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Capacitation Inducers on Thawed Stallion Sperm: Effect on Sperm Capacitation and Homologous Zona Pellucida Binding Assay

Inductores de Capacitación en Espermatozoides Descongelado de Semental: Efecto en la Capacitación Espermática y la Unión a la Zona Pelúcida en Ensayo Homólogo

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SUMMARY: Frozen-thawed spermatozoa from different mammalian species successfully undergo capacitation *in vitro* under specific and defined conditions, however the induction of *in vitro* capacitation of equine spermatozoa still needs optimizations. The aim of this study was to evaluate the effects of different culture media on induction of *in vitro* capacitation of thawed stallion sperm. To do this, the sperm were thawed and incubated with different combinations of capacitating inducers for 2 h. In our study five different media: no capacitation medium (NC), basal medium that included BSA and bicarbonate, basal mediium plus capacitating inducer dibutyryl cyclic-AMP (dbcAMP) as a PKA activator, a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) and a cholesterol acceptor, methyl-β-cyclodextrin (MβCD). The membrane fluidity, acrosome reaction and intracellular calcium levels were evaluated after 2 h of incubation, but we did not find any differences among groups. Different behavior between stallions was also observed. Finally, the sperm treatments were evaluated using a homologues zona pellucida binding assay. This assay showed that incubation in basal medium including MβCD increased the number of spermatozoa adhered to the zona pellucida of mare oocytes. Further studies are needed to evaluate the effect of these conditions on *in vitro* fertilization of equine species using thawed sperm.

KEY WORDS: Capacitation; Spermatozoa; Stallion; Zona pellucida.

INTRODUCTION

The development of equine *in vitro* assisted reproduction technologies has improved over time. However, there is still the need to improve and increase the efficiency of several processes. Reproductive technologies such as artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), cryopreservation of gametes and embryos have been developed (Leemans *et al*., 2016), although with low efficiency and a narrow window in its application in the equine industry (Gimeno *et al*., 2021). A frequent factor to treat in male gamete cryopreservation in mammal species, is the wide variability of the ejaculates among donors and from different ejaculates from same sire (Dowsett & Knott, 1996). This is particularly evident in the equine species, since the semen is highly variable in terms of sperm survival against after cryopreservation (Hoffmann *et al*., 2011). Using frozen semen provides significant laboratory efficiency benefits by eliminating the need for fresh semen, thereby avoiding the logistical challenges associated with accessing animals. It also reduces the risk of disease transmission, overcomes geographic limitations, and allows for the indefinite preservation of genetic material. It is also important to highlight that the success of cryopreservation is influenced by factors such as the stallion's genetics and age (Gimeno *et al*., 2021), making cryopreservation a particularly challenging process for stallion sperm.

On the other hand, sperm capacitation is a physiological process that spermatozoa must undergo to reach the fertilizing ability. It primarily occurs in the female reproductive tract after ejaculation and involves a series of biochemical and cellular changes in the spermatozoa (Shivaji *et al*., 2007). The sperm capacitation is associated with the increase of membrane fluidity due to cholesterol

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depletion, lipid raft aggregation, protein tyrosine phosphorylation, hypermotility and finally the ability to undergo the acrosome reaction (Leemans *et al*., 2016). All of these processes can be mimicked *in vitro* by incubating sperm to an environment containing bicarbonate, calcium, and albumin (Leemans *et al*., 2019). The induction of *in vitro* capacitation is vital for the success of IVF in mammals. Despite numerous attempts to establish IVF, consistent and repeatable protocols have not been achieved in the equine species (Leemans *et al*., 2019).

In other mammals IVF has been successfully enhanced by using capacitation inducers, molecules that promote changes associated with sperm capacitation. These inducers include methyl-β-cyclodextrin (MβCD), isobutylmethylxanthine (IBMX), and dibutyryl-cAMP (dbcAMP), which have been applied in species such as cattle and mice (Seita *et al*., 2009). IBMX increases intracellular cyclic AMP (cAMP) levels (Gadella & Harrison, 2002), while MβCD, a heptasaccharide, facilitates cholesterol efflux from the sperm cell membrane (Companyó *et al*., 2007). Additionally, dbcAMP acts as a cAMP analogue, further supporting capacitation (Maitan *et al*., 2021). These inducers collectively enhance fertilization rates in mammalian embryos.

