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Inhibition of phospholipase C reduces the capacitation of cryopreserved ovine sperm

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ABSTRACT

This study aimed to evaluate the effect of phospholipase C (PLC) on the capacitation of cryopreserved ovine semen. Sixteen semen samples were cryopreserved with diluent added by 0, 10, or 20 μM of U73122, a PLC inhibitor. The sperm kinetics of the thawed samples were evaluated using the "Computer-assisted Sperm Analysis" system, and the integrity of the plasma and mitochondrial membranes was evaluated using fluorescent probes. Additionally, sperm capacitation and the acrosome reaction with chlortetracycline hydrochloride were evaluated before and after capacitation induction. The results were analysed using analysis of variance and Tukey's test with a 95% probability. Concentrations of 10 or 20 μM of U73122 did not affect the kinetics or number of **sperm** with intact plasma and mitochondrial membranes. However, after thawing, 10 and 20 μM of the inhibitor reduced the percentage of capacitated and acrosome-reacted sperm. After induction of capacitation, there was a reduction in the number of non-capacitated **sperm** in all treatment groups, suggesting a reversible effect of U73122. In conclusion, U73122 at concentrations of 10 or 20 μM prevents premature capacitation and acrosome reaction induced by the freezing procedure, without affecting the kinetics and integrity of the sperm membranes.

1. Introduction

For decades, semen cryopreservation has been a technique of great interest because of its capacity to preserve the genetic material of different species [1]. However, freeze-thawing procedures induce several injuries to the sperm, reducing their lifespan [2]. Among the damages produced during cryopreservation, changes in membrane structure and function as a result of exposure to low temperatures [1,3] and the use of penetrating cryoprotectants [4] result in increased calcium influx, which can lead to premature capacitation [5].

Ovine **sperm** are more sensitive to this process because they have a lower cholesterol/phospholipid ratio [6] and greater plasma membrane permeability [7] than that of other species. Thus, low fertilization rates have been reported using cryopreserved ram **sperm** [8].

Capacitation is defined as a series of transformations that **sperm** must undergo to reach and bind to the pellucid zone and then fertilize the oocyte [9]. It involves several physiological events that occur in mammalian **sperm** during their path through the female reproductive tract [10], such as hyperactivation and preparation of sperm membranes to undergo an acrosomal reaction [5,11].

The role of calcium in the regulation of capacitation events is well documented [12–14]. Capacitation can also be induced in vitro by several mechanisms, including high calcium concentrations in the extracellular medium [15]. During capacitation, **sperm** become more permeable to the entry of calcium [16]. The entry of extracellular calcium activates phospholipase C (PLC), which in **sperm** and other cells, participates in the hydrolysis of membrane phospholipids, phosphatidylinositol biphosphate (PIP2), into two important intracellular messengers, diacylglycerol (DAG) and inositol triphosphate (IP3) [17,18]. After formation, DAG diffuses through the lipid bilayer in cell membranes, creating a spacing between the phospholipid heads, which makes the membranes more fluid and destabilized [19]. In addition,

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DAG translocate and activate protein kinase C (PKC) [20], which promotes the opening of voltage-gated calcium channels present in the membrane [21]. Several studies have suggested that the influx of calcium through channels activated by PKC is important for motility regulation, maintenance of hyperactivation, and acrosome reactions [20,22]. However, as PKC has several isoforms and its activity is regulated by DAG, phospholipids, and calcium, it is difficult to conduct studies to understand the function of this enzyme [22].

Unlike DAG, which acts on cell membranes and PKC, favoring the entry of extracellular calcium, IP3 acts on internal reserves, allowing the opening and release of calcium stored in the endoplasmic reticulum of cells [23–26]. In **sperm**, IP3 receptors are located in the neck region [27] and acrosome [28,29] and induce hyperactivation and acrosomal reactions, respectively [27].

The U73122 reagent (Sigma-Aldrich, MO, USA) is an amino steroid-penetrating cell membrane known to be a PLC inhibitor that inhibits the hydrolysis of PIP2 to DAG and IP3, leading to a decrease in free calcium concentration in the cytosol of cells. Since its action on PLC activity has been demonstrated in human neutrophils [30,31], this reagent has been extensively used in scientific research across a wide range of cell types for studying events secondary to the activation of PLCs, such as the release of intracellular calcium [32].

