

The effect of non-traditional cooling on dog sperm cryosurvival and ability to perform the acrosome reaction

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ABSTRACT. The objective of this study was to assess cryosurvival, plasma membrane fluidity, and capability of cryopreserved dog (*Canis lupus familiaris*) spermatozoa, cooled to -5°C before freezing, to perform the acrosome reaction under the effect of progesterone and calcium ionophore. In the first experiment, fresh spermatozoa diluted in Tyrode's medium plus albumin, lactate, and pyruvate (TALP) were incubated at 38°C in 5% CO_2 in air, with progesterone or calcium ionophore added at 2, 4, and 6 h after incubation and sampled 30 min later to assess the acrosome reaction. In the second experiment, diluted sperm were packaged in plastic straws, cooled to either $+5^{\circ}\text{C}$ or -5°C and cryopreserved. Progressive motility, plasma membrane integrity and fluidity, capacitation status and acrosome integrity were assessed before and after freeze-thawing. After thawing, sperm were assessed, resuspended in TALP and incubated to assess the acrosome reaction. Parameters for sperm cryosurvival were similar in sperm cooled to either $+5^{\circ}\text{C}$ or -5°C , except in the percentage of hyper-fluid membranes which was lower ($P<0.05$) in sperm cooled to -5°C . There were no differences in the percentages of frozen-thawed spermatozoa with acrosome reaction, induced by progesterone or calcium ionophore, between cooling treatments. In conclusion, cooling of dog spermatozoa to -5°C did not improve sperm cryosurvival but had a positive effect on plasma membrane fluidity.

Key words: dog semen, freeze-thawing, progesterone, calcium ionophore, membrane fluidity.

INTRODUCTION

Sperm cryopreservation results in different advantages in dog breeding, such as the movement of genetic material among different geographic regions, thus increasing genetic variability and the use of the best males (Eilts 2005). However, cryopreservation invariably reduces the fertilising capacity of spermatozoa (Watson 1995), therefore, the research aiming to elucidate the mechanisms responsible for damage to sperm physiology caused by low temperatures is of great importance (Eilts 2005). A phenomenon that sperm have to face during cryopreservation is the phase transition of lipids that comprise the plasma membrane. As the temperature decreases, the lipids progressively change from a liquid-crystalline phase to a gel phase. Consequently the membrane loses elasticity (Watson 1995). Most lipids undergo this change at temperatures above zero degrees, however, there is evidence that some additional phase transitions could occur below zero degrees (Crowe *et al* 1989). The lipid composition of the plasma membrane (cholesterol and unsaturated fatty acids) is directly related to the motility of the sperm and the fluidity of the membrane; the presence of unsaturated fatty acids gives the membrane greater fluidity. Lucio *et al* (2017) have reported the presence of both saturated (palmitic and stearic) and unsaturated fatty acids (arachidonic and oleic) in dog spermatozoa.

In addition, freezing affects the physical states of membrane lipids due to changes in the hydration level. Membrane phase transition may cause different effects on the membrane conformational disorder in the frozen state when the ice nucleation occurs at different sub-zero temperatures (Balasubramanian *et al* 2009).

Thus, spermatozoa are subjected to volume changes (i.e., intracellular water flow for ice formation), while their plasma membrane is suffering conformational rearrangements—changes in fluidity—due to lipid phase transitions. One approach that could assist the sperm plasma membrane to manage those stressors in order to maintain its viability is to extend the cooling of spermatozoa to sub-zero temperatures (-5°C) before freezing. In this way, the occurrence of severe changes in plasma membrane fluidity could be reduced. Some researchers have been exploring the effect of cooling to sub-zero temperatures on sperm cryosurvival (Garzon-Perez *et al* 2010, Contreras-Mendez and Medrano 2016, Alcantar-Rodriguez and Medrano 2017). However, they have obtained either positive or null effects on sperm cryosurvival.

Assessment of the sperm fertilising capacity by measuring the ability of the cell to suffer the acrosome reaction is considered a valuable test in fresh and cryopreserved spermatozoa (Graham and Foote 1987, Whitfield and Parkinson 1992). The objective of this work was to test further the effect of cooling to -5°C before freezing on dog sperm cryosurvival, plasma membrane fluidity and the sperm capability to carry out the acrosome reaction, comparatively employing 2 inductors: progesterone (P_4) and calcium ionophore (CI). In this work, 2 cooling protocols were compared by inducing the acrosome reaction on cryopreserved dog spermatozoa as a measure of the *in vitro* fertilising capacity of spermatozoa.

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MATERIAL AND METHODS

All experiments comply with the guidelines of the Institutional Subcommittee for the Care of Animals in Experimentation from the National Autonomous University of Mexico (Faculty of Superior Studies, Cuautitlan). Semen assessment and cryopreservation were carried out in the laboratory of Animal Reproduction (UIM-FESC), National Autonomous University of Mexico.

SEMEN COLLECTION AND PROCESSING

Semen was collected by manual stimulation from 3 dogs (first experiment, 6 ejaculates per male: 1 Shih-tzu and 2 mixed races, 3 to 5 years of age) and 5 dogs (second experiment, 3 ejaculates per male: 3 Belgian and 2 German Shepherd, 2 to 8 years of age) once a week from the same male, from January to August 2016 (20°N). Only 1 ejaculate from the corresponding dog was collected per each day of work, and each individual ejaculate was treated separately. Only ejaculates showing at least 80% progressive motility and 85% viability were included in these experiments.

Each ejaculate was diluted 1:1 (v/v) in a Tris/citric acid/glucose solution (TRIS 198.2 mM, citric acid 72.9 mM, glucose 8.9 mM, and kanamycin 0.83 mg/ml) to be transported at approximately 28 °C. Semen arrived at the laboratory within 30 min of collection.

SEMEN ASSESSMENT

Immediately after collection, semen was macroscopically assessed for volume, colour, consistency and presence of strange materials (hair, blood). Once in the laboratory, semen in the transport solution was left at room temperature (18 – 23 °C) for 45 min to adjust, then an aliquot was taken and diluted 1:6 (v/v) in phosphate-buffered saline PBS at 34 °C. Ten min later, sperm were microscopically assessed for progressive motility, sperm concentration, viability, sperm morphology, plasma membrane integrity, acrosome integrity, capacitation status and plasma membrane fluidity.