Thus, the objective of this study was to find a medium capable to effectively induce *in vitro* capacitation of thawed equine sperm, since it has not been possible to achieve this process in short incubation times. Considering the high demand for *in vitro* equine embryos, and the significant cost associated with producing equine embryos through ICSI, including the need for highly trained staff, it is essential to advance in the search of capacitation media and/or capacitation conditions to enhance IVF performance and improve fertilization rates.

MATERIAL AND METHOD

Ethical approval. The study protocol was approved by Bioethical Committee from Universidad de La Frontera (Approval number, Act N° 057/2016; Approval date, June 2016).

Chemicals. Unless otherwise stated, all reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Semen collection and preparation. Semen was collected from three Chilote stallions 3 to 8 years old with a "Colorado" artificial vagina twice a week during the reproductive season. Immediately after collection, the semen was diluted adding a commercial extender (Botusemen®, Botucatu, Brazil) in a 1:1 ratio and transported to the laboratory at room temperature within 15 min of semen collection. Sperm concentration was evaluated using an SDM1 equine photometer (Minitube, Valencia, Spain) diluting the sample 100 times. Semen was loaded in different 15 mL tubes for subsequent cryopreservation. At least four ejaculates per stallion were collected, cryopreserved, and analyzed.

Semen freezing. The semen was centrifuged at 600g for 10 min. The supernatant was discarded, and the pellet was diluted in freezing medium (Minitube (R) to a concentration of 200 x 10^6 sperm/mL at room temperature. The diluted semen was gradually cooled from room temperature to 4°C for 1 h (0.3 °C/min). The sperm suspension was stored in 0.5 mL straws at a concentration of 200 x 10^6 sperm/mL. The straws were placed in liquid nitrogen vapor atmosphere (NL_2) horizontally at 4 cm for 10 min. Subsequently, they were immersed and stored in NL_2 (-196 °C) for further evaluation.

Media Preparation. Base (non-capacitating) medium: Sperm TLP (NC) medium (100 mM NaCl, 3.1 mM KCl, 0.3 mM NaH2PO4, 21.6 mM sodium lactate, 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10.0 mM HEPES and 1.0 mM pyruvate) was used for post-thaw incubations (McPartlin *et al*., 2009). Capacitating medium: Sperm TALP (C) supplemented with bovine serum albumin (BSA 0.1 mg/mL) and 25 mM $NaHCO_3$ (McPartlin *et al*., 2009). Medium osmolarity was adjusted to 290 to 300 mOs and pH 7.4.

Fert-TALP: consisted of 114 mM NaCl, 3.2 mM KCl, 25 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.05 mM $MgCl_2$, 4.6 mM D-glucose, 10 mM HEPES, 10 mM sodium lactate, 0.5 mM sodium pyruvate, 1.5 mM $CaCl_2$, and 6 mg/mL BSA, at 290-305 mOsm and pH 7.3 after equilibration at 5 % CO_2 in according to Felix *et al*. (2022).

Semen thawing and incubation under capacitating conditions. The straws with semen from each of the stallions were thawed in a 37 °C water bath for 30 seconds; the semen was deposited in 1 ml Eppendorf tubes containing the base Sperm TLP medium at 37 °C. The sample was washed in non-capacitating (SPTL NC) medium and divided into aliquots of 200 x 10^6 sperm/mL and placed in 0.4 mL as described below. The treatments evaluated were the following: NC control non capacitating: C capacitating with BSA and $NaHCO_3$, C-I: capacitating with BSA + $NaHCO_3$ + 0.4 mM IBMX, C-CD: capacitating with BSA + $NaHCO_3$ +0.1 mM MβCD, C-D: capacitating with BSA + $NaHCO_3$ + 5 mM dbAMPc. Thawed spermatozoa in the different treatments were incubated for 120 min at 38°C in CO_2 in a saturated atmosphere.

Flow cytometry. Flow cytometry was conducted in a FACSCanto II flow cytometer (Becton, Dickinson and Company). A total of 10,000 events were used from each measurement. Acquisition was performed with a sample aspiration of 60 mL/min. Samples were acquired and analyzed using the FACSDivasoftware, version 6.1.3 (Becton, Dickinson and Company). Fluorophores were excited at 488 nm using an argon laser. Green fluorescence staining was detected using a 530/30 nm bandwidth filter. Orange fluorescence was detected using a 585/42 nm bandwidth filter. Three replicates of each foal were performed.

