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Capacitation and Acrosomal Reaction Parameters of Fresh and Cryopreserved Rooster Semen

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ABSTRACT

Background: Semen cryopreservation induces alterations in spermatozoa and changes in the plasma membrane, limiting the acrosome reaction and fertilizing ability. The research aim was to study the sperm capacitation indicators and acrosome reaction capacity in rooster semen, comparing fresh semen and post-thaw semen.

Methods: Fifty ejaculates were obtained from five roosters through dorsal-ventral massage. Semen was diluted and preserved fresh or cryopreserved in Beltsville Poultry Semen Extender. In both groups of preserved semen, basic seminal parameters, capacitation and acrosome reaction were assessed. The latter were evaluated both with and without a perivitelline layer as the acrosome reaction inducer, using chlortetracycline staining.

Result: The results revealed a higher percentage of sperm progressive motility in fresh semen (85.2±1.5%) compared with thawed semen (44.8±1.7%; P<0.05). The percentages were similar when comparing sperm with an acrosome reaction in fresh semen (10.6±1.1%) to sperm incubated without a perivitelline layer (13.2±1.7%; P>0.05). However, when incubated with a perivitelline layer for 40 minutes, fresh semen spermatozoa exhibited a higher percentage of acrosome reaction (59.2±2.1%) compared with thawed semen incubated with a perivitelline layer (40.6±2.3%; P<0.05).

Key words: Chlortetracycline, Cryopreservation, Fertility, Membrane, Rooster.

INTRODUCTION

Assisted reproduction technology in birds has contributed to the development of the poultry industry by enabling the conservation of genetic resources from roosters (*Gallus gallus)* (Łukaszewicz *et al*., 2020; Partyka and Niżañski, 2022). Nonetheless, cryopreservation of rooster sperm has shown apoptotic changes (Miranda *et al*., 2017), oxidative stress damage to mitochondrial function and decreased plasma membrane integrity (Cruz *et al*., 2023). Seminal conservation of fresh semen and freezing process induces changes in the proportion and distribution of carbohydrates associated with the gamete-recognition capacity in sperm plasma membrane (Peláez *et al*., 2011). These changes due to the freezing process may limit the sperms' capacity for acrosome reaction (AR), which occurs when the spermatozoon interacts with the perivitelline layer (PVL), thus limiting the sperm fertilizing ability (Camarillo *et al*., 2019).

In vitro studies suggest that a 40-min capacitating period is necessary for plasma membrane changes in rooster sperm to occur (Asano and Tajima, 2017), including carbohydrate and integral and peripheral glycoprotein distributions, cholesterol reduction, phospholipid composition and increased permeability in the acrosome region, inducing an increase of plasma membrane fluidity (Johnson *et al*., 2014), culminating in AR. Molecules such as progesterone may induce spermatic capacitation (SC) and AR by interacting with receptors in the sperm membrane and causing Ca^{2+} influx into the cell. Calcium channels are activated upon first contact of the spermatozoon with the PVL through the G protein-receptors stimulating phospholipase C. The latter, together with intracellular Ca^{2+}, alters the acrosome membrane and causes AR (Tulsiani and Abou-Haila, 2011).

The fluorescent antibiotic chlortetracycline (CTC) has been used to determine the *in vitro* SC and AR of sperm from different avian species (Ochoa *et al*., 2014; Herrera *et al*., 2017). The three characteristic CTC staining patterns have been defined depending on the Ca^{2+} distribution in the sperm cell that can aid in the identification of intact sperm cell populations or populations without capacitation, with SC and AR (Ibarra *et al*., 2020).

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Cryopreservation studies in poultry have shown the sperm membrane alterations can also alter its fertilizing capacity (Long *et al*., 2014; Camarillo *et al*., 2019). These changes in the membrane are similar that occur during semen capacitation. The aim of this study was to determine in rooster semen, capacitation and acrosome reaction indicators of sperm preserved in fresh and post thaw.

MATERIALS AND METHODS

The experimental birds were managed according to 062-ZOO-1999 Mexican Official Standard protocols for animal care and welfare. For the study, from February to May of 2022, 50 semen ejaculates were obtained by dorsal-ventral massage technique (González *et al*., 2019).from five adult Lohmann Brown roosters housed at the experimental farm of the Autonomous University of Chapingo, Estate of Mexico. The roosters were provided with a diet and water ad libitum and subjected to a photoperiod of 13 hours of light and 11 hours of darkness.

The ejaculates obtained were conserved and evaluated in the Reproduction Biochemistry Laboratory of the Xochimilco Metropolitan Autonomous University; CDMX.

The volume of the ejaculate was estimated and placed in 500 µL of Beltsville Poultry Semen Extender (BPSE), as described by Sexton (1977) and then stored at 5°C for 10 minutes. From each sample, 50% of the volume of the diluted semen was used for fresh semen evaluation, while the remainder was frozen for 30 days until assessment.