Progressive motility, the percentage of sperm showing progressive and linear movement was subjectively assessed under light microscopy (Leica DMLS) using the 20x objective.

Sperm concentration was estimated from a 1:200 dilution (semen: formaldehyde saline solution v/v) with the aid of the Neubauer chamber using the 40x objective.

Viability was assessed by counting live (non-stained) and dead (stained) spermatozoa (at least 200 hundred) in smears stained with eosin-nigrosine using the 100x objective. Sperm morphology was assessed by counting normal and abnormal (primary and secondary) cells (at least 200 hundred) in smears stained with eosin-nigrosine using the 100x objective (Feldman and Nelson 1996).

Plasma membrane integrity was assessed as follows: 50 µl of diluted spermatozoa (in PBS 1+5 v/v) were added to 5 µl of SYBR14 (100 nmol/L) and mixed. Immediately, 5 µl of propidium iodide (PI, 12 µmol/L) was added and mixed again for 10 s. Finally, 5 µl of glutaraldehyde (0.4%) was added to immobilise the spermatozoa (Garner and Johnson 1995). The percentage of live cells (SYBR14-positive and PI-negative) was calculated after counting 200 spermatozoa under fluorescence microscopy (Leica DMLS) using the 100x objective.

Acrosome integrity was assessed as follows: diluted sperm were smeared on a slide and air-dried, and then cells were permeabilised in alcohol for 60 min. Then, 50 µl of fluorescein-conjugated *Pisum sativum* agglutinin (PSA-FITC) lectin (L0770 Sigma St. Louis MO, USA) was spread on the slide and left in the dark for 10 min. Immediately, the slide was gently washed with distilled water and air-dried (Medrano *et al* 2009). One drop of an antifade solution (DABCO 220 mM in glycerol, D-2522 Sigma St. Louis MO USA) was put on the slide, and a coverslip was positioned on top. The percentage of cells showing a smooth and well-defined acrosome was calculated after counting 200 spermatozoa under fluorescence microscopy using the 100x objective.

Capacitation status was assessed by the chlortetracycline (CTC) assay as follows: 100 µl of diluted sperm (in PBS 1+5 v/v) were added to 100 µl of CTC solution (pH 7.8), mixed for 30 s and 20 µl of glutaraldehyde (0.2%) were added to immobilise the sperm (Green and Watson, 2001). CTC-stained sperm were mixed (1:1 v/v) with antifade solution on a slide, and a coverslip was positioned on top. Percentages of cells showing any of the CTC patterns were calculated after counting 200 spermatozoa under fluorescence microscopy using the 100x objective: F, with uniform fluorescence over the whole head (non-capacitated acrosome-intact spermatozoa); B, with fluorescence-free band in the post-acrosomal region (capacitated acrosome-intact spermatozoa); or AR, with almost no fluorescence over the whole head except for a band of fluorescence in the equatorial segment (acrosome-reacted spermatozoa) (Rota *et al* 1999).

To assess sperm plasma membrane fluidity, a Merocyanine 540 assay was carried out as follows: a stock solution of Merocyanine (5 mM) in dimethyl sulphoxide (DMSO, 154938 Sigma St. Louis MO, USA) was prepared and stored at room temperature (23 °C) protected from the light until use. Then, a working solution of Merocyanine (40 µM) in PBS (495 µL PBS + 5 µL Merocyanine (5 mM) in DMSO) was freshly prepared before use. One hundred and forty microlitres of sperm in PBS were added to 10 µL of the Merocyanine working solution, mixed, and left for 1 min to interact. Then, 22 µL of glutaraldehyde (0.4%) were added to fix the sperm. One drop of this mix and 1 drop of antifade solution (DABCO 220 mM in glycerol/PBS) were put on a warm glass slide, and a glass cover slide was positioned on top. Gentle pressure

was applied to the cover slide, with the aid of absorbent paper to eliminate the excess liquid. Percentages of cells showing either of the Merocyanine patterns, opaque (low fluidity) or brilliant (high fluidity - high-binding cells), were calculated after counting 200 spermatozoa under fluorescence microscopy (Leica DMLS) using the 100x objective (Steckler *et al* 2015).

EXPERIMENTAL DESIGN

Experiment 1. Validation of the use of 2 inductors of the acrosome reaction on fresh-incubated dog spermatozoa. This experimental stage was carried out to identify the optimum incubation time for each of the inductors of the acrosome reaction to produce the highest proportion of acrosome-reacted spermatozoa. Fresh spermatozoa, diluted in Tyrode's medium plus albumin, lactate and pyruvate (TALP), were incubated during 6.5 h, employing 2 inductors of the acrosome reaction: CI and progesterone (P4). Semen was collected, diluted and transported as mentioned. Immediately after adjustment at room temperature, diluted semen was centrifuged at 750 g for 5 min, supernatant was removed and TALP medium was added to reach 400×10^6 sperm/ml. Sperm in TALP were further diluted to reach either 100×10^6 sperm/ml (for CI experiment) or 75×10^6 sperm/ml (for P4 experiment). Immediately, sperm suspension was split in aliquots of 200 μ l each, which were incubated at 38 °C in 5% CO₂ in air, and sampled when required. Either CI (2.5 μ M, Sigma-Aldrich, USA) in Tyrode's medium (Szász *et al* 2000) or P4 (10 μ g/ml, Sigma-Aldrich, USA) in Tyrode's medium (Cheng *et al* 2005) were added at 2, 4 and 6 h of incubation and left for 30 min to interact before sampling. Progressive motility, plasma membrane integrity, capacitation status and acrosome integrity were assessed at 0, 2, 2.5, 4, 4.5, 6 and 6.5 h of incubation in the control and treated groups (figure 1).

Eighteen ejaculates from 3 dogs (6 from each male; 3 per each AR inductor) were used in this stage.

