and phospholipid identification, respectively. Establishment of these protocols should enable us to more precisely define the conditions that enable sperm capacitation and the acrosome reaction and consequently provide for a greater understanding of these physiologic processes.

2. Materials and methods

The semen samples were donated to the National Animal Germplasm Program from commercial studs and, therefore, no IACUC review and approval was required. To the best of our knowledge, however, all animal care and handling was performed in accordance with standard operating procedures that had been approved by the USDA CARR Animal Care and Use Committee. All animals were fed a complete ration to provide all nutritional needs, hay (cattle), and provided water ad libitum.

2.1. Boar sperm cryopreservation

Boars were between the ages of 18 and 26 months at the time of collection. Boar semen samples were collected from nine boars (nine different breeds) using the hand-glove technique and the sperm-rich fraction was diluted 1:1 (v:v) with Androhep-Plus (Minitube USA, Verona, WI) at 37 °C. Gross sperm motility was evaluated microscopically to ensure > 70% total sperm motility. The samples (individual, not pooled) were then cooled to 15 °C during a 2 h period and were maintained at this temperature during transport to the laboratory overnight. Upon arrival at the laboratory, the samples were centrifuged (10 min at 800 x *g*) and the supernatant was removed, leaving the sperm pellet and approximately 3 mL of fluid. The contents of the sperm pellet were resuspended and the percentages of motile sperm was determined using CASA (IVOS Version 10.9, Hamilton Thorne Bioscience, Beverly, MA, USA) while using the following parameters: 60 frames acquired, frame rate of 45 Hz, minimum contrast of 46, minimum cell size of seven pixels, VAP cutoff of 20 μm, progressive minimum VAP cutoff of 25 μm/s, VSL cutoff of 5 μm/s, straightness of 45%, static head size of 0.53–4.45, and magnification of 1.89 which were preset by the manufacturer. A minimum of seven fields and 1000 sperm were evaluated for motility analysis. Only samples having > 70% motile cells were used. The sperm concentration was determined using a spectrophotometer (Spectronic 20 Genesys, Thermo Fisher Scientific, Waltham, MA) calibrated for boar sperm. Samples were then diluted to 600×10^6 sperm/mL with glycerol-free BF-5 (Pursel and Johnson, 1975) or LEY (Almlid and Johnson, 1988) and cooled to 5 °C during a 2 h period. Samples were then diluted to 400×10^6 sperm/mL with a second fraction of respective diluent containing glycerol (6% by volume) and Equex paste (2.5% by volume) (Pursel and Johnson, 1975; Almlid and Johnson, 1988) resulting in final concentrations of 2% and 0.833%, by volume, respectively. Samples were then loaded into 0.5 mL wick and powder straws (Minitube USA), heat sealed, and frozen using a Cryo Bio System Mini Digitcool UJ400 programmable freezer (IMV Corporation, Minneapolis, MN) using the following cooling program: 5 °C to − 8 °C at − 20 °C per minute, − 8 °C to − 120 °C at − 69 °C per minute, − 120 °C to − 140 °C at − 20 °C per minute. Samples were then plunged and stored in liquid nitrogen. Contents of straws were thawed for 20 s in a 50 °C water bath (BF5, Pursel and Johnson, 1975) or 8 s in a 70 °C water bath (LEY, Almlid and Johnson, 1988) and the motility was evaluated as previously described.

2.2. Bull sperm cryopreservation

Semen from three mature Angus bulls was collected using an artificial vagina and the sperm motility and concentration determined, using procedures previously described in this manuscript, using CASA with there being the following parameters: 30 frames acquired, frame rate of 60 Hz, minimum contrast of 80, minimum cell size of five pixels, VAP cutoff of 30 μm, progressive minimum VAP cutoff of 50 μm/s, VSL cutoff of 15 μm/s, straightness of 70%, static head size of 0.53–4.45, and magnification of 1.89 which were preset by the manufacturer. A minimum of seven fields and 1000 sperm were evaluated for motility analysis. Only samples having > 80% motile cells were used. The samples were diluted to 120×10^6 sperm/mL with glycerol-free Tris-egg yolk-glycerol cryopreservation medium (Purdy and Graham, 2004) and cooled to 5 °C during a 2 h period. The samples were then diluted 1:1 (v:v), with the same medium containing 14% glycerol by volume (7% glycerol and 60×10^6 sperm/mL, final concentrations) loaded into 0.5 mL wick and powder straws and sealed with PVC powder. Contents of straws were placed on a rack 4.5 cm above liquid nitrogen and frozen in LN2 vapor for 10 min. The samples were then plunged into the liquid nitrogen and stored in LN2 until evaluation 7 days later. Semen straws were thawed in a 37 °C water bath for 30 s and the motility was evaluated as previously described in this manuscript.

2.3. Flow cytometric analysis of sperm capacitation

Thawed sperm samples (0.5 mL) were diluted in 4 mL of Tris buffered media (bull, Purdy and Graham, 2004) or Beltsville Thawing Solution (Pursel and Johnson, 1975) and centrifuged at 500 x *g* for 5 min. Boar sperm were resuspended in a bicarbonate capacitation medium (Harrison et al., 1996) and bull sperm were resuspended in either 10 mM Calcium TALP (TALP-Ca; Graham et al., 1986) or CFDM1 (De La Torre-Sanchez et al., 2006) to 50×10^6 sperm/mL. Sperm were then incubated at 39 °C for as long as 3 h. Subsamples were removed at designated times (0,15, 30 and 45 min for boar sperm and 0, 60, 120 and 180 min for bull sperm) for analysis.

