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Decapacitating effect of oviductal proteins in rooster spermatozoa *in vitro*

Efecto descapacitante de proteínas oviductales en espermatozoides de gallo *in vitro*

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ABSTRACT

In birds, the process of sperm maturation has not been fully described. The objective of this study was to determine *in vitro* parameters for the decapacitating effect of oviductal proteins on rooster spermatozoa. Aliquots with spermatozoon were incubated to *in vitro* induce metabolic states: capacitation, decapacitation, and the acrosomal reaction to again determined percentages of live spermatozoa, motility, and concentrations of Malondialdehyde, reduced Glutathione and Adenosine triphosphate. The percentages of motility and live sperm in fresh and capacitated semen were similar (P>0.05), but higher (P<0.05) than those determined in decapacitated semen and with acrosome reaction. The determination of nmol/ml of MDA in fresh semen (1.59) was higher (P<0.005) than in capacitated semen (1.05), with acrosomal reaction (1.07) and decapacitated semen (1.05). GSH nmol/ml were similar (P>0.05) in fresh (72.6) and capacitated (62.04) semen, similarly (P<0.05) between acrosome reaction (99.09) and decapacitated (86.07) semen. The highest concentration of ATP was in capacitated semen with 69.9 µmol/ml, with similar concentrations (P<0.05) in fresh, with acrosomal reaction, and decapacitated semen. The parameters, determined, demonstrated that the protein fraction of the utero-vaginal junction, *in vitro*, produces decapacitation and successfully maintains sperm viability.

Keywords: adenosine triphosphate, reduced glutathione, malondialdehyde, oxidation, semen.

RESUMEN

En las aves, no se ha descrito totalmente el proceso de maduración espermática. El objetivo del estudio fue determinar parámetros *in vitro*, del efecto descapacitante de las proteínas oviductales, en espermatozoides de gallo. Alícuotas con espermatozoides, fueron incubadas *in vitro* para inducir estados metabólicos de capacitación, descapacitación y reacción acrosomal para determinar porcentajes de espermatozoides vivos, su movilidad y concentraciones de Malondialdehído, Glutation reducido y Adenosin tri fosfato, como parámetros del estado metabólico de los espermatozoides. Los resultados mostraron

porcentajes de movilidad y espermatozoides vivos en semen fresco y capacitado similares (P>0.05), pero mayores (P<0.05) a los determinados en espermatozoides con reacción acrosomal. Los nmol/ml de MDA en semen fresco (1.59) fue mayor (P<0.005) que en semen capacitado (1.05), con reacción acrosomal (1.07) y en semen descapacitado (1.05). Los nmol/ml de GSG fueron similares (P>0.05) en semen fresco (72.6) y capacitado (62.04), y entre semen con reacción acrosomal (99.09) y descapacitado (86.07). La mayor concentración de ATP fue en semen capacitado con 69.9 µmol/ml, con concentraciones similares (P<0.05) en semen fresco, con reacción acrosomal y descapacitados. Los parámetros determinados, demostraron que la fracción proteica de la unión útero-vaginal, *in vitro* produce descapacitación y mantiene la viabilidad espermática.
Palabras clave: adenosin trifosfato, glutation reducido, malondialdehido, oxidación, semen

INTRODUCTION

Birds present specific morphophysiological reproductive characteristics that are distinct from those of mammals. In the rooster's reproductive tract, these include the absence of accessory glands that supply fluids to the ejaculate (Álvarez *et al*., 2020), and of a structure like the mammal's epididymis, which participates in spermatic maturation and storage (Asano & Tajima, 2017). There are reports that rooster semen "does not require spermatic capacitation" to achieve fertilizing capacity (Lemoine *et al*., 2011), but a separate study identified a necessary period of spermatic capacitation of 40 minutes duration that can be induced *in vitro* using a medium enriched with Ca^{2+}.

Spermatozoa storage tubules (SST) have been identified in the junction uterus vaginal (JUV) in hen's oviduct, that contribute to sperm decapacitation for storage by metabolizing fatty acids or other lipids by the sperm (Long & Conn, 2012; Sasanami, 2013); recent research has demonstrated an *in vitro* decapacitating effect of the oviductal proteins, but has not described any characteristic parameters of spermatic metabolism (Camarillo *et al*., 2019). Biochemical changes and modifications to cell membrane fluidity occur during capacitation sperm, in addition to intercellular pH shifts, increased permeability to Ca^{2+} ions, modification of protein phosphorylation patterns and lipid composition. Sperm cell capacitation is a pre-requisite for the acrosome reaction in order to attain their fertilizing ability. In the reproductive tract of avian, the SST have been described, in the JUV, the cellular secretions of these structures can induce decapacitation of spermatozoa for storage (Sasanami, 2013; Camarillo *et al*., 2019; González *et al*., 2019).