For freezing, semen aliquots containing 100×10^6 sperm were prepared with BPSE with 64.1 M dimethylsulfóxide adjusting to in 0.25 ml straws. The samples were cooled at a rate of -5°C/minute, equilibrated for one minute at 5°C and then frozen for 10 minutes in liquid nitrogen vapors at 3 cm above from the surface. The aliquots were then immersed in liquid nitrogen for at least 30 days before thawing, which was performed at 37°C for 30 sec for further seminal analysis (Camarillo *et al*., 2019).

The percentage of sperm with progressive rectilinear movement in a 10 µL sample was estimated using an optical microscope. The sperm concentration was quantified using a Neubauer chamber, utilizing a 10 µL sample. Additionally, 10-µL aliquots of the sample were stained with eosin and nigrosin for live-dead estimation. For each sample, 100 spermatozoa were analyzed using an optical microscope with a 100× objective, to assess the viability and morphology of the sperm (Blanco *et al*., 2012).

The patterns of SC, intact sperm (IS) and sperm with an AR were determined in both the fresh semen (FS) and thawed semen (TS) by making aliquots of 5×10^6 sperm that were then incubated with 0.9 mM CTC at 38°C in a dark environment according to Ochoa *et al*. (2014), in all conditions to preserved and post thaw, with and without PVL 25 µg/ ml for 30 min (Camarillo *et al*., 2019).

Experiments

1) In fresh semen just ejaculated and in thawed semen, the IS, SC and with AR parameters were determined.
2) The proportion to IS, CS and sperm with AR was determined in semen fresh and thaw after 5, 10, 20, 30 and 40 min of incubation with CTC and with and without PVL.

After the incubation of both experiments, the samples were placed on slides and studied under a fluorescence microscope with a 100x objective using 488-nm excitation and emission wavelengths >560 nm (Fig 1). In each evaluation, a total of 200 spermatozoa were counted and in agreement with the fluorescence pattern the proportion of IS CS and sperm with AR were determined (Ochoa *et al*., 2014; Herrera *et al*., 2017).

Analysis of variance (ANOVA) was conducted to identify the differences between fresh and thawed semen and to discern differences in parameters across various storage times. The significance of these differences was determined using a Tukey test with a p-value <0.05.

RESULTS AND DISCUSSION

The basic assessment parameters (Table 1) showed an average sperm concentration of 579.2×10^6 sperm/mL in the rooster ejaculate. A decrease of approximately 50% in sperm motility was observed in the TS ($P<0.05$). The percentage of viable sperm and of sperm with morphological alterations showed no significant differences between the FS and TS conditions ($P>0.05$).

The variance analysis showed an effect ($P<0.05$) on the percentage of IS in the interaction between the type of semen (FS and TS) and the experimental condition. In the semen assessment prior to incubation, a higher percentage of IS was observed in fresh semen (70.4± 11.21%) as compared to thawed semen (57.2±7.51%; $P<0.05$), while the incubation without PVL for 40 min did not induce changes in the FS and TS (Fig 2A). Nonetheless, following the 40-min incubation with PVL, the IS decreased in both FS and TS ($P<0.05$) but without any significant difference between FS and TS ($P>0.05$). Fig 2B shows non-significant differences ($P>0.05$) in the percentage of SC in the FS and TS without incubation and incubated with and without PVL. Among the three treatments, the TS showed approximately

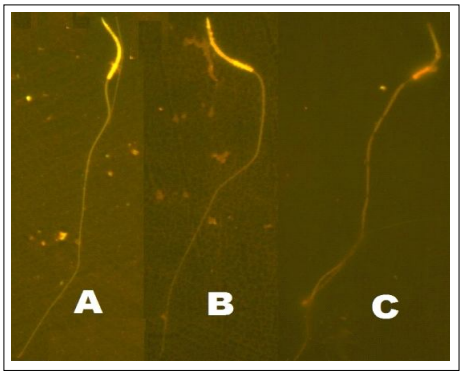


Fig 1: Patterns of fluorescence for sperm stained with chlortetracycline: A) Intact, B) Capacitated and C) With acrosome reaction.

Table 1: Evaluation parameters of fresh and post-thaw semen.