Experiment 2. Sperm cryopreservation and post-thawing incubation. This experimental stage was carried out to assess both sperm cryosurvival after cooling to 2 pre-freeze temperatures (+5 °C vs. -5 °C) and the capacity of frozen-thawed spermatozoa to perform the acrosome reaction employing calcium ionophore A23187 and progesterone (figure 2). Semen was collected, diluted and transported as previously mentioned. Immediately after adjustment at room temperature, diluted semen was centrifuged at 750 g for 5 min, supernatant was removed and egg yolk-Tris (EYT) medium containing 3% glycerol (Peña and Linde Forsberg 2000) was added to reach 400×10^6 sperm/ml. Fifteen ejaculates (6 straws: 3 for each cooling temperature) from 5 dogs were used in this stage.

Diluted sperm were slowly cooled from 23 °C to 5 °C in approximately 2 h (0.17 °C/min). Then, EYT medium

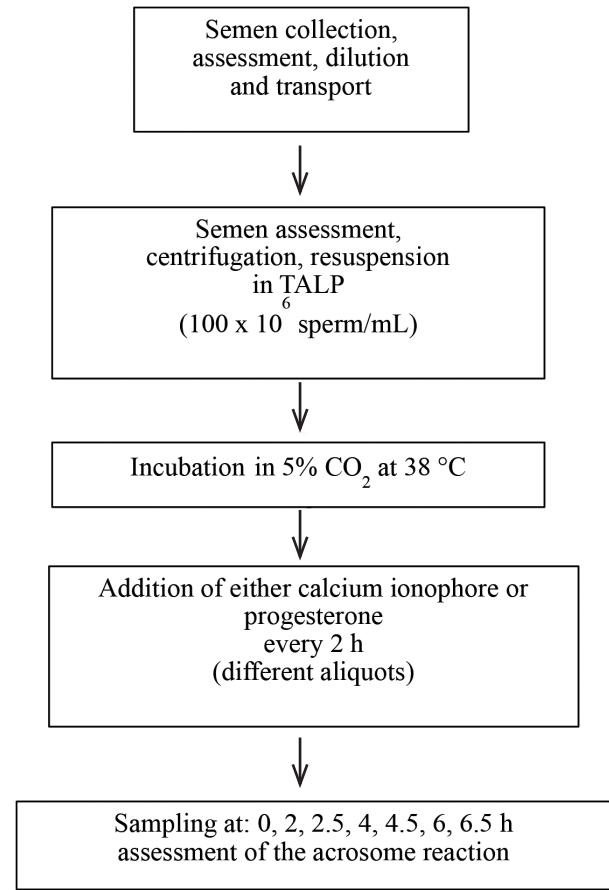


Figure 1. Flow chart of Experiment 1. Validation of the use of 2 inductors of the acrosome reaction on fresh-incubated dog spermatozoa.

containing 7% glycerol was added to reach a final concentration of 200×10^6 sperm/ml and 5% glycerol, and diluted sperm was packaged into 0.5 ml plastic straws, which remained in equilibrium for 30 min before freezing. Half of the straws at +5 °C (control) were frozen in 4 cm of nitrogen vapour over liquid nitrogen levels for 15 min and stored in liquid nitrogen. The other half at +5 °C were further cooled to -5 °C at approximately 0.04 °C/min (experimental), frozen and stored as mentioned. To cool the straws to sub-zero temperatures, an insulated box filled with crushed saline ice (10% w/v) at -12 °C was used. This method had been previously validated in our laboratory (Alcantar-Rodríguez and Medrano 2017). Temperature was carefully monitored with the aid of a thermocouple (HANNA Instruments, USA) positioned inside a monitor straw containing EYT diluent (5% glycerol). Readouts were stored in a computer using a special software (HANNA Instruments, USA).

Thawing was performed by immersing straws (3 per treatment) in water at 38 °C for 30 s. The content of each straw was poured in dry tubes into the water bath (4.5 l volume). Each straw was assessed separately. Variables

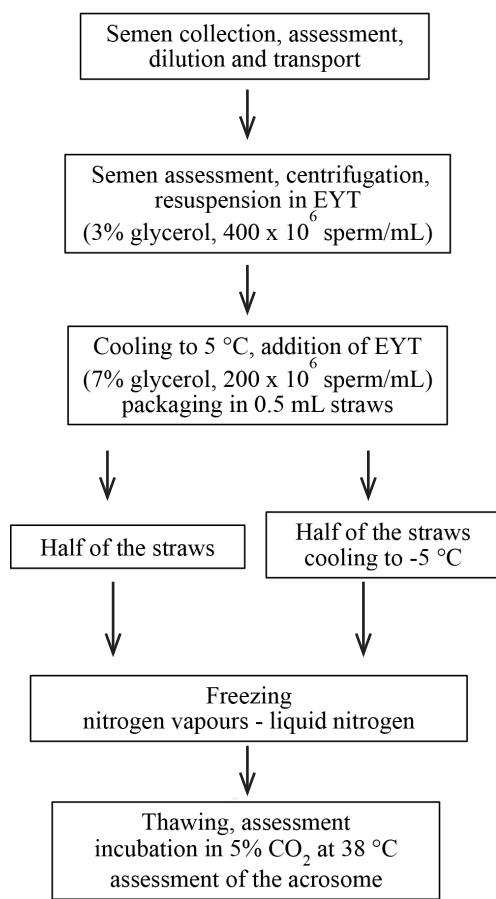


Figure 2. Flow chart of Experiment 2. Sperm cryopreservation and post-thawing incubation.

were progressive motility, viability, plasma membrane integrity, acrosome integrity, capacitation status, and plasma membrane fluidity.

Induction of the acrosome reaction on cryopreserved dog spermatozoa. Straws (3 per treatment) were thawed, and sperm was assessed as mentioned. Then, diluted sperm from the 3 straws were pooled and centrifuged at 300 g for 4 min, supernatant was removed and the pellet was resuspended in TALP to reach 75×10^6 sperm/ml. Sperm suspension was split into 5 aliquots of 200 μ l each that were incubated at 38 °C in 5% CO₂ in air, and sampled at (i) 0 h – control, (ii) 4 h – control and (iii) 4:30 h – control, CI and P₄. CI and P₄ were added after 4 h of incubation and left for 30 min to interact with the sperm. Then, sperm acrosome and plasma membrane integrity were assessed.