These fatty acids, which can be obtained in the birds' diet (Olubowale *et al*., 2014; Ashraf *et al*., 2020) contribute to maintaining spermatozoa viability and guaranteeing fertilization of the oocytes for up to seven days after copulation (Bakst, 2010).

Studies have not revealed any differences in spermatozoa and their morphometry as they pass through the rooster's reproductive tract but, in contrast, there are extensive descriptions of the glandular secretions in the hen's oviduct, associated primarily with the formation of the egg (Zhong *et al*., 2020). However, spermatozoa must complete their transit through this tract to reach the fertilization site (Álvarez *et al*., 2017). There are very

few studies concerning the conditions of the hen's reproductive tract in relation to the storage, maturation, and activation of spermatozoa.

Reproductive failure can be associated up to 50% with males; it is known that oxidative stress determined by an increase in Malondialdehyde (MDA) in the semen, can be associated with damage of the axoneme, produce morphological alterations in the midpiece and decreased sperm motility (Kurkowska *et al*., 2020); glycolysis and mitochondrial oxidation contribute to adenosine triphosphate (ATP) production via oxidative phosphorylation for the maintenance of flagellar motility in sperm cook (Setiawan *et al*., 2021) and the positive effect of glutathione (GSH) has also been shown to protect spermatozoa from oxidative stress (Masoudi *et al*., 2019).

While the rooster's reproductive efficacy is well-known due to its adequate percentages of fertility (Nakamura, 2017), implementing artificial insemination on production farms can reduce production costs by lowering the number of roosters required. Moreover, semen could be transported to various stations or locations under conditions of greater biosecurity that ensure the conservation of the germplasm of primary grandfather and great-grandfather germinal lines (Nakamura, 2017). To date, artificial insemination has utilized two means of seminal conservation, called the Beltsville and Lake Poultry Semen Extenders, which were developed over 40 years ago mainly for use with turkeys. The only subsequent work conducted with these two methods has been designed to modify the concentration of their components, thus reflecting the modest advances in our knowledge of the reproductive biology of this species and the scarce utilization of that knowledge for biotechnological development (Asano & Tajima, 2017).

Against this background, the aim of the present study was to determine *in vitro* parameters for the decapacitating effect of the hen's oviductal proteins on rooster sperm. This research will contribute to developing new means of semen conservation that will increase the viability of spermatozoa during *in vitro* handling and storage.

MATERIALS AND METHODS

Experiment

Fresh semen, in each metabolic state, basic evaluation and measurement of Malondialdehyde (MDA), reduced glutathione (GSH) was performed to confirm its membrane function and ATP levels to verify energetic activity; With this was evaluations the viability of spermatic and parameters to sperm maturity and decapacitation.

Use of animals

The study complied fully with the animals' nutritional requirements by offering commercial feed with 17 % crude protein, that supplies the nutrients required for breeding birds for zootechnical purposes; the animals were maintained with water *ad libitum*. They were housed individually in cages of 90 X 90X 120 cm, with a perch height of 90 cm (Applegate & Angel, 2014). Adequate housing conditions were provided throughout the study. The study subjects were five roosters of the Lohman breed. To obtain fluids from the oviduct,

10 hens in the second third of posture were utilized, housed individually in similar conditions.

Obtaining semen

Ejaculates were obtained by dorsoventral massage performed three times per week on each rooster (Camarillo *et al*., 2019). Ejaculates were combined to form a total of 25 pool semen, depositing in the same vial the semen of the five roosters. The semen was collected from the cloaca by aspiration using an SL10-1000 micropipette (RANIN™, USA). Each semen pool was then mixed with Lake medium containing 0.6% fructose, 1.92% sodium glutamate, 0.08% magnesium acetate, 0.51% sodium acetate, and 0.128% potassium citrate, pH 7.2 and 330 mOms. Only ejaculates that satisfy the normal seminal criteria: 5% motility, 90% live sperm, and < 5% abnormal sperm were used (Fattah *et al*., 2017).