Assessment of acrosome integrity. The acrosome integrity was assessed using the FITC-PNA/PI-assay. Frozen spermatozoa were thawed as described earlier. Aliquots of 500 µL of sperm suspension (3-5 x10^6 cells/mL) were stained with 2 µL of FITC-PNA (60 µg/ml stock solution) for 15 min at 38 °C in darkness. Then, 2 µL of PI (2.4 mM stock solution) was added and incubated for 5 min before the analysis by flow cytometry. This staining led to the identification of four different populations of spermatozoa: (1) non-viable spermatozoa with intact acrosome (PNA-/PI+), (2) non-viable spermatozoa with reacted acrosome (PNA+/PI+), (3) viable spermatozoa with intact acrosome (PNA-/PI-) and (4) viable spermatozoa with reacted acrosome (PNA+/PI-). Results were expressed as the percentage of viable spermatozoa with intact and reacted acrosome.

Assessment of plasma membrane integrity and fluidity. To evaluate plasma membrane integrity and changes in fluidity (lipid disorder) there was use of Merocyanine 540 (MC540; Sigma M6390) and Sytox Green staining (Molecular Probes, Life Technologies, Carlsbad, USA). For each analysis, 0.5 mL of sperm suspension containing 2×10^6 cells/mL was incubated during 15 min at 37.5 °C with 2.7 µM MC540. Spermatozoa were subsequently washed at 350 g for 5 min and suspended in 500 µL of Sperm TLP medium. Finally, 0.5 µM of Sytox Green was added and incubated at 37.5 °C for 5 min. Analyses were performed by flow cytometry. Results were expressed as the percentage of sperm positive to MC540 and negative to Sytox Green.

Assessment of intracellular calcium i(Ca^{2+}) levels. To assess the i(Ca^{2+}) levels, spermatozoa were loaded with 2 µM Fluo-3 AM (Molecular Probes, Eugene, OR, USA) and incubated for 30 min at 37.5 °C and protected from light. Then, 2 µM of PI were added, and the suspension was incubated for an additional 5 min. Samples were washed once by centrifugation at 350 g for 10 min and then resuspended in Sperm TLP medium. Sperm samples were evaluated using flow cytometry, and the results are expressed as mean fluorescence intensity of viable spermatozoa (arbitrary unit; AU).

Zona pellucida (ZP) binding assay. Zona pellucida (ZP) binding assay was performed as previously reported (Bromfield *et al*., 2014) with some modifications. Briefly, equine ovaries were collected at a local slaughterhouse (Frigorifico Nueva imperial, Imperial and Matadero Cholchol, Cholchol, Chile) and transported to the laboratory in a warmed 0.9 % saline solution within 1 h of slaughter. Immature oocytes were recovered by follicular aspiration and scrapping, selecting those with homogenous granular cytoplasm and more than 5 layers of cumulus oophorus cells. A maximum of 50 oocytes were incubated in 500 mL of maturation medium for 30 h at 38.5° C, 5 % CO_2 in a saturated atmosphere. After maturation, oocytes were stripped of cumulus cells by treatment with 1 mg/mL of hyaluronidase and pipetting. Oocytes presenting extrusion of the first polar body were selected. Stallion semen straws were thawed according to the protocol described above, washed in PBS, and diluted to 1 x10^6 sperm/mL. Oocytes were cultured in drops of non-capacitating medium previously equilibrated at 38° C and 5 % CO_2 for 1 h, and the sperm suspension at concentration of 1 x 10^6 sperm/mL were added to each drop and the gametes were co-incubated for 1 h at 38°C and 5 % CO_2. Sperm-oocyte complexes were washed thoroughly 3 times with a washing medium (SPTL NC) supplemented with 0.2 % PVA to remove loosely attached spermatozoa stained with Hoechst (Molecular Probes, Eugene, OR, USA) (10 mg/mL in PBS) for 30 min and mounted on slides with a mounting medium. The number of ZP-bound sperm was recorded under an epifluorescence microscope (Eclipse TS100F, Nikon Instruments, New York, NY, USA) at 200x magnification. Results were expressed as the average number of spermatozoa attached to the oocytes.