STATISTICAL ANALYSIS

Data from the first experiment (incubation times and the effect of AR inductors) was analysed using the Friedman test of analysis of variance (ANOVA, repeated measures), Wilcoxon matched pair test and the “t” test (Snedecor and

Cochran 1989). Data from the second experiment (sperm cryopreservation and post-thawing incubation) was analysed by (i) ANOVA (fresh semen variables), with variables expressed in percentages arcsine-transformed to normalise them, (ii) “t” test (frozen-thawed sperm, cooled before freezing at either +5 or –5 °C) and (iii) Friedman ANOVA (incubation times of frozen-thawed sperm). To compare treatments (Control, CI, and P₄) at 4:30 h of incubation, ANOVA and Tukey tests were used as well as the software SPSS v15.0 (2006, Chicago, USA). CITAR ATRAS

RESULTS

EXPERIMENT 1. VALIDATION OF THE USE OF 2 INDUCTORS OF THE ACROSOME REACTION ON FRESH-INCUBATED DOG SPERMATOZOA

Stage 1. Calcium ionophore A23187 (CI). Sperm motility decreased constantly as incubation progressed. At 2.5 and 6.5 h of incubation, there were no differences between the control and CI, however, at 4.5 h of incubation they were different ($P < 0.05$) (figure 3). The number of plasma membrane-intact spermatozoa decreased constantly as incubation progressed; there were no differences between the control and CI at any of the incubation times (figure 3). The number of acrosome-intact spermatozoa decreased as incubation progressed; there were no differences between 4 and 4.5 h as well as between 6 and 6.5 h. At 2.5, 4.5 and 6.5 h of incubation, there were differences ($P < 0.05$) between the control and CI 3 (figure 3).

The number of non-capacitated, acrosome-intact spermatozoa (CTC, F Pattern) decreased constantly as incubation progressed. At 2.5 and 4.5 h of incubation, there were differences ($P < 0.05$) between the control and CI, however, at 6.5 h of incubation, there was no difference (figure 4). Number of capacitated, acrosome-intact spermatozoa (CTC, B Pattern) increased constantly as incubation progressed, reaching top values at 4 and 4.5 h and decreasing at 6.5 h of incubation ($P < 0.05$). At 2.5 h of incubation, there was no difference between the control and CI, however, at 4.5 and 6.5 h of incubation, they were different ($P < 0.05$) (figure 4). The number of acrosome-reacted spermatozoa (CTC, AR Pattern) increased constantly as incubation progressed. At 2.5, 4.5 and 6.5 h of incubation, there were differences ($P < 0.05$) between the control and CI (figure 4).

Stage 2. Progesterone (P4). Sperm motility decreased constantly as incubation progressed. At 2.5 and 6.5 h of incubation, and there were no differences between the control and P₄, however, at 4.5 h of incubation they were different ($P < 0.05$) (figure 5). The number of plasma membrane-intact spermatozoa decreased constantly as incubation progressed; there were no differences between the control and P₄ at any of the incubation times (figure 5). The number of acrosome-intact spermatozoa decreased as