Collection and quantification of the protein fraction of the oviductal fluid

The cloaca was everted to insert a 12-caliber probe to a depth of 3 cm in the oviduct and deposit 3 ml of Lake medium in the uterovaginal union (UVU), followed by the collection of approximately 1.54 ml of liquid from the oviduct to form a pool. The fluid extracted from the 10 hens was maintained at 2°C for handling (Ito *et al.,* 2011). It was filtered through a 70 µm cell strainer and then centrifuged for 30 minutes at 1500 x g. The supernatant was stored at -20ºC until use (Sedaghat *et al*., 2021). The protein fraction from the UVU was precipitated using 4 volumes of acetone at 2°C for 30 min, followed by centrifugation at 14000 x g for 10 minutes. The sediment was recovered by evaporating the supernatant in liquid nitrogen (Sedaghat *et al*., 2021). The spectrophotometry technique was utilized to quantify the protein concentration, at a wavelength of 595 nm (Ku *et al.,* 2013).

To describe the protein fraction by unidimensional electrophoresis, 20 µg of the protein obtained were placed in a buffer of the sample (Tris 0.5 M at pH 6.8, SDS 10%, glycerol, 0.5% bromophenol blue, and 5% β-mercaptoethanol) and maintained at 100°C for 3 min. Electrophoresis required preparing a separation gel at 10% and a compaction gel at 4% with 30% acrylamide/Bis at 37.5:1 (2.6% C). Separation was conducted in an electrophoresis chamber at 200 volts for 45 min. The buffer solution of the electrode was prepared with 0.025 M Tris, 0.192 M glycine, and SDS at 0.1% (p/v) to a pH of 8.3 (Sajjadi *et al*., 2019).

Induction of metabolic states in the spermatozoa

Aliquots of $200X10^6$ spermatozoa in 1 ml of Lake medium were used in each metabolic condition; fresh semen was evaluated before 10 min post-ejaculation had elapsed (Camarillo *et al*., 2019). Capacitation spermatozoa were induced by incubating the semen 1:1 in Lake medium for 40 min at 38ºC.

To induce decapacitation, spermatozoa were incubated for 40 min at 37.5°C, and then diluted 1:1 in Lake medium supplemented with 200 µg/ml of the protein fraction from the UVU under darkness condition (Camarillo *et al.*, 2019). The acrosomal reaction was determined in spermatozoa that were co-incubated with 20 µg of PVL to induce the acrosome reaction (Lemoine *et al*., 2011; Camarillo *et al*., 2019).

Basic sperm evaluation

The percentage of sperm with straight progressive motility in 15 µl semen was estimated by microscopy (OLYMPUS BX51) with a 40X objective at 37.5°C. In addition, 10 µL of semen was stained with eosin-nigrosine (QCA, 996518, USA) and 100 spermatozoa from each sample were analyzed to assess viability and morphology under an optical microscope (OLYMPUS BX51) (Jabbar *et al.*, 2015; Fischer *et al*., 2015).

Metabolic parameters of the spermatozoa

Malondialdehyde (MDA). The nMol concentration of MDA/ml was quantified (Najafi *et al*., 2021). In this case, 1 ml of trichloroacetic acid at 20% was added to one aliquot of semen with $100X10^6$ spermatozoa and centrifuged for 15 min1500 x g. The supernatant was removed and 1 ml of thiobarbituric acid (TBA) at 0.67% was added for incubation at 100°C for 10 minutes. Under conditions of low pH and high temperature, MDA reacts with TBA to generate an MDA-TBA adduct that can be read in a spectrophotometer at 532nm, utilizing butanol as the target.

Reduced glutathione (GSH). To determine the GSH concentration, a fluorescence reaction and spectrofluorometry were utilized *(*Najafi *et al*., 2021*)*. Aliquots of 250 µl of ejaculated semen cointaining $200X10^6$ were homogenized in a vortex for 3 min to lyse the spermatozoa. Next, 3.70 ml of phosphate buffer at pH 8 were added, followed by 1 ml of metaphosphoric acid at 25%. This was centrifuged at 1500 x g. for 30 minutes at 4ºC to release the GSH. This allowed the recovery of 0.5 ml of supernatant, which was adjusted with 4.5 ml of phosphate buffer to pH 8. After that, 100 µL of the previous mixture were recovered and 1.8 mL of phosphate buffer at pH 8 were added. This was mixed in a vortex, and 100 µL of OPT at 0.1% were added to homogenize and stabilize the mixture for 15 min under conditions of darkness. The determination was performed in a spectrofluorometer at an emission rate of 420 nm, excitation of 350 nm, and 5 SLIT for 5 seconds.