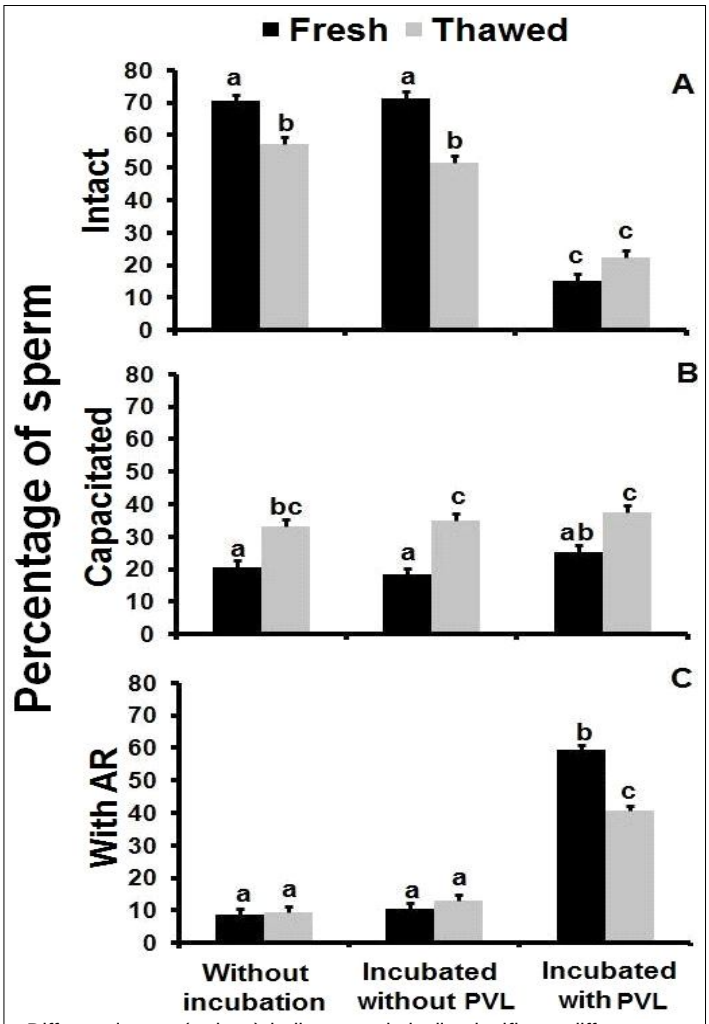
	Fresh Medians±SD	Thawed Medians±SD
N	25	25
Motility %	85.2±9.7[a]	44.8±8.8[b]
Live sperm %	91.4±10.3	85.8±4.4
Normal morphology %	91.7±7.0	80.8±4.9
Abnormal morphology %	8.9±6.4	9.6±4.8
Abnormality in the nucleus %	3.2±1.9	3.2±1.4
Abnormality in the neck %	2.9±3.2	3.0±2.0
Abnormality in the tail %	2.8±2.19	3.4±1.9

*Values represent the mean±standard deviation. [a, b]Different superscripts indicate statistically significant differences (P<0.05).

one third higher in the proportion of sperm with an SC fluorescence pattern compared with FS (P<0.05).

Nonetheless, when incubated with PVL (Fig 2C), a significant increase (P>0.05) in the percentage of sperm with AR pattern was observed for both fresh and thawed semen. It is important to note that FS incubated with PVL showed a higher proportion in the fluorescence pattern of AR (59.3±2.1%) than TS (40.4±2.3%; P<0.05).

The percentage of IS decreased (P<0.05) progressively after 5 minutes of incubation in both FS and TS (Fig 3A). The percentage of SC was increased in FS after 5 minutes of incubation with PVL compared with the control getting its higher proportion after 10 minutes of incubation. However, SC decreased gradually, reaching its lowest percentage at



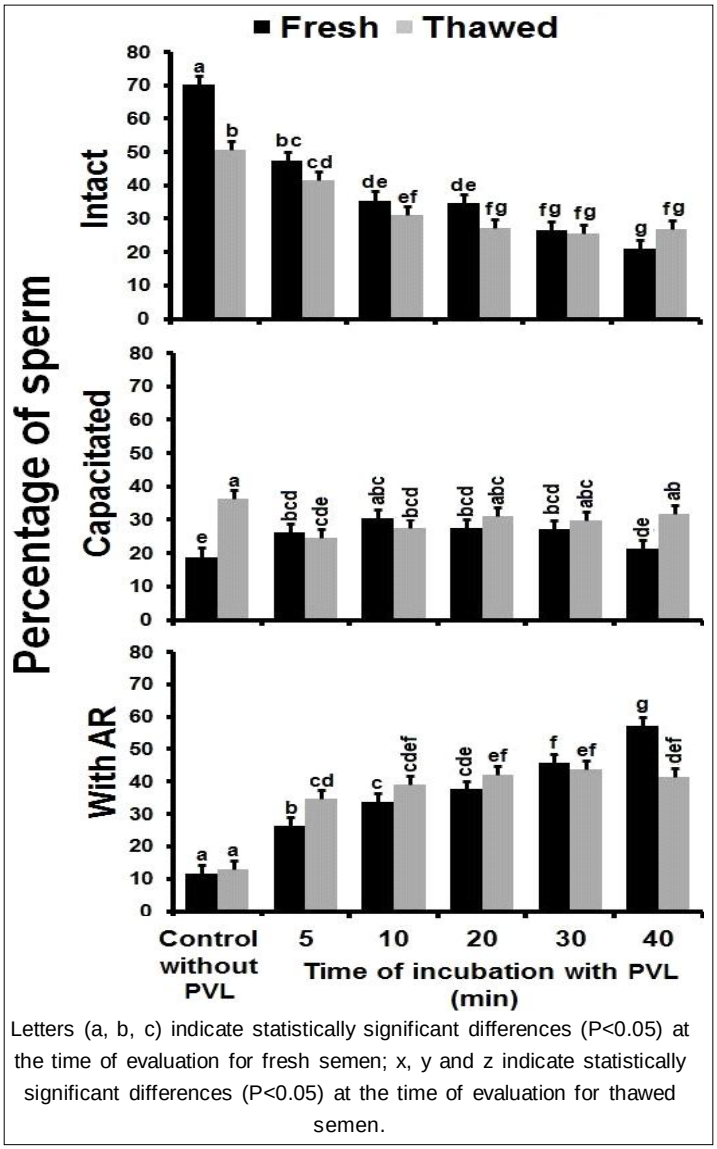
Different letters (a, b, c) indicate statistically significant differences (P<0.05) at the time of evaluation (fresh or thawed) or by evaluation condition (without incubation, incubated without PVL or incubated with PVL).