Statistical analysis. Statistical analyses were performed with GraphPad Prism®, version 8.0.1 (GraphPad Software, San Diego CA). Data for the functional parameters evaluated by flow cytometry are presented as mean ± standard deviation (S.D.). The homoscedasticity of the data was analyzed using the Shapiro Wilk test. Differences between treatments were analyzed using ANOVA. To determine differences between groups, a post hoc analysis was performed using the Tukey or Kruskal Wallis test according to the normality of the data. Differences were considered significant at $P < 0.05$.

RESULTS

Acrosome integrity, plasma membrane fluidity and intracellular calcium i(Ca^{2+}) levels. Evaluation of sperm acrosome integrity, plasma membrane fluidity, and i(Ca^{2+}) levels showed no differences ($P > 0.05$) between groups in each of the stallion analyzed (Figs. 1 to 3). Similarly, when pooling all the data from the stallions, no significant differences were observed (Panel D of Figs. 1 to 3).

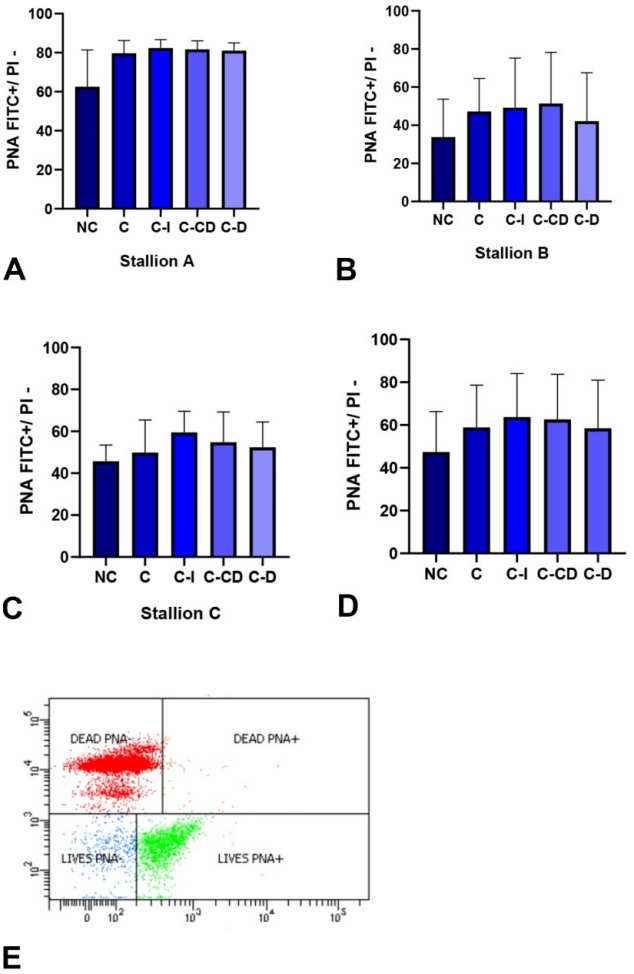

Fig. 1. Analysis of the effect of incubating thawed spermatozoa with different capacitating conditions on the acrosome membrane integrity. Panels A, B, C: acrosome membrane integrity (FITC-PNA/PI staining) of each stallion. Panel D: average of data from all stallions. Panel E: Corresponds to two-dimensional fluorescence dot plot of results conducted with the FITC-PNA/PI staining.
NC control: non-capacitating, C: capacitating with BSA and $NaHCO_3$ C-I: capacitating with BSA + $NaHCO_3$ + IBMX, C-CD: capacitating with BSA + $NaHCO_3$ + MβCD, C-D: capacitating with BSA +$NaHCO_3$ + dbAMPc. Three biological replicates from each one.