Adenosine triphosphate (ATP). Ascertaining the concentration of ATP in the spermatozoa required the Adenosine 5´-triphosphate Bioluminescence Assay Kit (ATP) (SIGMA Life Science), which determined that the luminous emission was directly proportional to the amount of ATP present (Nguyen *et al*., 2016). This was quantified in a

Perkin Elmer LS spectrofluorometer using the concentration application. An aliquot of semen with 200X10^6 spermatozoa was lysed to recover 50 µl of the lysis, which were placed in a spectrophotometer cell with 50µL of reagent from the ATP Bioluminescence Assay Kit. The spectrophotometer determined the concentration of ATP in each sample and compared it to the intensity emitted by the standard solution of 100 micromoles of ATP that the kit contains.

Statistical analyses

The results were subjected to the goodness test and the Jaque-Bera fit, to verify the normality of the data; for which the analysis of variance (ANOVA) was applied to determine differences between the variables ($P<0.05$). A Tukey test was used to determine differences between the mean and the values of the variables in the different metabolic states. All statistical analyses were performed with the free-access PAST software (Hammer *et al*., 2001).

RESULTS AND DISCUSSION

Basic evaluation parameters. The percentages of spermatic motility (Table 1) were similar ($P>0.05$) in the fresh semen and in the capacitated spermatozoa at 96.8 and 91.5%, respectively. These percentages were higher ($P<0.05$) than those determined for the spermatozoa with the acrosomal reaction (61%), and the decapacitated spermatozoa (40%). Results for the latter two were similar ($P>0.05$).

Regarding the decapacitated spermatozoa and those with the acrosomal reaction, although motility was evident and similar, it was lower than in the other metabolic states. However, the percentages of live spermatozoa demonstrated spermatic viability in all the conditions evaluated.

In the absence of an estral cycle characteristic of mammals, which synchronizes copulation with ovulation, reproduction in birds depends on sperm storage in the female's oviduct (Bakst, 2010) to ensure that spermatozoa with fertilizing capacity will be available when the oocyte is present for fertilization in the infundibulum. In the presence of tubules sperm storage, the spermatozoa deposited in the female's reproductive apparatus can survive for 2-15 weeks in domestic birds like chicken and turkey hens (Sasanami, 2013).

The percentages of live spermatozoa (Table 1) were similar ($P>0.05$) between the fresh semen (97.6%) and the capacitated spermatozoa (91.6%), and significantly higher ($P<0.05$) than in the spermatozoa with the acrosomal reaction (72.9%) and the decapacitated samples (64.4%). The latter two conditions showed similar percentages ($P>0.05$).

In general, the percentages of motility and live spermatozoa determined in this study were high and similar in the ejaculated and capacitated spermatozoa. This finding concurs with reports by other authors (Restrepo *et al.*, 2016), who observed a high motility called hyperactivity in spermatic capacitation, which is characteristic of this metabolic state. Spermatic hyperactivation is also characterized by an intense, non-progressive motility with a low frequency of tail wagging.

Table 1. Parameters *in vitro* of basic evaluation spermatic in the different metabolic states

	Semen metabolic condition (X±EE) n=25			
Spermatozoa	Fresh	Capacitated	With acrosome reaction	Decapacitated
Motility %	96.8±0.4 [a]	91.5±2.4 [a]	61.0±8.8 [b]	40.0±6.5 [b]
Live %	97.6±1.4 [a]	91.6±1.6 [a]	72.9±3.4 [b]	64.4±4.2 [b]

Different literal in super index between columns, indicates statistical difference (P<0.005).
(X±EE): Average and standard error

Indicators of oxidative stress. Studies have shown that the fluidity of the membrane can be altered by modifications of such molecules as cholesterol and phospholipids, which are related to the proteins involved in gametic recognition and membrane fusion. The role of these molecules has also been demonstrated in sperm storage in tubules for spermatic storage in the UVU located in the hen's reproductive. Currently, this is understood as suggesting their effect as decapacitating factors to maintain spermatic viability *in vivo* (Sasanami, 2013; Matsuzaki & Sasanami, 2022).

The concentration of MDA (Table 2) in the fresh semen was 1.59 nmol/ml, significantly higher (P<0.05) than in the capacitated spermatozoa (1.05 nmol/ml), those with the acrosomal reaction (1.07 nmol/ml), and the decapacitated spermatozoa (1.05 nmol/ml). It is important to note that the concentrations determined for the latter three were similar (P>0.05).