At each timepoint, multiple subsamples (one for each flow cytometric assessment to be made) of 2.5×10^6 sperm, were removed and diluted into 0.25 mL of respective capacitation medium, resulting in a sperm concentration of 10×10^6 sperm/mL and the sperm stained to evaluate different sperm attributes.

Intracellular calcium was determined by staining the sperm with Fluo-3AM (2.5 μL of a 1 mM solution in DMSO). Protein tyrosine phosphorylation was measured using a monoclonal anti-phosphotyrosine-FITC conjugate (PTYR; 1 μL of the stock solution) according to Pommer et al. (2003). Protein kinase C activity was measured using a monoclonal anti-PDK1 antibody (PDK, Invitrogen) with a goat anti-mouse IgG conjugated to Alexa Fluor 488 (Invitrogen) as a secondary antibody (30 μL of the primary antibody which was diluted 1:200 in Tris buffer (Purdy and Graham, 2004) and 1 μL of the stock solution of the secondary antibody, modified protocol from Kumaresan et al., 2011). Acrosomal integrity was assessed using FITC-PNA (1 mg/mL in water, Purdy, 2008). These samples were all counter stained with 2.5 μL of propidium iodide (PI, 1 mg/mL in distilled water) to identify sperm with damaged plasma membranes. In addition, membrane phospholipid organization (order/disorder) was assessed using the combination of Merocyanine 540 (M540) and Yo-Pro-1. These samples were initially stained with 5 μL of Yo-Pro-1 (0.1 mM in DMSO) for 10 min, after which 5 μL of M540 (50 μM solution in DMSO/PBS, Guthrie and Welch, 2005) was added and the samples were analyzed exactly 75 s after addition of the M540.

Samples were analyzed using a CYAN-ADP flow cytometer (Beckman Coulter, Miami, FL, USA) and at least 10,000 sperm were analyzed per sample. The flow cytometer was equipped with a 488-nm argon laser at 150 mW of power for excitation, a 95% reduction

filter, a 530/40 nm band pass filter to detect intracellular calcium (Fluo-3AM), protein tyrosine phosphorylation (PTYR), protein kinase C activity (PDK, only used with bulls), membrane protein organization (Quant-IT, only used with boars) or acrosomal integrity (FITC-PNA). The PI was detected using a 613/20-nm band pass filter. The M540 emission was detected using the 530/40 nm band pass filter and Yo-Pro-1 emission was detected using the 613/20 nm band pass filter. The median fluorescence of the plasma membrane intact population (M540 positive/Yo-Pro-1 negative) were recorded for each sample. Increases in the median of M540 indicate greater fluorescence and, consequently, greater phospholipid disorder (increased membrane fluidity). All data were collected on log scale histograms at a rate of 500–600 events per second. Populations were gated first using forward/side scatter (linear scale) to identify sperm and then by their fluorescent properties (collected in log scale) in accordance with the properties of the fluorophore. Control samples of each stain, single and in combination with the membrane integrity stain (PI or Yo-Pro 1) were analyzed prior to experiments to establish the gates for each stain and then immediately prior to analyses. Calibration of the flow cytometer was performed at the beginning of each day of analysis using CytoFLEX Daily QC Fluorospheres (Beckman Coulter, Miami, FL, USA) and the CYAN calibration software of the flow cytometer. Compensation was performed using the automatic feature of the CYAN software.

2.4. Statistical analysis

Statistical analyses were performed using SAS (Statistical Analysis Systems, 1985) and the repeated measures GLM-ANOVA to determine the effects of incubation medium, time, and their interaction on boar and bull sperm physiologic characteristics (intracellular calcium, protein tyrosine phosphorylation, protein kinase C activity, membrane protein and phospholipid organization, and acrosomal or plasma membrane integrity). All percentage data were log transformed to ensure normality, but the actual means ± SEM are presented in the figures and text. Boar and bull ejaculates were treated as random effects. Means were separate using Student–Newman–Keuls *post-hoc* test. There were considered to be mean differences when there was a $P < 0.05$.

3. Results

3.1. Boar sperm analyses

The post thawed boar sperm total and progressive motility for samples frozen in LEY were 53% and 43%, respectively (range 34–70 and 26–61, respectively) and for samples frozen in BF5 were 33% and 26%, respectively (range 18–45 and 13–37, respectively). Boar sperm viability after cryopreservation (evaluated by intact plasma membranes) was greater when sperm were cryopreserved in the LEY medium as compared with the BF5 medium ($P < 0.05$), but the cryopreservation medium did not affect acrosomal membrane integrity ($P > 0.05$; Fig. 1). In addition, boar sperm frozen in LEY medium had lesser intracellular calcium concentrations ($P < 0.05$, Fig. 2), as well as a plasma membrane protein organization ($P < 0.05$, Fig. 3), a plasma membrane phospholipid order ($P < 0.05$, Fig. 3), and a protein tyrosine phosphorylation pattern ($P > 0.05$; Fig. 2) that had not been disrupted as a result of cryopreservation. The main effect of incubation time was a source of variation for plasma membrane integrity ($P < 0.002$, Fig. 1), acrosomal membrane integrity ($P < 0.0001$, Fig. 1) intracellular calcium ($P < 0.0001$, Fig. 2), protein tyrosine phosphorylation ($P < 0.0001$, Fig. 2), and plasma membrane protein organization ($P < 0.0005$, Fig. 3), however, there were no effects on plasma membrane phospholipid order ($P > 0.05$, Fig. 3). Boar was a source of variation when all analyses were conducted ($P < 0.003$), but there was no interaction of freezing medium x time when all the analyses were conducted ($P > 0.05$).