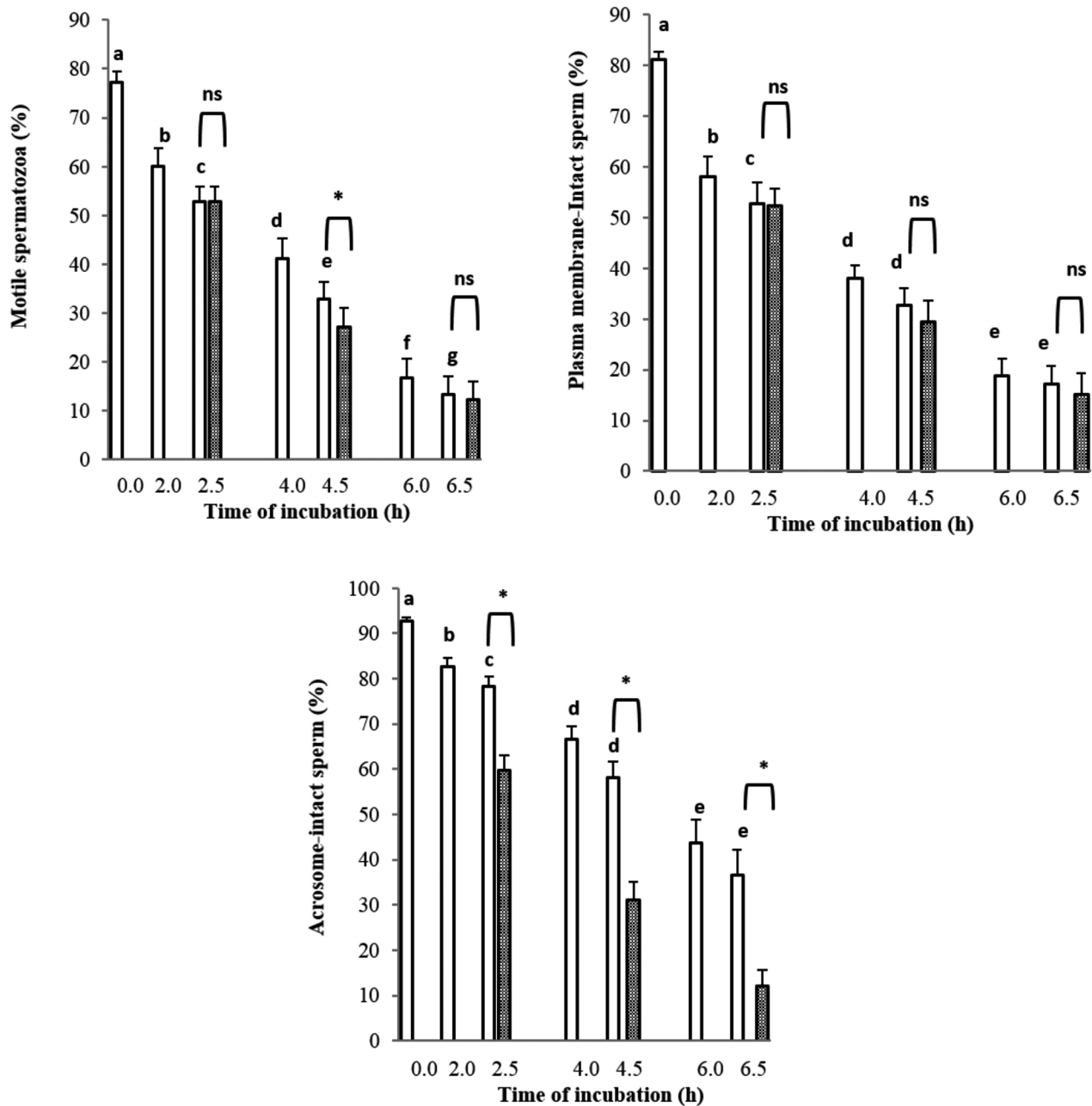


Figure 3. Motile, plasma membrane-intact, and acrosome-intact dog spermatozoa incubated at 37 °C during 6.5 h under the effect of calcium ionophore. Values are means + SEM.

Control: open bars (a-g), calcium ionophore: shadowed bars.

Control vs calcium ionophore: * $P < 0.05$, ns = non significant.

incubation progressed. At 2.5, 4.5 and 6.5 h of incubation, there were differences ($P < 0.05$) between the control and P_4 (figure 5).

Number of non-capacitated, acrosome-intact spermatozoa (CTC, F Pattern) decreased constantly as incubation progressed; there were significant differences ($P < 0.05$) between the control and P_4 at 2.5, 4.5 and 6.5 h of incubation (figure 6). The number of capacitated, acrosome-intact spermatozoa (CTC, B Pattern) increased constantly as

incubation progressed, reaching top values at 4 and 4.5 h and decreasing at 6.5 h of incubation ($P < 0.05$). At 2.5 h of incubation, there was no difference between the control and P_4 ; however, at 4.5 and 6.5 h of incubation, they were different ($P < 0.05$) (figure 6). The number of acrosome-reacted spermatozoa (CTC, AR Pattern) increased constantly as incubation progressed. At 2.5, 4.5 and 6.5 h of incubation, there were differences ($P < 0.05$) between the control and P_4 (figure 6).

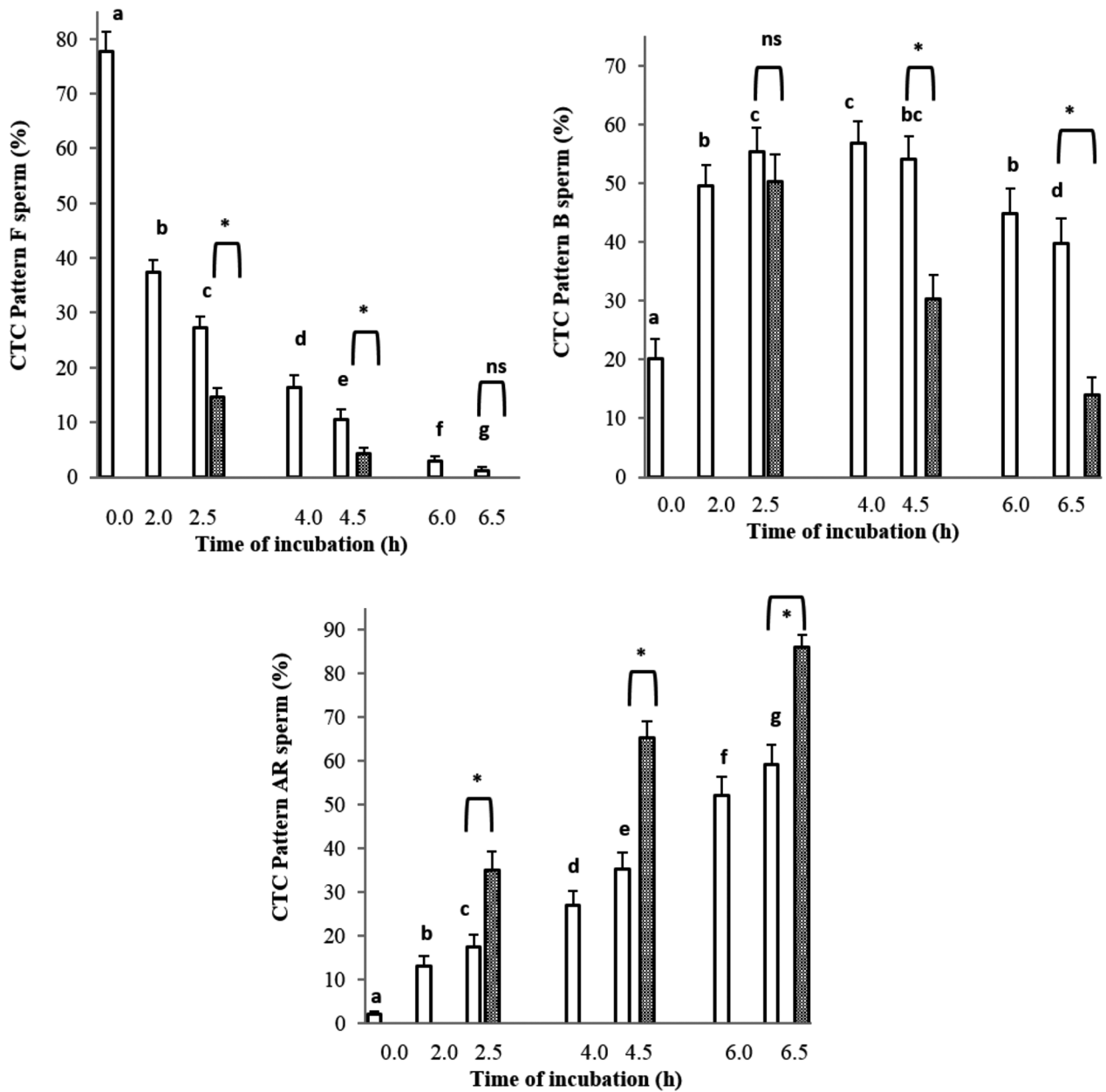


Figure 4. Capacitation status of dog spermatozoa incubated at 37 °C during 6.5 h under the effect of calcium ionophore. Values are means + SEM.