In mammalian sperm, the PLCβ, PLCγ, and PLCδ isoforms are frequently described and they seem to be involved in the acrosomal reaction process [17,33]. Additionally, the PLCζ isoform was identified and has since been recognized as the main sperm factor responsible for triggering calcium oscillations in the egg after fertilization [17,34].

Although U73122 is the most widely used PLC inhibitor, its mechanism of action remains poorly understood and is believed to involve several PLCs [32]. The reduction of intracellular calcium was evidenced in human **sperm** incubated with U73122 at concentrations of 2 μM [35], 5 μM [35], and 10 μM [36], even in the presence of calcium-inducing substances. In thawed bovine **sperm**, the use of 10 μM of U73122 in an incubation medium with the cAMP inducer showed the number of cells in a state of capacitation and hyperactivation [37]. Studies have focused on improving the results of the insemination of cryopreserved semen in sheep. In ovine, there are no reports on the use of this inhibitor; however, studies have indicated that the reduction in premature capacitation could improve the results of insemination with cryopreserved semen [2,5].

We hypothesized that premature capacitation in cryopreserved ram **sperm** can be prevented by inhibiting PLC. Therefore, the aim of this study was to evaluate the supplementation of U73122 in a freezing extender and its effects on sperm parameters.

2. Materials and methods

This study was approved by the Ethics Committee on the Use of Animals in Experimentation at Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, Rio de Janeiro, Brazil (No. 374867/2013).

All reagents used in this study were purchased from Sigma-Aldrich, except when indicated.

2.1. Animals and experimental design

The semen samples of four fertile rams Santa Inês breed, 20–24 months old and located in Campos dos Goytacazes - RJ, Brazil (21° 76′ 30″ S, 41° 29′ 18″ W), were collected twice weekly with the aid of an artificial vagina, totaling 16 semen samples. In natura samples were examined subjectively by the same evaluator for mass movement (0–5), total motility (0–100%), and displacement speed (0–5). The sperm concentration was measured using a Neubauer chamber (400X). Semen samples with mass movement ≥3, motility ≥80%, and displacement speed ≥3 were used in this study.

Semen samples were diluted to a concentration of 60×10^6 **sperm**/mL in a commercial extender (BotuBov®, Botupharma, Botucatu, São Paulo, Brazil) in a single fraction and supplemented with 0 (control), 10, or 20 μM of U73122. The semen was packed in 0.5 mL straws and placed in a Styrofoam box (30 × 19 × 39 cm) containing 6 L of crushed ice for 2.5 h on a platform 5 cm above the ice surface (5 °C). Subsequently, the platform with the straws was transferred to another Styrofoam box of the same dimensions, positioned horizontally and maintained at 5 cm from the surface of liquid nitrogen (-120 °C), for 15 min. The straws were then dipped in liquid nitrogen and stored in cryogenic containers for up to six months.

For evaluations, the straws were thawed at 37 °C for 30 s and the contents were deposited in a preheated microvial. The straws were used for each evaluation.

2.2. Sperm kinematics

Immediately after thawing, the semen was diluted in BotuBov® fraction I, without cryoprotector (v:v 1:10), at 37 °C. Aliquots of 10 μL were deposited between the preheated coverslip and glass slide for sperm count in five random fields with the aid of the Computer-assisted Sperm Analysis system, using Sperm Class Analyze (Microptic, Automatic Diagnostic Systems, Barcelona, Spain) coupled to a Nikon Eclipse microscope (Nikon, Tokyo, Japan), under a magnification of 400X. Sperm movement was observed under negative phase-contrast optics and videotaped in five different fields. Software settings were adjusted for ram **sperm** and the parameters were evaluated as described by Olivares et al. [38]. The following parameters were evaluated: total motility (%); progressive motile (%); **sperm** rapid (%); curvilinear velocity (μm/s); straight-line velocity (μm/s); average path velocity (μm/s); linearity (%); straightness (%); head displacement (%); beat/-cross frequency (%); and hyperactivity (%).