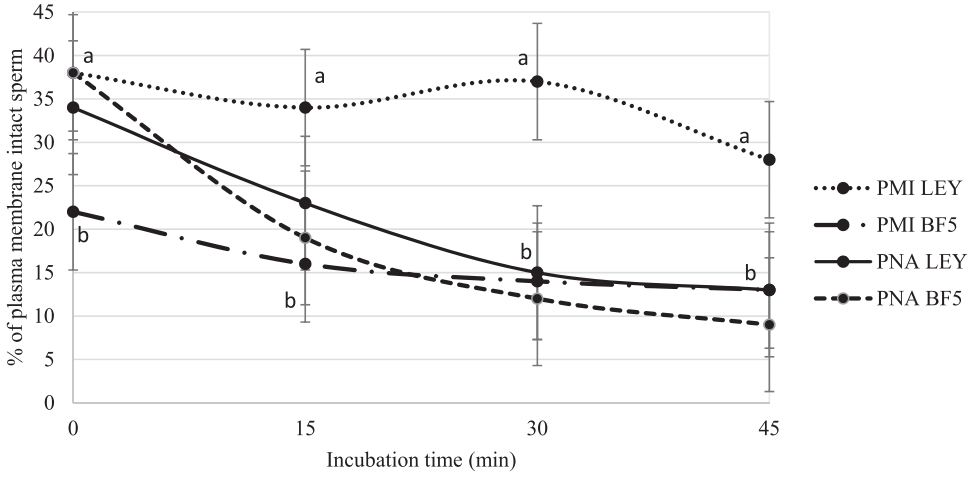

Fig. 1. Percentages (± SEM) of boar sperm with intact plasma membranes (PMI) and intact acrosomes (PNA) when sperm from nine boars were cryopreserved in Beltsville Freezing Extender 5 (BF5) or a lactose egg-yolk (LEY) medium, and then thawed, and incubated at 39 °C for up to 45 min; Data points with different superscripts (a,b) indicate sperm cryopreserved in BF5 and LEY are different within the PMI and PNA analyses ($P < 0.05$).

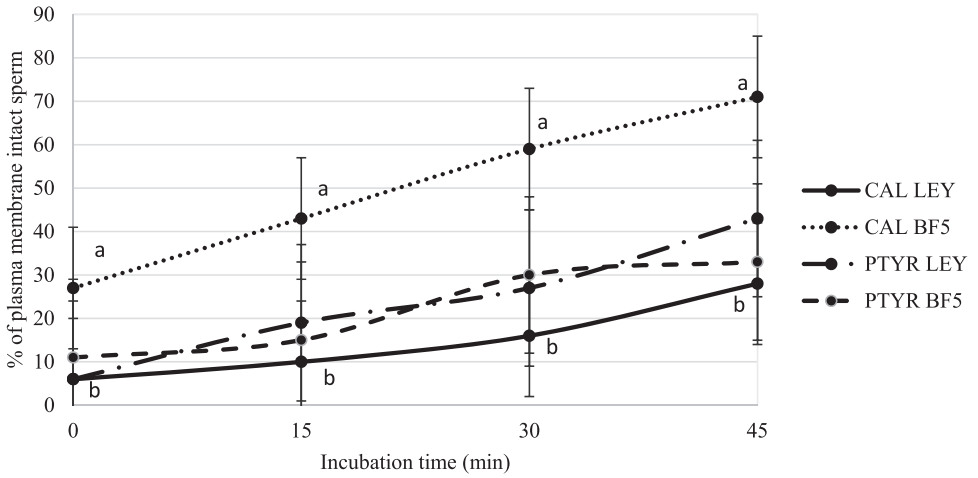

Fig. 2. Percentages (± SEM) of boar sperm with high intracellular calcium (CAL) and active phosphotyrosine phosphorylation (PTYR) in intact cells, after sperm from nine boars were cryopreserved in either Beltsville Freezing Extender 5 (BF5) or a lactose-egg yolk medium (LEY) and then thawed and incubated at 39 °C for as long as 45 min; Data points with different superscripts (a,b) indicate cryopreservation diluents resulted in different intracellular calcium concentrations during incubation ($P < 0.05$).