Control: open bars (a-g), calcium ionophore: shadowed bars.

Control vs calcium ionophore: * $P < 0.05$, ns = non significant.

EXPERIMENT 2. SPERM CRYOPRESERVATION AND POST-THAWING INCUBATION

Stage 1. Sperm cryosurvival. After thawing, there was significant difference ($P < 0.05$) in the percentage of Merocyanine high-binding (hyper-fluidity) cells between sperm cooled to either +5 °C (69.5 ± 3.30 Mean \pm SEM) or -5 °C (63.8 ± 3.00 Mean \pm SEM). There were no differences between cooling temperatures in the other sperm characteristics (table 1).

Stage 2. Induction of the acrosome reaction on cryopreserved dog spermatozoa. The number of acrosome-intact spermatozoa from +5 °C and -5 °C cooling treatments decreased as incubation progressed. At 4.5 h of incubation in each cooling treatment, there were differences between the control and the experimental groups (CI and P_4), but there was no difference between CI and P_4 . The number of plasma membrane-intact spermatozoa from +5 °C and -5 °C cooling treatments decreased significantly ($P < 0.05$) as incubation progressed. However, at 4.5 h of incubation,

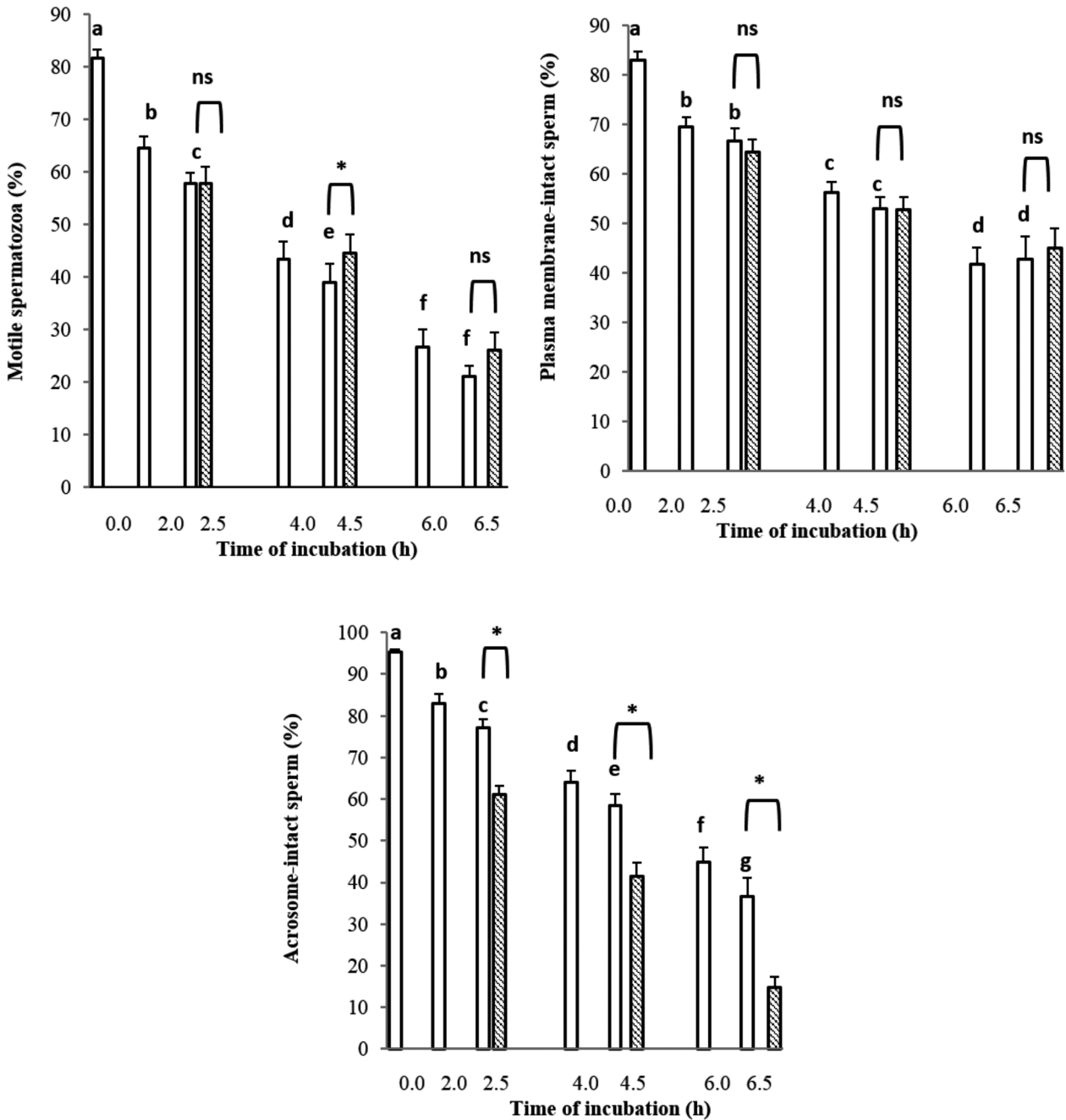


Figure 5. Motile, plasma membrane-intact, and acrosome-intact dog spermatozoa incubated at 38 °C in 5% CO₂ in air during 6.5 h under the effect of Progesterone. Values are means + SEM.

Control: open bars (a-g), Progesterone: shadowed bars.

Control vs Progesterone: * $P < 0.05$, ns = non significant.

there were no differences between the control, CI and P₄ of each cooling treatment. Figure 7 comparatively shows the percentages of acrosome-intact and plasma membrane-intact spermatozoa from +5 °C and -5 °C cooling treatments. There were no differences in any pair of values (+5 °C vs. -5 °C) incubated at the same time with an inductor of the acrosome reaction (CI & P₄) or without (control).