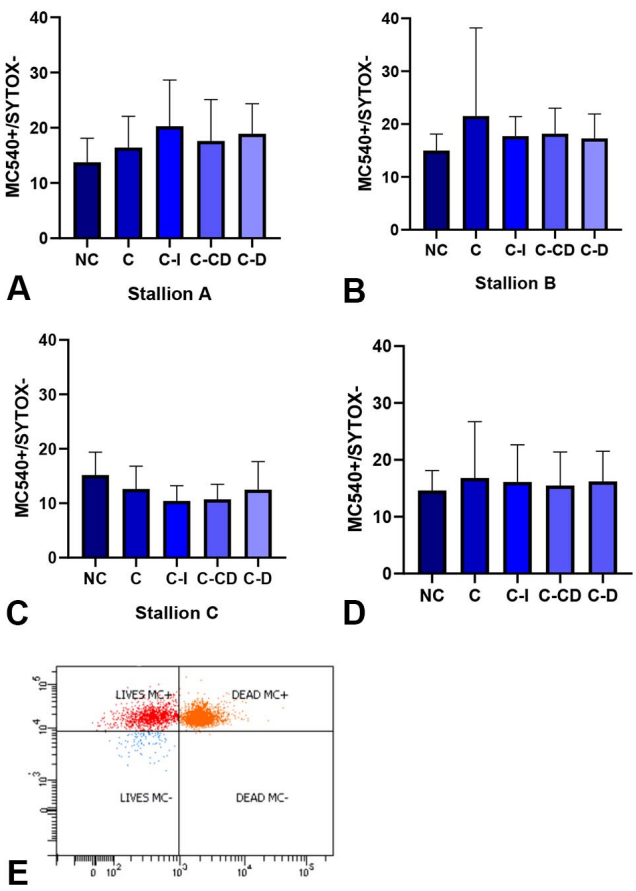

Fig. 2. Analysis of the effect of incubating thawed spermatozoa with different capacitating conditions on membrane fluidity. Panels A, B, C: membrane fluidity (MC540/ SYTOX staining) of each stallion. Panel D: average of data from all stallions. Panel E: Corresponding to two-dimensional fluorescence dot plot of results from conducting the MC540/ SYTOX staining. NC control: non-capacitating, C: capacitating with BSA and $NaHCO_3$ C-I: capacitating with BSA + $NaHCO_3$ + IBMX, C-CD: capacitating with BSA + $NaHCO_3$ + MβCD, C-D: capacitating with BSA +$NaHCO_3$ + dbAMPc. Three biological replicates from each one.

Evaluation of plasma membrane fluidity after two hours of incubation in different capacitating treatments showed no differences ($P > 0.05$; Fig. 2) between the capacitating treatments and the non-capacitating control in each of the stallion analyzed. Similarly, the average of all foals did not show significant differences either (Fig. 2 Panel D). Evaluation of i (Ca^{2+}) levels after two hours of incubation in different capacitating treatments showed no differences ($P > 0.05$; Fig. 3) between the capacitating treatments and the non-capacitating control in each of the stallion analyzed. Similarly, when grouping (pooling) all the data from the stallions, no significant differences were also observed (Fig. 3 Panel D).

Homologues zona pellucida (ZP) binding assay. Stallion sperm was incubated in different capacitation conditions including inducers of capacitation. Incubation in capacitation medium including MβCD showed significant differences ($P < 0.05$) with the non-capacitation (NC), capacitation (C) and capacitation medium with IBMX (C-I) (Fig. 4). Figure 5 shows representative images of the spermatozoa bound to mare oocytes. It is important to note that this zona pellucida (ZP) binding assay was performed with mare oocytes, therefore we could demonstrate the induction of capacitation in stallion spermatozoa with these conditions.

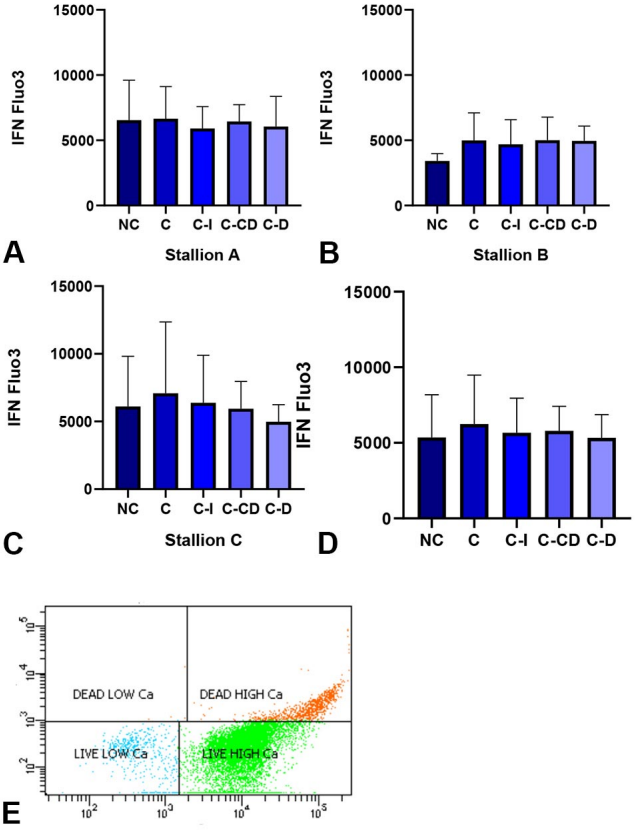

Fig. 3. Analysis of the effect of incubating thawed spermatozoa with different capacitating conditions on the concentrations of intracellular calcium. Panels A, B, C: Concentrations of i(Ca^{2+}) (Fluo-3/PI staining) of each of the stallion. Panel D: average of data from all stallions. Panel E: Corresponds to two-dimensional fluorescence dot plot of results from conducting the Fluo-3/PI staining.