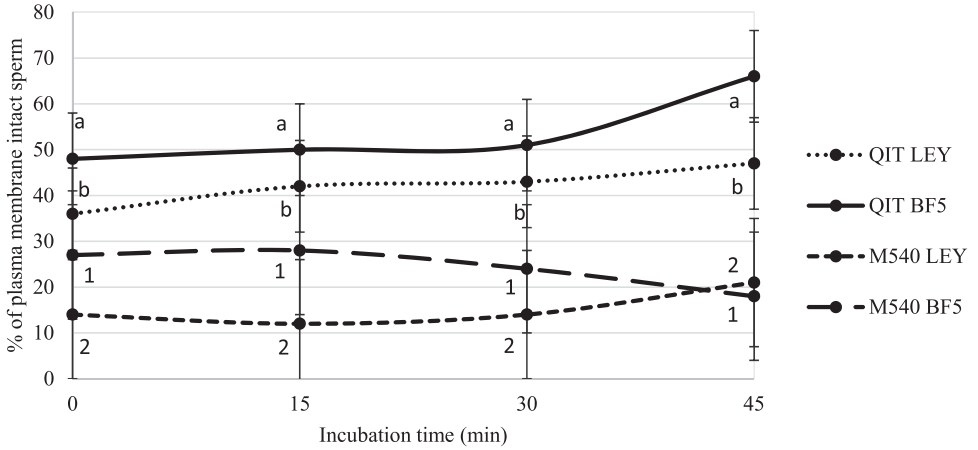

Fig. 3. Percentages (± SEM) of boar sperm with reorganized membrane protein (QIT) and phospholipids (M540) in intact cells, after sperm from nine boars were cryopreserved in either Beltsville Freezing Extender 5 (BF5) or a lactose-egg yolk medium (LEY) and then thawed and incubated at 39 °C for up to 45 min; Data points with different superscripts indicate cryopreservation diluents resulted in different percentages of sperm with reorganized protein (a,b) or with reorganized lipids (1,2) ($P < 0.05$).

3.2. Bull sperm analyses

The post thawed bull sperm total and progressive motility was 46% and 24%, respectively, (range 25–72 and 6–29, respectively). Bull sperm incubated in TALP-Ca for 3 h had greater percentages of cells with intact plasma membranes than sperm incubated in CFDM1 ($P < 0.05$ Fig. 4). In addition, there were greater percentages of sperm that were incubated in the TALP-Ca medium ($P < 0.05$) than in CFDM1 that had capacitated sperm characteristics, including a greater intracellular calcium, protein tyrosine phosphorylation (Fig. 5), plasma membrane phospholipid organization (Fig. 5), PDK1 second messenger signaling (Fig. 6), and acrosome reaction (Fig. 6). Similar to boar sperm, the main effect of incubation time was a source of variation ($P < 0.05$) for plasma membrane integrity (Fig. 4), intracellular calcium (Fig. 4), PDK1 second messenger signaling (Fig. 6), and acrosomal membrane integrity (Fig. 6). Incubation time, however, did not affect protein tyrosine phosphorylation (Fig. 5) or membrane phospholipid organization (Fig. 5, $P > 0.05$). The effect of bull was a source of variation for intracellular calcium and PDK1 second messenger signaling ($P < 0.05$), but not for plasma membrane integrity, protein tyrosine phosphorylation, plasma membrane phospholipid disorder, or acrosomal membrane integrity ($P > 0.05$).

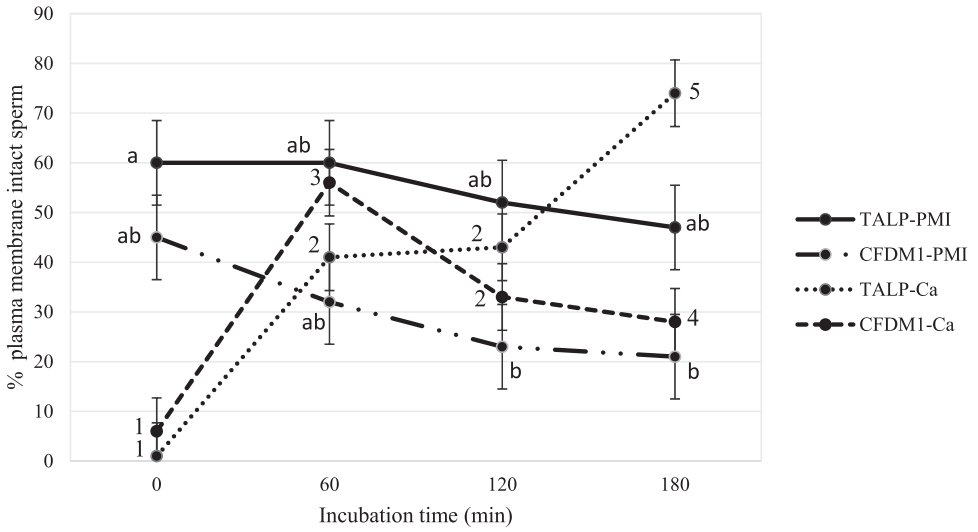

Fig. 4. Percentages (± SEM) of bull sperm with intact plasma membranes (PMI) and large intracellular calcium concentrations (Ca) when frozen-thawed sperm from three bulls were incubated in 10 mM Ca-TALP (TALP) or CFDM1 medium for up to 180 m; Data points with different superscripts indicate that the capacitation medium resulted in different percentages of sperm with intact plasma membranes (a,b) or with large concentrations of intracellular calcium (1,2) ($P < 0.05$).