Fig 2: Percentage of intact sperm (A), capacitated sperm (B) and sperm with acrosome reaction (C) evaluated in fresh and thawed semen without incubation and following incubated with and without perivitelline membrane (PVL).

40 min. In the case of TS, non-significant changes were observed throughout the incubation with PVL (Fig 3B). An increase in the percentage of sperm with PVL induced AR was observed in FS after 5 minutes; in contrast, TS showed an abrupt increase in the percentage of sperm with AR after 5 minutes of incubation that remained unchanged for the remainder of the incubation (Fig 3C).

This study showed that cryopreservation of rooster semen induces changes in the sperm membrane, causing a SC and the AR. Prior to incubation, a 20% lower IS was observed in TS compared with FS. Similarly, independent of treatment, TS had a higher percentage of SC than FS; these results agree with other mammalian sperm studies showing a decreased IS percentage and an increased capacitation percentage (Yánez *et al*., 2022). This finding suggests that our report of high percentages of sperm with capacitation in TS is due to damage to the membrane integrity caused by the freezing process leading to a fluorescence pattern that of CS (Benko *et al*., 2022).

The different percentages of sperm with patterns of CTC staining were found for FS and TS across the incubation period. Although there was a linear reduction in the percentage of IS in both FS and TS over time, a linear increase in the percentage of sperm with AR was observed only in FS. In contrast, an increase was observed between 5 and 10 minutes of incubation in TS that remained unchanged for the remainder of the incubation. In accordance with other authors regarding the damage from buffalo sperm cryopreservation on the permeability of the plasma membrane (Kadirvel *et al*., 2012), the cryopreservation can cause an atypical influx of Ca^{2+} to the cytosol, leading to a process of AR and may decrease the fertilization capacity of the sperm.



Letters (a, b, c) indicate statistically significant differences (P<0.05) at the time of evaluation for fresh semen; x, y and z indicate statistically significant differences (P<0.05) at the time of evaluation for thawed semen.

Fig 3: Percentages of sperm in different conditions and times of incubation that were intact (A), capacitated (B) or showing acrosome reaction (C).

Additionally, FS had 18 % higher percentage of AR than TS after 40 minutes of incubation with PVL. However, other authors have reported a 50% decrease in the percentage of sperm with PVL-induced AR between FS and TS of roosters (Svoradova *et al*., 2021). Studies have reported that cryopreservation modifies the sperms capacity to undergo AR due to failure in cAMP production, Ca^{2+} motility, oxidative stress, or changes in the proportions of cholesterol and phospholipids in the membranes prior to AR (Benko *et al*., 2022). For instance, bovine TS shows lower cAMP and higher free cytosolic Ca^{2+} concentrations compared with FS (Zhang *et al*., 2022).

These results explain our findings regarding the differences in the percentage of sperm with AR in FS and TS. The *in vitro* PVL induced AR is a good indicator for the *in vivo* fertilizing potential of spermatozoa (Lemoine *et al*., 2011). However, in TS, AR detected with a CTC stain could be due to a damaged membrane, risks the fertilizing capacity of such sperm. Our results support those of previous studies showing that incubation with PVL causes AR in rooster sperm.

CONCLUSION

It is concluded that the cryo capacitation and the acrosomal reaction induced *in vitro* are positive indicators of its fertilizing capacity after thawing.

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Conflict of interest

The authors declare that there is no conflict of interest.

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