2.3. Membrane integrity and mitochondrial activity test

Thawed semen samples were diluted in Ca^{2+}- and Mg^{2+}- (1:3) to obtain a concentration of 20×10^6 **sperm**/mL. Aliquots of 50 μL were stained using 1 μL of propidium iodide (0.5 mg/mL in PBS) and 7 μL of JC-1 (153 μM in DMSO) to evaluate the integrity of plasma and mitochondrial activity, respectively.

From each sample, 200 **sperm** were counted in moist preparations between coverslips and glass slides using an epifluorescence microscope (Eclipse TE 300, Nikon) at a magnification of 400X. The **sperm** were classified as presenting an injured plasma membrane, the sperm nuclei emitting red fluorescence, with injured acrosome, those with a yellowish-green acrosome, those without mitochondrial function, and those who had an intermediate emitting green fluorescence, while the red emission indicated high mitochondrial activity.

2.4. Capacitation and acrosome reaction

Sperm capacitation and acrosomal reactions were evaluated as described by Fraser et al. [13]. After thawing, the volume of each straw was transferred to a microvial along with 1 mL of PBS free from Ca^{2+} and Mg^{2+} (1:2), at 37 °C and centrifuged (600 g) for 2 min in a microcentrifuge (MiniSpin Plus, Eppendorf, Hamburg, Germany). The supernatant was discarded and the pellet was resuspended with 1 mL of PBS at 37 °C followed by centrifugation (600 g for 2 min). The supernatant was again discarded and 500 μL of heated PBS was added to the pellet, and 50 μL of this homogenized liquid was used for dilution with the chlortetracycline hydrochloride (CTC; 1:1) fluorescent marker in a new microvial. Subsequently, 8 μL of this suspension was mixed with 8 μL of DABCO and 0.2 μL of paraformaldehyde.

For each sample, a total of 200 **sperm** were counted under a magnification of 600X using an epifluorescence microscope (Eclipse TE 300, Nikon), using a 440 nm excitation filter and 470 nm emission filter. Sperm with fully fluorescent surfaces were classified as non-capacitated,

those that presented fluorescence loss in the post-acrosomal region were classified as capacitated, and those with fluorescence loss in the post-acrosomal and acrosomal regions, and fluorescence only in the intermediate part, were classified as **sperm** with reacted acrosomes.

2.5. Induction of in vitro capacitation

After thawing, 25 0 μL of semen was deposited in a microvial containing 800 μL of discontinuous gradient mini Percoll (400 μL for each density: 90 and 45%) and centrifuged (600 g) for 15 min. The supernatant was discarded and the pellet was resuspended with 1 mL of PBS at 37 °C, and centrifuged again (600 g for 2 min); the supernatant was then discarded.

To induce the capacitation, the pellet was resuspended in 200 μL of Talp sp medium containing 100 mM NaCl (S5886), 0.3 mM NaH_2PO4 (S0751), 3.1 mM KCl (P5405), 21.6 mM Lactic Acid 98% (L4263), 10 mM Hepes (H4034), 25 mM $NaHCO_3$ (S6297), 2.2 mM $CaCl_2$ $2H_2O$ (C3881), 0.4 mM $MgCl_2$ $6H_2O$ (M0250), 100 UI/mL penicillin (P3032), 100 μg/mL streptomycin (S01277), and 0.6% Bovine Serum Albumin Fraction V (A9418) supplemented with heparin (2 μg/mL; H3149). This medium was prepared 3 h before use and incubated at 5% CO2 and 37 °C.

The **sperm** diluted in Talp sp – heparin was stored for 4 h at 37 °C in a 5% CO_2 incubator. After this period, the samples were centrifuged (600 g for 2 min) and the pellet was resuspended in 1 mL of PBS at 37 °C. A second centrifugation was performed under the same conditions and the pellet was resuspended in 200 μL of PBS. An aliquot of 50 μL was diluted with a CTC (1:1) fluorescent marker in a new microvial. The preparation of samples for the evaluation of capacitated sperm and acrosome reaction was performed as described in Section 2.3.3.