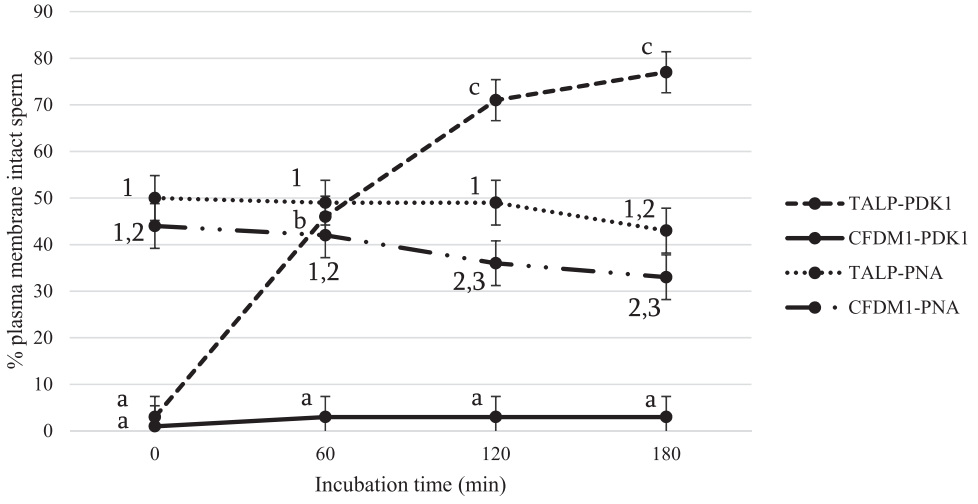

Fig. 5. Percentages (± SEM) of bull sperm with protein tyrosine phosphorylation (PTYR; dashed lines) and plasma membranes with disordered phospholipid bilayers (high fluidity, M540m, solid lines) when frozen-thawed sperm from three bulls were incubated in 10 mM Ca-TALP (TALP) or CFDM1 medium for up to 180 m; Data points with different superscripts indicate that the capacitation medium resulted in different percentages of sperm with PTYR activity (a,b) or with large amounts of plasma membrane phospholipid disorder (1,2) ($P < 0.05$).

4. Discussion

The research presented here was not designed to test an overall hypothesis, but to illustrate the application of flow cytometric techniques and perspectives that Dr. Garner pursued. Some of the assays and fluorophores utilized in this research, such as PI, FITC-PNA, and Fluo-3 AM have become standard, for assessing sperm quality, physiology, and capacitation status. Others, such as M540, which allow for quantification of lipid scrambling within a membrane, can be used to evaluate sperm capacitation status (Guthrie and Welch, 2005), are not often used due to challenges in working with this compound (i.e., sensitivity to photo-bleaching, utilization requires tremendous attention to detail for there to be repeatable results, and findings may be difficult to interpret). The other fluorescent stains used in these experiments to monitor intracellular phosphorylation cascades that are indicative of capacitation (PTYR, Pommer et al., 2003) and the acrosome reaction (PDK1, Jungnickel et al., 2007; Breitbart et al., 2010, Macías-García et al., 2019), have not been used previously to assess sperm that had not undergone fixation procedures. These antibodies, however, can be used with viable sperm, if the staining is performed when there are conditions that include sufficient calcium and bicarbonate to induce

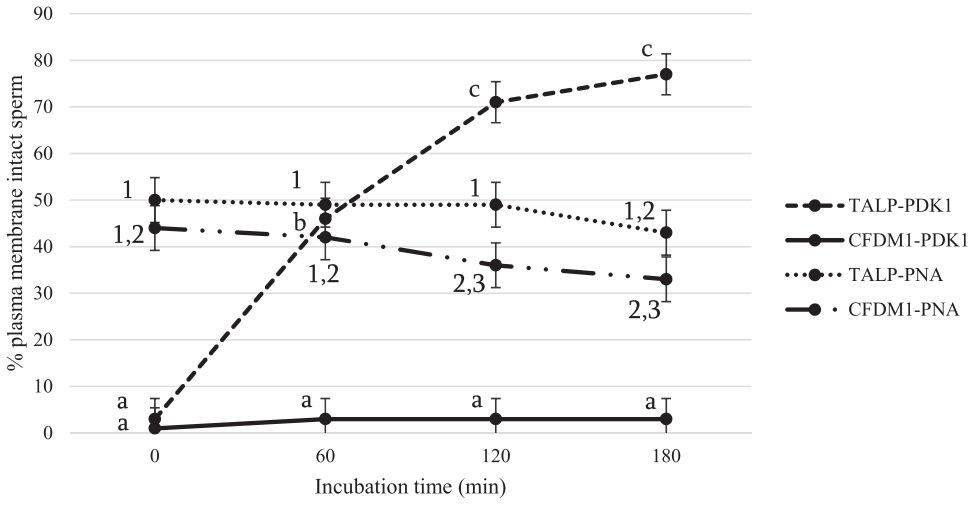

Fig. 6. Percentages (± SEM) of bull sperm demonstrating PDK1 second messenger activity (PDK1) and the percentage of acrosome reacted sperm (PNA) when frozen-thawed sperm from three bulls were incubated in 10 mM Ca-TALP (TALP) or CFDM1 medium for as long as 180 m; Data points with different superscripts indicate that the capacitation medium resulted in different percentages of sperm with PDK1 activity (a-c) or acrosome reacted cells (1–3) ($P < 0.05$).

increased plasma membrane fluidity and rearranged protein and phospholipid organization leading to capacitation and the acrosome reaction (Flesch et al., 1999). When there are conditions that permit sperm plasma membrane proteins and phospholipids to reorient in the plasma membrane, plasma membrane fluidity increases, and this in turn enables the second messengers (PTYR and PDK1) to be labeled with fluorescently conjugated antibodies and analyzed using flow cytometry procedures in real-time (Flesch et al., 1999; Harayama and Nakamura, 2008). Utilizing a variety of probes, in these experiments, there was assessment of multiple sperm characteristics that change during capacitation and the acrosome reaction, on individual samples. Similarly, Dr Garner and others (Graham, 2001) advocated for using a variety of probes to the greatest extent possible to gain a further understanding of sperm physiology.