DISCUSSION

This work was conducted to compare the effect of 2 pre-freeze cooling temperatures, +5 °C vs. 5 °C, on the *in vitro* fertilising capacity of cryopreserved dog spermatozoa assessed by the sperm capacity to perform the acrosome reaction, CI and P₄. In the first experiment, the induction

Table 1. Effect of cooling to +5 °C and -5 °C before freezing on dog sperm cryosurvival.

Cooling target temperature	Progressive motility (%)	MC540 high-binding cells (%)	Acrosome integrity (%)	Capacitation status (CTC patters %)			Plasma membrane integrity (%)
				F	B	AR	
+5 °C	30.0 ± 3.09	69.5 ± 3.30 ^a	80.9 ± 0.99	11.4 ± 1.21	73.4 ± 1.29	15.2 ± 0.82	34.0 ± 2.68
-5 °C	28.7 ± 2.15	63.8 ± 3.00 ^b	82.3 ± 0.95	12.5 ± 0.90	73.4 ± 1.00	14.1 ± 0.91	36.3 ± 2.79

Values are Means ± SEM. Different letters in columns indicate significant differences (p<0.05). Progressive motility, visual; plasma membrane fluidity, merocyanine (MC540); acrosome integrity, PSA-FITC; capacitation status, CTC assay; plasma membrane integrity, SYBR14/PI.

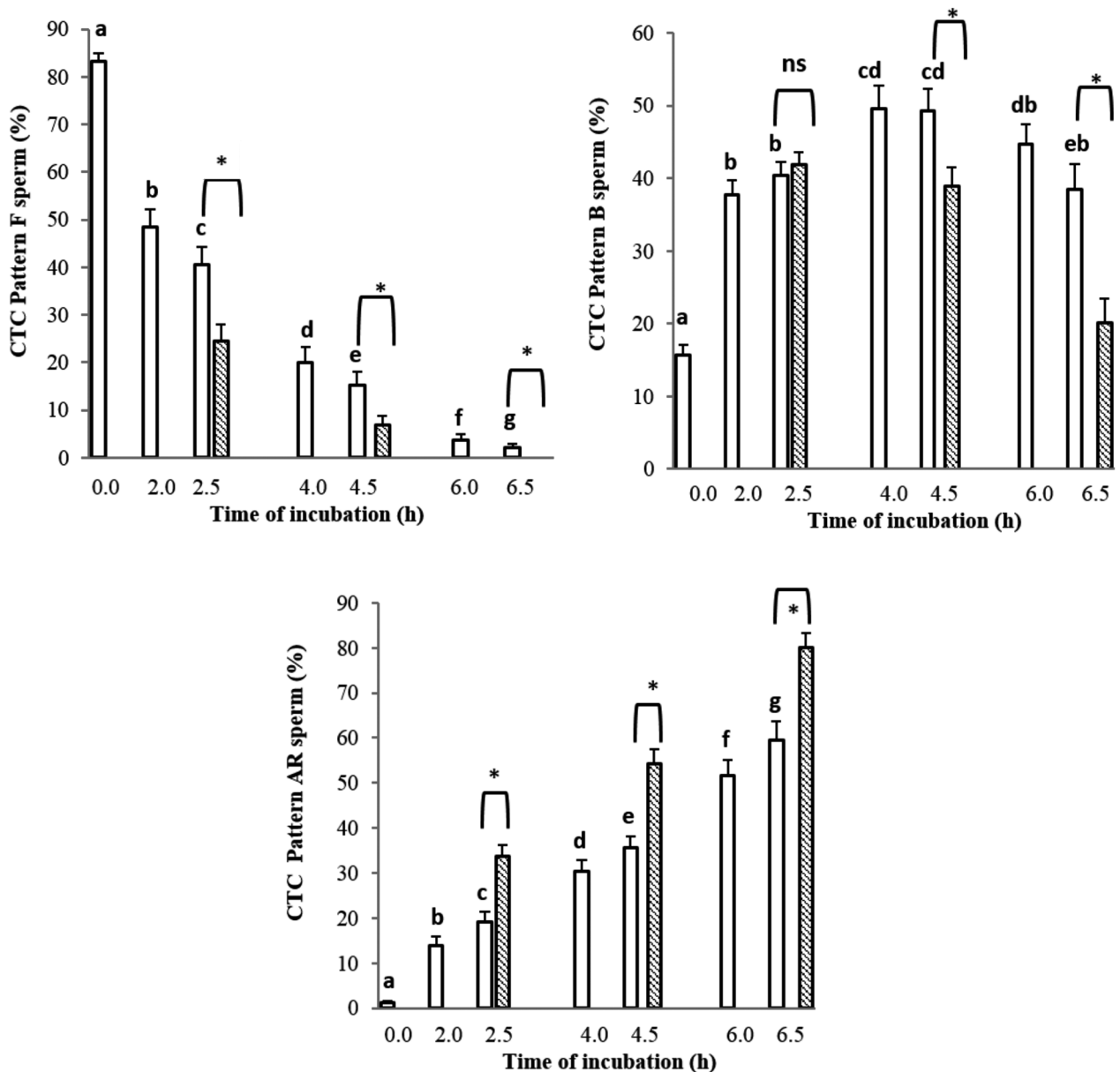


Figure 6. Capacitation status of dog spermatozoa incubated at 38 °C in 5% CO₂ in air during 6.5 h under the effect of Progesterone. Values are means + SEM.

Control: open bars (a-g), Progesterone: shadowed bars.

Control vs Progesterone: *P<0.05, ns = non significant.