2.6. Statistical analysis

A completely randomized design with a 3 × 4 factorial arrangement (concentrations of inhibitor × breeders) was used with four repetitions (semen samples). Data obtained from the kinetic parameters of frozen semen, membrane integrity, capacitation, and the acrosome reaction were subjected to normality (Lilliefors Test) and homoscedasticity (Cochran & Bartlett Test) tests, not meeting the assumptions of one-way analysis of variance (ANOVA). Therefore, the data were transformed using the square root (X+0.5) transformation to meet the assumptions of ANOVA and are presented in their untransformed form.

The observed variables did not have a significant effect on the relationship between the inhibitor concentration and breeders, thereby preventing the use of a test protected by ANOVA. Tukey's honestly significant difference test ($p < 0.05$) was used to compare the independent effects of inhibitors and breeding levels.

3. Results

The use of U73122, at concentrations of 10 and 20 μM, for the cryopreservation of ovine semen did not affect ($p > 0.05$) kinetic parameters. In addition, no differences were observed in plasma membrane integrity or mitochondrial activity after thawing (Table 1).

Treatment with 10 or 20 μM of inhibitor reduced the percentage of capacitated sperm and acrosome-reacted after thawing (Fig. 1). However, no significant differences were observed between the two inhibitor concentrations ($p > 0.05$).

After the induction of capacitation in vitro, a reduction was observed in the percentage of non-capacitated sperm in all the experimental groups ($p < 0.05$). Consequently, there was an increase in the percentage of capacitated sperm or acrosome reaction (Fig. 1), proving the reversible effect of the inhibitor on acrosome capacity and reaction.

Table 1
Effects of U73122 during the cryopreservation of sheep sperm, on spermatozoa kinetics and the integrity of the plasma membranes and mitochondrial activity, determined by the propidium iodide fluorescent probes or JC-1.

	Unit	Concentration of U73122		
Parameters		0 μM	10 μM	20 μM
TM	(%)	68,40 (±19,40)	67,60 (±18,13)	70,90 (±14,20)
PM	(%)	31,04 (±19,43)	31,10 (±15,18)	31,10 (±14,44)
RAP	(%)	21,96 (±15,97)	22,43 (±12,18)	20,11 (±11,34)
MED	(%)	16,03 (±11,31)	16,21 (±11,64)	17,96 (±9,02)
SLO	(%)	30,37 (±13,73)	28,90 (±13,49)	32,80 (±12,68)
STA	(%)	31,60 (±20,18)	32,43 (±18,25)	29,10 (±14,09)
VCL	(μm/s)	59,94 (±17,98)	55,38 (±14,15)	53,21 (±11,89)
VSL	(μm/s)	44,60 (±21,37)	41,10 (±16,06)	38,31 (±14,07)
VAP	(μm/s)	50,23 (±21,16)	46,10 (±16,01)	43,35 (±14,05)
LIN	(%)	71,15 (±15,03)	71,85 (±13,99)	69,52 (±12,57)
STR	(%)	86,73 (±7,43)	87,41 (±8,48)	86,79 (±6,24)
ALH	(%)	1,74 (±0,30)	1,66 (±0,39)	1,71 (±0,36)
BCF	(%)	9,16 (±1,67)	8,75 (±1,54)	8,67 (±1,17)
HYP	(%)	3,13 (±2,28)	4,63 (±3,34)	4,25 (±3,07)
IPM	(%)	30,5 (±9,4)	30,4 (±11,0)	27,37 (±7,8)
MF	(%)	33,9 (±9,2)	34,0 (±10,5)	31,9 (±8,6)

Total motility (TM); progressive motility (PM); rapid sperm (RAP); medium (MED), slow (SLO), or static displacement sperm (STA); curvilinear velocity (VCL); straight line velocity (VSL); average path velocity (VAP); linearity (LIN); straightness (STR); amplitude of lateral head displacement (ALH); beat-cross frequency (BCF); hyperactivated (HYP); Intact plasma membrane (IPM); mitochondrial function (MF).
*Averages (±SD) followed by the same uppercase letter on the variable columns do not differ according to the DMS test $P \leq 0.05$, $n = 16$ (ejaculated).