In the first experiment, there was use of flow cytometric analysis to determine how different cryopreservation media (LEY and BF5) affected boar sperm capacitation and the acrosome reaction after thawing. The results indicate that boar sperm frozen in LEY survived the cryopreservation process to a greater extent than sperm frozen in BF5, because sperm frozen in LEY had greater percentages of intact plasma and acrosomal membranes (Fig. 1) and in many reports there would not be additional data evaluated. Assessing beyond membrane integrity, however, these data indicate sperm frozen in LEY do not undergo capacitation as rapidly as sperm frozen in BF5, as indicated by differences in intracellular calcium activity, second messenger signaling (PTYR), and plasma membrane protein (QIT) and phospholipid (M540) reorganization (Figs. 2 and 3), and although both media are used to cryopreserve boar sperm, these types of data may provide important insights to those involved in swine reproduction for making choices to better suit their needs. For example, based on these findings, if cryopreserved boar semen is to be used for artificial insemination, there should be consideration of freezing the sperm in LEY, because the numbers of sperm are greater that are viable after the freezing and thawing processes and these sperm undergo capacitation relatively slowly which is an important consideration for artificial insemination. If the sperm, however, are to be used for IVF, one might choose to freeze the sperm in BF5 because these sperm undergo capacitation more readily and fewer sperm are needed for IVF than for artificial insemination.

The second experiment was conducted to evaluate the capacity of bull sperm to capacitate in a modified TALP medium and CFDM1. These media were selected because both have been found to induce capacitation and the acrosome reaction in bull sperm (Graham et al., 1986; De La Torre-Sanchez et al., 2006), but have very different compositions. With use of both media, there was a relatively greater increase in intracellular calcium concentrations (Fig. 4), plasma membrane fluidity (Fig. 5), protein tyrosine phosphorylation (Fig. 5), activation of PKC second messenger signaling (PDK1; Fig. 6) and acrosome reactions (Fig. 6). These effects, however, were greater for sperm incubated in TALP-Ca compared with CFDM1. There are interesting differences between the sperm incubated in TALP-Ca and CFDM1 for intracellular calcium concentrations and PDK1. At 60 min of incubation, sperm intracellular calcium concentrations were increased in cells incubated in both media, however, sperm incubated in CFDM1 had a greater capacity for restoration of the lesser calcium concentrations as the duration of the incubation period increased, whereas sperm incubated in TALP-Ca did not have this capacity for restoration of intracellular calcium concentrations (Fig. 4), probably due to the greater concentrations of calcium in the TALP medium.

Perhaps more interesting, > 75% of the sperm incubated in TALP-Ca had an induction of PDK1 activity during the incubation period, while very few sperm incubated in CFDM1 had this induction in activity. These data provide important foundational information for planning additional experiments to understand why there are these effects on PDK1 activity, and what effect this may have on the capacity of sperm to fertilize oocytes. Investigations into understanding sperm second messenger systems would not be as feasible, however, if there was only analysis of these sperm with probes that allowed for assessment of membrane integrity.

The body of work from Dr. Garner and his colleagues has left a foundation of research which andrologists have utilized to advance

the understanding of sperm functions. Although the focus of this manuscript is on Dr. Garner's development and application of flow cytometry stains and techniques, his research also had an impact in facilitating the basic understanding of the biological and physiological effects of seminal plasma (Garner et al., 2001), and the relationship of flow cytometric sperm analyses with classical sperm morphology, motility, and non-return (fertility) rates (Ericsson et al., 1993). Those experiments were foundational to research focused on understanding the biological characteristics of sperm that are relevant to fertility using flow cytometric analysis, which has progressed to include imaging flow cytometry, a technology that combines simultaneous flow cytometric analysis with fluorescent microscopy. Applications of that technology enable simultaneous evaluation of sperm physiology (flow cytometry) and morphology (imaging of the cells analyzed flow cytometrically) and has allowed for identification of sperm characteristics that correlate with boar fertility (Kerns et al., 2020). The application of that type of flow cytometry is clearly in its infancy. Dr. Garner and his colleagues clearly envisioned the potential for flow cytometry in evaluations of as many sperm variables as possible to assess sperm throughout the periods when assisted reproductive technology processes are utilized for animal breeding. It is not difficult to envision that utilization of imaging flow cytometry will lead to an increased understanding of sperm membrane reorganization, phenotypes, and provide a foundation of understanding for combining these assays with proteomic and genomic analyses. This will lead to a more complete understanding of sperm biology and fertilization capacity of sperm with flow cytometric analysis being an integral procedure allowing these accomplishments.

5. Conclusion

Using many flow cytometric assays allows a more robust understanding of all the capacitation processes to be understood, including information about aspects of second messenger signaling, which may be a more subtle indicator of sperm quality and function. The ability to monitor many aspects of sperm physiology, including intracellular signaling, using flow cytometry may lead to improvements of methods to treat sperm for industry applications and understanding sperm physiology.