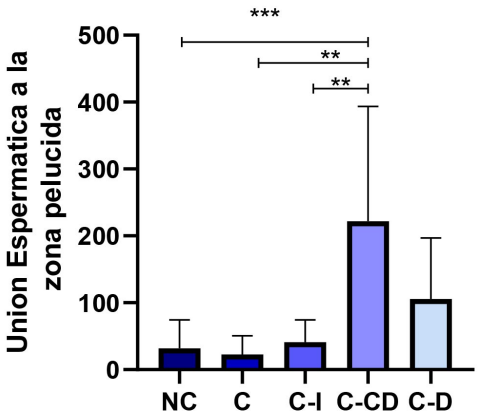

Fig. 4. Analysis of the effect of incubating thawed spermatozoa with different capacitating conditions on the number of bound spermatozoa to the zona pellucida of mare oocytes stained with Hoechst. NC control: non-capacitating, C: capacitating with BSA and $NaHCO_3$, C-I: capacitating with BSA + $NaHCO_3$ + IBMX, C-CD: capacitating with BSA + $NaHCO_3$ + MbCD, C-D: capacitating with BSA + $NaHCO_3$ + dbcAMP. *Above the bars indicate significant differences ($P < 0.05$; Three biological replicates).

NC control: non-capacitating, C: capacitating with BSA and $NaHCO_3$ C-I: capacitating with BSA + $NaHCO_3$ + IBMX, C-CD: capacitating with BSA + $NaHCO_3$ + MβCD, C-D: capacitating with BSA +$NaHCO_3$ + dbcAMP. Three biological replicates from each one.