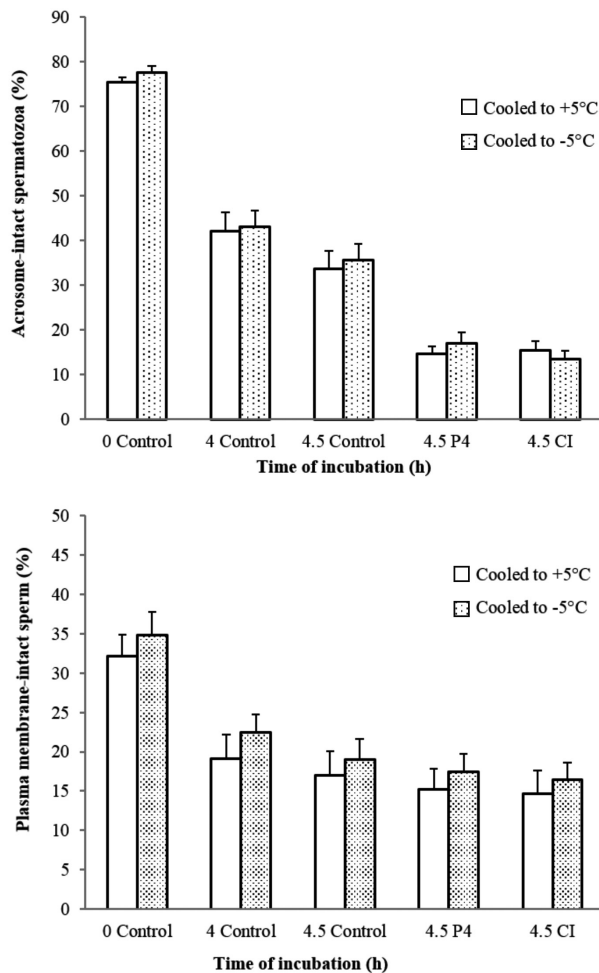


Figure 7. Acrosome-intact and plasma membrane-intact dog spermatozoa after freeze-thawing and post-thaw incubation for 4.5 h at 38 °C in 5% CO₂ in air, without or with Progesterone (P₄)/Calcium ionophore, to induce the acrosome reaction. Sperm were cooled to two target temperatures before freezing: +5 °C or -5 °C. Values are means ± SEM.

of acrosome reaction in fresh-incubated spermatozoa using CI and P₄ was validated. Taking into account CTC capacitation patterns (i.e., B and AR) and the acrosome status, 4 and 4.5 h of incubation were chosen as the optimum time of incubation for the sperm to display the acrosome reaction. Thus, in the next experiments, those times of incubation were used.

The second experiment assessed sperm cryosurvival. One important finding was that after thawing, there was a significant difference in the number of Merocyanine high-binding (hyper-fluidity) cells between sperm cooled to either +5 °C or -5 °C, with the value of the former larger than that of the latter. This observation agrees with the proposed hypothesis that cooling of spermatozoa to sub-zero temperatures, around the freezing point (of diluted spermatozoa), favours sperm plasma membrane reorganisation after lipid phase transition takes place, thus avoiding an excessive increase of membrane fluidity (Watson 1995, Holt

2000). We are aware that this hypothesis disagrees with the general concept that an increase in membrane fluidity before freezing favours sperm cryosurvival (Giraud *et al* 2000, Aboagla and Terada 2003). Increased plasma membrane fluidity may benefit some sperm characteristics, such as motility (Giraud *et al* 2000), but also seems to be related to the incidence of premature sperm capacitation (Watson 1995). In physiological conditions, plasma membrane fluidity increases during sperm capacitation to prepare sperm membranes to suffer the acrosome reaction and facilitates the sperm-oocyte interaction (Flesch and Gadella 2000); however, in cryopreserved sperm, hyper-fluidity shortens the window of sperm fertility (Watson 1995). Studying the cryopreservation of dog spermatozoa, Alcantar-Rodriguez and Medrano (2017) found no differences in sperm quality and plasma membrane fluidity between sperm cooled to either +5 °C or -5 °C. In that work, approximately 52% of spermatozoa were classified as Merocyanine high-binding cells (hyper-fluidity). In contrast, in our work, values were 69.5 and 63.8% for +5 °C and -5 °C, respectively. In the study by Alcantar-Rodriguez and Medrano (2017), sperm were frozen on the next day after collection. In contrast, in our work sperm were collected and frozen on the same day. Thus, it could be the long storage at +5 °C and the stress of cryopreservation itself that minimised any positive effect of cooling to sub-zero temperatures on dog sperm fluidity. Plasma membrane fluidity may be modified during cryopreservation by both removal of cholesterol from the plasma membrane and by lipid peroxidation (Moein-Vaziri *et al* 2014), however, in our work, we were not able to discriminate between these 2 mechanisms.

Regarding the induction of the acrosome reaction on frozen-thawed spermatozoa, there were no differences between CI and P₄ or between cooling temperatures (+5 °C or -5 °C) in the proportion of spermatozoa that carried out that process. CI and P₄ have been previously used to induce the acrosome reaction in fresh and cryopreserved dog spermatozoa (Szász *et al* 2000, Cheng *et al* 2005), however, to our knowledge, CI and P₄ have not been comparatively used in frozen-thawed dog spermatozoa. In our work, both induced the AR after 4.5 h of incubation; thus, either of them may be employed for that purpose in future research.

In this work, we partially proved our hypothesis that cooling of spermatozoa to -5 °C favours sperm plasma membrane reorganisation, thus avoiding an excessive increase of plasma membrane fluidity. The membrane phase behaviour of different cellular types (human prostate tumour cells, porcine smooth muscle cells and human dermal fibroblasts) during freezing seems to depend on the ice nucleation temperature (Balasubramanian *et al* 2009). In these cellular types, membrane phase transition causes different effects on the membrane conformational disorder in the frozen state when the ice nucleation occurs at either -3 °C (favours dehydration) or -10 °C (intracellular ice formation). Balasubramanian *et al* (2009) reported that ice nucleation at -6 °C (between temperature for dehydration

and that for intracellular ice formation) enables a fraction of cellular and membrane bound water to stay in the cell and thus promotes cell viability. This may be the mechanism by which cooling to -5°C before freezing favours sperm cryosurvival.

At this point, we do not know whether cooling to sub-zero temperatures improves the fertilising capacity of frozen-thawed dog spermatozoa. A test of fertility by artificial insemination employing cryopreserved spermatozoa would provide further insights on that issue. It should also be considered that semen from different dog breeds may show variations in sperm cryosurvival when freeze-thawing protocols are modified (Yu *et al* 2002).

In conclusion, cooling to -5°C did not improve dog sperm cryosurvival but produced a positive effect of plasma membrane fluidity, and the amount of Merocyanine high-binding (hyper-fluidity) cells was smaller than that of sperm cooled to $+5^{\circ}\text{C}$.

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