CRediT authorship contribution statement

Phillip H. Purdy: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **James K. Graham:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Hymerson C. Azevedo:** Conceptualization, Methodology, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that we have no competing interests.

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References

Almlid, T., Johnson, L.A., 1988. Effects of glycerol concentration, equilibration time and temperature of glycerol addition on post-thaw viability of boar spermatozoa frozen in straws. J. Anim. Sci. 66, 2899–2905. https://doi.org/10.2527/jas1988.66112899x.

Breitbart, H., Rotman, T., Rubinstein, S., Etkovitz, N., 2010. Role and regulation of PI3K in sperm capacitation and the acrosome reaction. Mol. Cell. Endocrinol. 314, 234–238. https://doi.org/10.1016/j.mce.2009.06.009.

De La Torre-Sanchez, J.F., Preis, K., Seidel, G.E., 2006. Metabolic regulation of in-vitro-produced bovine embryos. I. Effects of metabolic regulators at different glucose concentrations with embryos produced by semen from different bulls. Reprod. Fert. Dev. 18, 585–596. https://doi.org/10.1071/RD05063.

Downing, T.W., Garner, D.L., Ericsson, S.A., Redelman, D., 1991. Metabolic toxicity of fluorescent stains on thawed cryopreserved bovine sperm cells. J. Histochem. Cytochem. 39, 485–489. https://doi.org/10.1177/39.4.1900872.

Ericsson, S.A., Garner, D.L., Redelman, D., Ahmad, K., 1989. Assessment of the viability and fertilizing potential of cryopreserved bovine spermatozoa using dual fluorescent staining and two-flow cytometric systems. Gamete Res. 22, 355–368. https://doi.org/10.1002/mrd.1120220402.

Ericsson, S.A., Garner, D.L., Thomas, C.A., Downing, T.W., Marshall, C.E., 1993. Interrelationships among fluorometric analyses of spermatozoal function, classical semen quality parameters and the fertility of frozen-thawed bovine spermatozoa. Theriogenology 39, 1009–1024. https://doi.org/10.1016/0093-691x(93)90002-m.

Flesch, F.M., Colenbrander, B., van Golde, L.M.G., Gadella, B.M., 1999. Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. Biochem. Biophys. Res. Comm. 262, 787–792. https://doi.org/10.1006/bbrc.1999.1300.

Garner, D., Thomas, C., Gravance, C., 1999. The effect of glycerol on the viability, mitochondrial function and acrosomal integrity of bovine spermatozoa. Reprod. Domest. Anim. 34, 399–404. https://doi.org/10.1111/j.1439-0531.1999.tb01392.x.

Garner, D.L., 2009. Hoechst 33342: the dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology 71, 11–21. https://doi.org/10.1016/j.theriogenology.2008.09.023.

Garner, D.L., Johnson, L.A., 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol. Reprod. 53, 276–284. https://doi.org/10.1095/biolreprod53.2.276.

Garner, D.L., Thomas, C.A., 1999. Organelle-specific probe JC-1 identifies membrane potential differences in the mitochondrial function of bovine sperm. Mol. Reprod. Dev. 53, 222–229. https://doi.org/10.1002/(sici)1098-2795(199906)53:2%3C222::aid-mrd11%3E3.0.co;2-l.

Garner, D.L., Gledhill, B.L., Pinkel, D., Lake, S., Stephenson, D., Van Dilla, M.A., Johnson, L.A., 1983. Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry. Biol. Reprod. 28, 312–321. https://doi.org/10.1095/biolreprod28.2.312.

Garner, D.L., Johnson, L.A., Lake, S., Chaney, N., Stephenson, D., Pinkel, D., Gledhill, B.L., 1984. Morphological and ultrastructural characterization of mammalian spermatozoa processed for flow cytometric DNA analyses. Gamete Res. 10, 339–351. https://doi.org/10.1002/mrd.1120100402.

Garner, D.L., Johnson, L.A., Allen, C.H., 1988. Fluorometric evaluation of cryopreserved bovine spermatozoa extended in egg yolk and milk. Theriogenology 30, 369–378. https://doi.org/10.1016/0093-691x(88)90184-7.

Garner, D.L., Dobrinsky, J.R., Welch, G.R., Johnson, L.A., 1996a. Porcine sperm viability, oocyte fertilization and embryo development after staining spermatozoa with SYBR-14. Theriogenology 15, 1103–1113. https://doi.org/10.1016/0093-691x(96)00067-2.

Garner, D.L., Johnson, L.A., Allen, C.H., Palencia, D.D., Chambers, C.S., 1996b. Comparison of seminal quality in Holstein bulls as yearlings and as mature sires. Theriogenology 45, 923–934. https://doi.org/10.1016/0093-691x(96)00022-2.

Garner, D.L., Thomas, C.A., Allen, C.H., Senger, P.L., Sasser, R.G., 1997a. Effect of cryopreservation on bovine sperm viability as determined by dual DNA staining. Reprod. Domest. Anim. 32, 279–283. https://doi.org/10.1111/j.1439-0531.1997.tb01296.x.

Garner, D.L., Thomas, C.A., Joerg, H.W., DeJarnette, J.M., Marshall, C.E., 1997b. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 57, 1401–1406. https://doi.org/10.1095/biolreprod57.6.1401.