4. Discussion

To the best of our knowledge, this is the first study to evaluate the effect of U73122, a phospholipase C inhibitor, on cryopreserved ovine semen. Cellular alterations are inherent to the cryopreservation technique [39], which increases the permeability of **sperm** to calcium intake. As a result, they become more susceptible to capacitation [1,16]. Premature capacitation occurs when the process of capacitation is activated ahead of time, causing **sperm** to lose their fertilizing capacity before encountering an egg. This phenomenon is particularly relevant in the case of cryopreserved semen samples.

In the present study, the activity of PLC was regulated by U73122 in an attempt to avoid premature capacitation of cryopreserved ovine **sperm**. This enzyme acts on PIP2 hydrolysis, forming DAG, which translocates and activates PKC (an enzyme that promotes the opening of calcium channels in the plasma membrane) [10], and IP3, which releases calcium stored in the endoplasmic reticulum [17,40,41].

In previous tests, we observed that the use of 10 μM of U73122 did not alter the sperm kinetics of fresh **sperm** incubated at 37 °C for 4 h, with or without the presence of penetrating cryoprotectant (data not shown), which encouraged us to complement the commercial extender with 10 or 20 μM of U73122.

Sperm motility can be altered by the concentration of intracellular calcium. During capacitation, **sperm** acquire a motility pattern characterized by asymmetric flagellar beats of greater amplitude [42], known as hyperactivation [9]. In the present study, no kinetic parameters were affected by PLC inhibition.

The PKC activation of calcium channels, induced by an increase in DAG concentration, enhances sperm motility [43]. However, the intracellular calcium concentration in **sperm** is also regulated by other calcium channels such as CatSper [13]. In a murine study, CatSper was the most important channel in the regulation of normal sperm motility, the progressive one, since **sperm** from animals without the genes for this channel presented slow flagellar beating and non-progressive motility and were not able to fertilize the oocytes [13]. Thus, the CatSper channel, which may have contributed to the unchanged kinetic patterns, would compensate for the inhibition of PLC by U73122. In addition, the

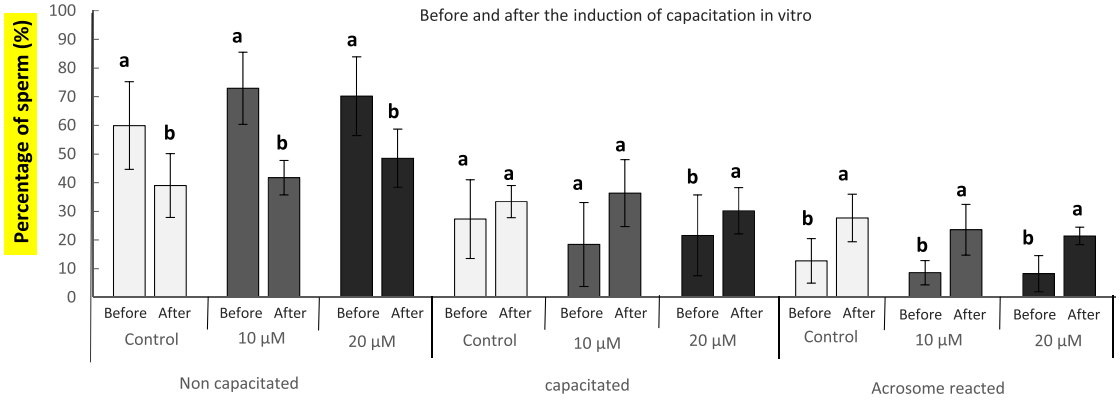

Fig. 1. Effect of the different concentrations of U73122 (0, 10, 20 µM) on the number of non-capacitated sperm (NC), capacitated sperm (C) or with acrosome reacted (AR) of cryopreserved ram sperm samples. The evaluations were before (i.e., immediately after thawing), and after the in vitro induction of capacitation, where the cells were incubated during 4 h at 37 °C. The averages followed by the same lowercase do not differ between the categories after thawing and after the induction of capacitation. The average percentages of each treatment were compared using the DMS Tukey test, at the significance level of 5%.

mobilization of intracellular calcium stores by IP3 appears to be essential for sperm hyperactivation [27]. Our results confirm this fact and are consistent with the findings of Márquez et al. [15], who observed hyperactivation of approximately 90% of mouse sperm by activating PLC using thimerosal. In contrast, Alonso et al. [37] incubated thawed bovine **sperm** in the presence of 10 µM of U73122 and did not observe hyperactivation.