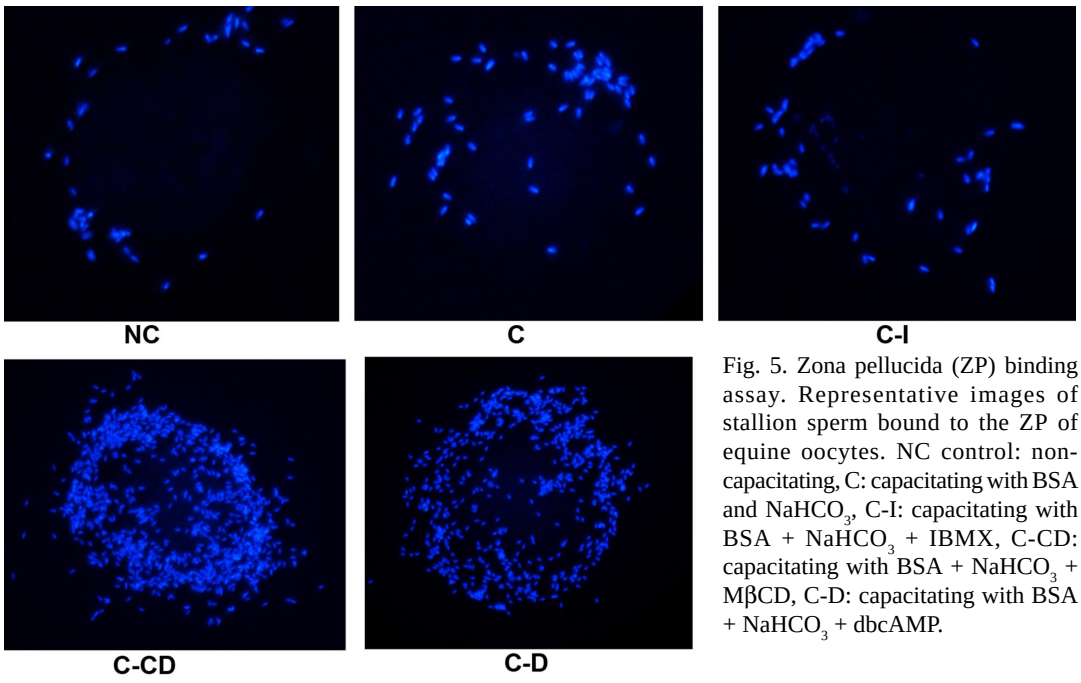

Fig. 5. Zona pellucida (ZP) binding assay. Representative images of stallion sperm bound to the ZP of equine oocytes. NC control: non-capacitating, C: capacitating with BSA and $NaHCO_3$, C-I: capacitating with BSA + $NaHCO_3$ + IBMX, C-CD: capacitating with BSA + $NaHCO_3$ + MβCD, C-D: capacitating with BSA + $NaHCO_3$ + dbcAMP.

DISCUSSION

Failure of *in vitro* capacitation of stallion sperm has been mentioned as a major limiting factor in the development of conventional IVF in the horse. Although previous studies have reported successful induction of sperm-zona pellucida interaction between equine gametes (Macías-García *et al*., 2015), media developed to support gamete interaction have not yielded repeatable IVF success (Mugnier *et al*., 2009).

In our study, we evaluated different capacitation conditions and inducers, analyzing the data both individually for each stallion and by pooling the sires. We observed that each stallion responded differently to the treatments, a phenomenon widely reported in the literature, where stallion sperm often exhibit variable behavior when analyzed separately. Stallion sperm are particularly variable in their resistance to freeze-thaw processes, especially regarding sperm survival post-thawing (Hoffmann *et al*., 2011). Despite this variability, when frozen-thawed semen was incubated with different capacitation inducers and analyzed both individually and in pooled data, we found no significant differences between the control and capacitation media.

A significant population of spermatozoa undergoing acrosome reaction was observed after two hours of incubation in both non-capacitating and capacitating media. Notably, different sensitivity to this process was observed among the stallions evaluated, with stallion A exhibiting a higher percentage of spermatozoa with reacted acrosomes compared to others, including a considerable proportion in the non-capacitating medium, which lacks bicarbonate and BSA. Previous studies have confirmed that cryopreserved semen often shows reduced sperm motility and fertility, with notable differences observed between individual stallions (Bedford *et al*., 1995). Pommer *et al*. (2003) suggested that sperm longevity might be compromised by premature capacitation-related changes associated with sperm storage, indicating that cryopreserved sperm may be more sensitive to stimuli, which could explain their shorter lifespan compared to fresh sperm. Furthermore, signs of premature capacitation associated with cryopreservation have been demonstrated in both bovine and equine sperm (Cormier *et al*., 1997).

For the capacitation process to occur, sperm must be maintained in an environment containing HCO_3-, Ca^{2+}, and BSA (Maitan *et al*., 2021). Additionally, agents such as calcium ionophore A23187, progesterone, or heparin are employed to induce the acrosome reaction (Varner *et al*., 1987). In our study, we used media containing HCO_3-, and BSA, supplemented single or combined with capacitation inducers: dbcAMP as a PKA activator, the phosphodiesterase inhibitor IBMX, and the cholesterol acceptor MβCD. Our data showed that after two hours of incubation in different capacitating media, there were similar levels of acrosome integrity, plasma membrane fluidity, and i(Ca^{2+}) levels, among groups. Similarly, when the data were pooled across all stallions, no significant differences were observed. Fuentes *et al*. (2024), working with fresh semen obtained promising results, showing an early response to tyrosine phosphorylation in the sperm within 4 hours after incubation in capacitating conditions including inducers of capacitation. In contrast to this data, our study employing cryopreserved semen, failed to show an increase in plasma membrane fluidity and increase of intracellular calcium levels. In a different study by Contreras *et al*. (2022), stallion sperm was cryopreserved with membrane stabilizers (cholesterol-loaded cyclodextrin and cholestanol-loaded cyclodextrin) after 4 hours of post-thaw incubation in capacitating conditions.

These findings suggest that the capacitation treatments used in this study did not produce the expected changes in sperm function parameters within the two-hour incubation period. Specifically, the lack of significant alterations in plasma membrane fluidity and intracellular calcium levels indicates that the spermatozoa did not undergo the typical capacitation-related modifications, such as increased membrane fluidity and calcium influx, which are essential for successful fertilization. One possible explanation for these results is that the cryopreserved semen may have a reduced capacity to respond to capacitating stimuli due to cryopreservation-induced damage or altered membrane properties. This is supported by the study of Fuentes *et al*. (2024), which demonstrated that fresh semen responded more readily to capacitation inducers, showing noticeable changes in tyrosine phosphorylation and membrane fluidity within 2 hours, an effect not observed in our study using cryopreserved semen.