The concentrations of GSH (Table 2) in the fresh semen and capacitated spermatozoa were similar at 72.6 and 62.04 nmol GSH/ml, respectively (P>0.05). This concentration in the spermatozoa with the acrosomal reaction was 99.09 nmol GSH/ml, similar (P>0.05) to that of the decapacitated spermatozoa, at 86.07 nmol GSH/ml. However, those spermatozoa maintained levels of oxidative stress, MDA, and GSH that demonstrated membrane stability (Setiawan *et al*., 2021).

During capacitation and acrosomal reaction changes occur in the membrane of sperm; our studies showed that concentrations of MDA and GSH maintain membrane integrity by associating their viability with the ATP concentration also determined in capacitated and decapacitated sperm.

In another significant finding, the energy levels determined by the concentration of ATP were similar in the capacitated spermatozoa and those with the acrosomal reaction,

illustrating the decapacitating effect *in vitro* of the protein fraction of the secretions from the UVU.

The concentrations of ATP (Table 2) in the fresh semen, capacitated spermatozoa, spermatozoa with the acrosomal reaction, and decapacitated spermatozoa were 59.41, 69.9, 66.49, and 51.79 µmol ATP/ml, respectively. These concentrations are statistically similar (P>0.05). In rooster spermatozoa, the production of ATP via glycolysis has been demonstrated (Dávila *et al*., 2015), so finding similar concentrations of ATP in the different metabolic conditions evaluated demonstrates sperm viability in our study.

The *in vitro* spermatic viability of the decapacitated spermatozoa in these study conditions was demonstrated by determining that the energy levels required by the spermatozoa during the fertilization process are similar, regardless of their activity or metabolic state, capacitation, acrosomal reaction, and/or decapacitation (Nguyen *et al.,* 2015). In addition to maintaining their viability and the percentages of live spermatozoa and those with motility, the study evidenced the *in vitro* capacity for the acrosomal reaction in the presence of PLV as a natural inducer of that reaction in decapacitated spermatozoa. This demonstrated spermatic viability, which is known to be useful *in vitro* parameter for predicting the *in vivo* fertilizing capacity of spermatozoa (Lemoine *et al*., 2011). This represents an opportunity to rescue genetic lines of zootechnical interest, with the implementation of assisted reproduction techniques in avians (Romo *et al*., 2022).

Table 2. Parameters of oxidative stress in semen with different metabolic states *in vitro*

	Semen metabolic condition (X±EE)			
	Fresh	Capacitated	With acrosome reaction	Decapacitated
Malondialdehyde (MDA) nMol/ml	1.59±0.09 ^{a}	1.12±0.08 ^{b}	1.07±0.12 ^{b}	1.05±0.02 ^{b}
Reduced glutation (GSH) nMol/ml	72.6±7.7 ^{ac}	62.04±5.1 ^{a}	99.09±3.6 ^{b}	86.07±8.1 ^{bc}
Adenosine riphosphate (ATP) µMol/ml	59.41±8.9 ^{a}	69.9±7.3 ^{a}	66.49±8.7 ^{a}	51.79±5.0 ^{a}

Different literal in super index between columns, indicates statistical difference (P<0.005)
Each average was determined with the parameters obtained from five tests performed (n=5)
(X±EE): Average and standard error

Has been reported, after the process of spermatic capacitation, motility decreases due to the limited energy of the spermatozoa (Ferramosca, 2014). In contrast, our studies demonstrated similar ATP concentrations in capacitated, decapacitated and acrosomal reaction sperm. This reveals an evident need to develop means of sperm conservation that limit or reduce metabolic energy expenditures by the spermatozoa. The important

findings of this study demonstrate a reduction of motility in the spermatozoa in which the metabolic state of decapacitation was induced.

CONCLUSIONS

The results of this study demonstrate metabolic parameters in spermatozoa that are associated with *in vitro* spermatic decapacitation induced by the protein fraction of fluid from the UVU. This protein fraction can be included as an ingredient *in vitro* spermatic preservation media to reduce metabolism and maintain sperm viability.

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Authors contribution:

CVC. Conceived the original idea of the manuscript and its realization.

GBA. Supervision and design of experiments

QSJ. Methodological proposals and their implementation

HBJ. Conceived the original idea of the manuscript and interpretation of results.

PRJ. Statistical analysis and critical review of the manuscript

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Errata Erratum

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