Sperm capacitation is correlated with several cellular events, including increased membrane fluidity [44]. PKC has also been reported to upregulate mitochondrial membrane potential [45]. Once formed, DAG is diffused by the double lipid layer of the cell membranes, making them fluid and destabilized [19], which is related to the greater resistance of cells to cryopreservation [46]. Although intracellular DAG increases are generally observed in cryopreserved semen samples [47], the use of the U73122 inhibitor did not affect plasma membrane integrity or mitochondrial activity. Further studies are needed to understand the role of DAG in cryopreserved sperm membranes.

The use of U73122 at both concentrations was efficient in reducing premature capacitation and anticipating the acrosome reaction in ovine **sperm** subjected to cryopreservation. Our results are with that of previous studies, where the same inhibitor, when used at concentrations of 6 and 10 µM in human **sperm**, reduced the acrosome reaction [35] and intracellular calcium levels in these cells [36]. In addition, Alonso et al. [37] reported that when incubating bovine **sperm** in a medium supplemented with AMPc, a capacitation inducer with 10 µM of U73122, there was a reduction in the number of cells in a capacitating state when compared to samples without the inhibitor.

Although PLC inhibitors have already been used in some sperm research methodologies [35–37], their use as additives in cryopreservation extenders is unknown. Thus, by inducing in vitro capacitation of cryopreserved sperm in the presence of the inhibitor, we verified that the effect of U73122 on capacitation and the acrosome reaction was reversible, which allows its safe use in semen extenders.

Gillan et al. [8], when analysing fresh and cryopreserved ovine semen, observed that fresh **sperm** displayed mainly the non-capacitated pattern (61.3%), becoming capacitated (54%) and reacted acrosome (41%) patterns after incubation (6 h at 37 °C). In contrast, frozen **sperm** displayed the capacitated pattern (65.9%), becoming the reacted acrosome pattern (64·2%) with incubation.

There is no estimate in the literature regarding the ideal percentage of non-capacitated sperm. Therefore, a cryopreserved semen sample is considered adequate for insemination. However, knowing that the capacitation and acrosome reaction processes should occur in the reproductive tract of females [48] and these events can interfere with fertilization [49], it is desirable that sperm samples contain the lowest percentage of capacitated sperm after thawing, with which it is expected to obtain a higher pregnancy rate.

Thus, the use of U73122 reagent to regulate the premature capacitation and acrosomal reaction of **sperm** can benefit the artificial insemination (AI) of sheep and other species, such as bovines, especially when using sexed semen, which appears to be susceptible to premature capacitation [50].

This study paves the way for the use of other biochemical inhibitors or regulators to address specific sperm traits in this species. However, in vivo studies are needed because the inhibition or regulation of intracellular mechanisms cannot compromise fertilization and pregnancy outcomes.

Therefore, we concluded that the regulation of PLC by the U73122 reagent at concentrations of 10 or 20 µM prevents premature capacitation and, consequently, the acrosomal reaction induced by cryopreservation in a reversible way, without changing the kinetics and integrity of the sperm membranes. However, we recommend conducting in vivo studies to evaluate its efficiency in improving the pregnancy rates in females subjected to AI with cryopreserved semen.

CRediT authorship contribution statement

Aline Matos Arrais: Data curation, Formal analysis, Investigation, Methodology, Data curation, Formal analysis, interpretation of data, Writing – original draft. **Angelo José Burla Dias:** Funding acquisition, Data curation, Formal analysis, Investigation, Methodology, Formal analysis, interpretation of data. **Cláudio Luiz Melo de Souza:** Investigation, Methodology, Formal analysis. **Alinne Glória Curcio:** Conceptualization, Investigation, Methodology, Formal analysis. **Marco Roberto Bourg de Mello:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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