On the other hand, we employed a zona pellucida (ZP) binding assay, based on earlier studies that demonstrated enhanced acrosomal exocytosis in response to media containing capacitation inducers compared to non-capacitating conditions. This technique, previously used to assess the capacitation status of stallion sperm by Coutinho da Silva *et al*. (2012), differs in our study as we used mare oocytes. In our assay, we observed a significant increase in the number of sperm bound to the ZP of mare oocytes under capacitating conditions, a result similar to that reported by Fuentes *et al*. (2024) with fresh semen.

Interestingly, the average number of stallion sperm bound to mare oocytes was particularly high with a capacitating medium supplemented with MβCD. The results

from Fuentes *et al*. (2024), provide evidence of the effects of HCO_3-, BSA, dbcAMP, IBMX, and MβCD on capacitation-related parameters in equine sperm, which could be valuable in future research aimed at reducing the time required for sperm capacitation in IVF protocols, as compared to those reported by Felix *et al*. (2022). Our findings are consistent with theirs, showing that the medium containing MβCD produced a significant increase in sperm binding compared to both the non-capacitating medium and the capacitating media without inducers.

The observed increase in the number of spermatozoa bound to mare oocytes with the addition of MβCD suggests that this inducer could be a viable option for improving *in vitro* fertilization with frozen spermatozoa. It is important to note that our study utilized thawed spermatozoa, which are known to suffer from cryopreservation-induced damage. Cryopreservation has been shown to cause sperm DNA fragmentation (Baumber *et al*., 2000), translocation of phosphatidylserine, decreased mitochondrial membrane potential, acrosome damage (Thomas *et al*., 2006), and increased intracellular ROS generation. Specifically, stallion sperm generates superoxide anions (O_2-), which rapidly convert to hydrogen peroxide (H_2O_2), a ROS with significant cytotoxic effects (Burnaugh *et al*., 2007).

CONCLUSIONS

Our results showed that the capacitation conditions evaluated in freeze-thawed stallion sperm did not support capacitation-like changes in stallion sperm after 120 min incubations in basal capacitation medium containing capacitation inducers. Interestingly, basal capacitation conditions containing bicarbonate and BSA were not sufficient to achieve binding to the ZP. In fact, MbCD was crucial to improve the interaction of the sperm with the eggs, confirming the important role of this inducer in *in vitro* fertilization.

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RESUMEN: Los espermatozoides congelados-descongelados de diferentes especies de mamíferos experimentan con éxito la capacitación *in vitro* en condiciones específicas y definidas, sin embargo, la inducción de la capacitación *in vitro* de espermatozoides equinos aún requiere optimizaciones. El objetivo de este estudio fue evaluar los efectos de diferentes medios de cultivo en la inducción de la capacitación *in vitro* de espermatozoides de semental descongelados. Para ello, los espermatozoides se descongelaron e incubaron con diferentes combinaciones de inductores capacitantes durante 2 h. En nuestro estudio se utilizaron cinco medios diferentes: medio sin capacitación (NC), medio basal que incluía BSA y bicarbonato, medio basal más el inductor capacitante dibutiril AMP cíclico (dbAMPc) como activador de PKA, un inhibidor de la fosfodiesterasa, 3-isobutil-1-metilxantina (IBMX) y un aceptor de colesterol, metil-b-ciclodextrina (MβCD). Se evaluaron la fluidez de la membrana, la reacción acrosómica y los niveles de calcio intracelular después de 2 h de incubación, pero no encontramos diferencias entre los grupos. También se observó un comportamiento diferente entre los sementales. Finalmente, los tratamientos de los espermatozoides se evaluaron utilizando un ensayo de unión de homólogos a la zona pelúcida. Este ensayo mostró que la incubación en medio basal que incluía MβCD aumentó el número de espermatozoides adheridos a la zona pelúcida de los ovocitos de yegua. Se necesitan más estudios para evaluar el efecto de estas condiciones en la fertilización *in vitro* de especies equinas utilizando espermatozoides descongelados.

PALABRAS CLAVE: Capacitación; Espermatozoide; Semental; Zona pelúcida.

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