Garner, D.L., Thomas, C.A., Gravance, C.G., Marshall, C.E., DeJarnette, J.M., Allen, C.H., 2001. Seminal plasma addition attenuates the dilution effect in bovine sperm. Theriogenology 56, 31–40. https://doi.org/10.1016/s0093-691x(01)00540-4.

Graham, J.K., 2001. Assessment of sperm quality: a flow cytometric approach. Anim. Reprod. Sci. 68, 239–247. https://doi.org/10.1016/s0378-4320(01)00160-9.

Graham, J.K., Foote, R.H., Parrish, J.J., 1986. Effect of dilauroylphosphatidylcholine on the acrosome reaction and subsequent penetration of bull spermatozoa into zona-free hamster eggs. Biol. Reprod. 35, 413–424. https://doi.org/10.1095/biolreprod35.2.413.

Guthrie, H.D., Welch, G.R., 2005. Effects of hypothermic liquid storage and cryopreservation on basal and induced plasma membrane phospholipid disorder and acrosome exocytosis in boar spermatozoa. Reprod. Fertil. Dev. 17, 467–477. https://doi.org/10.1071/rd05003.

Harayama, H., Nakamura, K., 2008. Changes of PKA and PDK1 in the principal piece of boar spermatozoa treated with a cell-permeable cAMP analog to induce flagellar hyperactivation. Mol. Reprod. Dev. 75, 1396–1407. https://doi.org/10.1002/mrd.20882.

Harrison, R.A., Ashworth, P.J., Miller, N.G., 1996. Bicarbonate/CO_2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. Mol. Reprod. Dev. 45, 378–391. https://doi.org/10.1002/(SICI)1098-2795(199611)45:3%3C378::AID-MRD16%3E3.0.CO;2-V.

Jungnickel, M.K., Sutton, K.A., Wang, Y., Florman, H.M., 2007. Phosphoinositide-dependent pathways in mouse sperm are regulated by egg ZP3 and drive the acrosome reaction. Dev. Biol. 304, 116–126 https://dx.doi.org/10.1016%2Fj.ydbio.2006.12.023.

Kerns, K., Jankovitz, J., Robinson, J., Minton, A., Kuster, C., Sutovsky, P., 2020. Relationship between the length of sperm tail mitochondrial sheath and fertility traits in boars used for artificial insemination. Antioxidants 9, 1033. https://doi.org/10.3390/antiox9111033.

Kumaresan, A., Siqueira, A.P., Hossain, M.S., Johannisson, A., Eriksson, I., Wallgren, M., Bergqvist, A.S., 2011. Quantification of kinetic changes in protein tyrosine phosphorylation and cytosolic Ca2+ concentration in boar spermatozoa during cryopreservation. Reprod. Fertil. Dev. 24, 531–542. https://doi.org/10.1071/RD11074.

Macías-García, B., García-Marín, L.J., Bragado, M.J., González-Fernández, L., 2019. The calcium-sensing receptor regulates protein tyrosine phosphorylation through PDK1 in boar spermatozoa. Mol. Reprod. Dev. 86, 751–761. https://doi.org/10.1002/mrd.23160.

Pinkel, D., Garner, D.L., Gledhill, B.L., Lake, S., Stephenson, D., Johnson, L.A., 1985. Flow cytometric determination of the proportions of X- and Y-chromosome-bearing sperm in samples of purportedly separated bull sperm. J. Anim. Sci. 60, 1303–1307. https://doi.org/10.2527/jas1985.6051303x.

Pommer, A.C., Rutllant, J., Meyers, S.A., 2003. Phosphorylation of protein tyrosine residues in fresh and cryopreserved stallion spermatozoa under capacitating conditions. Biol. Reprod. 68, 1208–1214. https://doi.org/10.1095/biolreprod.102.011106.

Purdy, P.H., 2008. Ubiquitination and its influence in boar sperm physiology and cryopreservation. Theriogenology 70, 818–826. https://doi.org/10.1016/j.theriogenology.2008.05.044.

Purdy, P.H., Graham, J.K., 2004. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology 48, 36–45. https://doi.org/10.1016/j.cryobiol.2003.12.001.

Purdy, P.H., Barbosa, E.A., Praamsma, C.J., Schisler, G.J., 2016. Modification of trout sperm membranes associated with activation and cryopreservation. Implications for fertilizing potential. Cryobiology 73, 73–79. https://doi.org/10.1016/j.cryobiol.2016.05.008.

Pursel, V.G., Johnson, L.A., 1975. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. J. Anim. Sci. 40, 99–102. https://doi.org/10.2527/jas1975.40199x.

Statistical Analysis Systems. SAS User's Guide, 1985. Statistical Analysis Systems Institute, Inc. Cary, North Carolina.

Steele, H., Makri, D., Maalouf, W.E., Reese, S., Kölle, S., 2020. Bovine sperm sexing alters sperm morphokinetics and subsequent early embryonic development. Sci. Rep. 10, 6255. https://doi.org/10.1038/s41598-020-63077-6.

Thomas, C.A., Garner, D.L., DeJarnette, J.M., Marshall, C.E., 1997. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. Biol. Reprod. 56, 991–998. https://doi.org/10.1095/biolreprod56.4.991.

Thomas, C.A., Garner, D.L., DeJarnette, J.M., Marshall, C.E., 1998. Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry. Biol. Reprod. 58, 786–793. https://doi.org/10.1095/biolreprod58.3.786.