ORIGINAL ARTICLE

*Veterinary Research Forum. 2020; 11 (3) 207 – 211*
doi: 10.30466/vrf.2019.95854.2300
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

# *In vitro* sperm storage with poultry oviductal secretions

**Ana Karen Vargas Ibarra[1], Samantha Anahi Carcoba Pérez[1], Alejandro Avalos Rodríguez[2], Ana María Rosales Torres[2], Fernanda Rodríguez Hernández[2], Ricardo Camarillo Flores[3], José Antonio Quintana López[4], José Antonio Herrera Barragán[2]***

*[1] Metropolitan Autonomous University - X, Mexico City, Mexico; [2] Department of Agricultural and Animal Production, Metropolitan Autonomous University - X, Mexico City, Mexico; [3] Metropolitan Autonomous University - I, Mexico City, Mexico; [4] Department of Avian Medicine and Zootechnics, College of Veterinary Medicine and Zootechnics, National Autonomous University of Mexico, Mexico City, Mexico.*

**Article Info**

**Article history:**

Received: 24 October 2018
Accepted: 02 February 2019
Available online: 15 September 2020

**Keywords:**

Acrosome
Decapacitation
Hen
Oviduct
Semen

## Abstract

In the hen oviduct, tubules have been identified that preserve the sperm, maintaining viability for up to 15 weeks. This study aimed to evaluate the physiological status of rooster sperm when preserved *in vitro* with uterus vaginal junction secretions (UVJS). Males and females of the Rhode Island breed were used. Sperm aliquots were prepared using Lake extender and Lake extender with UVJS (10.00%, 30.00%, 60.00%, and 90.00%). Subsequently, a basic sperm evaluation was performed and sperm physiological status was determined through the presence and distribution of $Ca^{2+}$ and its acrosomal reaction capability via perivitelline layer (PVL) co-incubation. It was observed that motility was decreased in sperm preserved with UVJS at 6 and 24 hr) compared to 40 min and fresh semen. The sperm decapacitation percentage was increased when preserved with UVJS at 40 min, 6 and 24 hr compared to fresh semen. The acrosomal reaction was increased in sperm co-incubated with PVL, even when preserved with UVJS. It was concluded that UVJS induced physiological changes in sperm by inducing a decapacitation process, which increased sperm viability when preserved *in vitro*.



## Introduction

In avian, the preservation mechanism of sperm storage tubules (SST) is not clear.[1,2] It has been reported that, in SST, sperm can be stored with viability and fertilization capability preserved for up to 15 weeks.[2] Nevertheless, little is known about the cellular or biochemical mechanisms involved in sperm storage.[3] The physiological condition of preserved sperm is not well-understood and this can be compared to sperm decapacitation status.[4] It has been suggested that sperm in SST metabolizes endogenous fatty acids or other lipids derived from SST apical microvilli.[1,2,5] Apical epithelial cell microvilli (MVB) in SST play a possible role in sperm survival as it has been reported that MVB provides lipids and proteins used by host sperm to prevent functions that may be associated with sperm capacitation and fertilization capacity. Furthermore, the MVB stabilizes the sperm plasmalemma and contributes to liquid transportation in SST epithelial cells.[4,6,7] It is known that phospholipids are involved in sperm maturation and motility. In particular, high cholesterol content has been proposed to inhibit capacitation, whereby it is suggested that sperm undergo a decapacitation process, and subsequently it can capacitate to undergo the acrosome reaction (AR) associated with its fertilization capacity.[4,5] Thus, it is possible that SST uterus vaginal junction secretions (UVJS) cause *in vitro* physiological changes in sperm by increasing decapacitation parameters to maintain viability. The present study aimed to evaluate rooster sperm physiological status when preserved *in vitro* with UVJS.

## Materials and Methods

**Poultry.** To obtain semen and uterus vaginal junction secretions, five males and five females Rhode Island breed were used. The birds were 32 weeks old and clinically healthy. Food was provided according to the National Research Council and water was available *ad libitum*.[8] Birds were maintained with a photoperiod of 14 hr light and 10 hr darkness. All animal welfare commissioned norms were met (NOM-062-ZOO-1999).[9]

***Correspondence:**

José Antonio Herrera Barragán. DVM, PhD
Department of Agricultural and Animal Production, Metropolitan Autonomous University - X, Mexico City, Mexico
**E-mail:** jherrerab@correo.xoc.uam.mx

**Semen collection and uterus vaginal junction secretions.** Ejaculation was performed using the dorso-ventral massage technique.[10] Semen was collected as a 1:3 solution with Lake extender (sodium glutamate 0.46 M, sodium acetate 1.86 M, fructose 22.20 M, potassium acetate 25.47 M, pH: 7.00 and osmolarity of 7.10).[11] All reagents used were obtained from Sigma Aldrich, St. Louis, USA. UVJS was obtained using a probe to perform an internal wash of the uterus vaginal junction with 3.00 mL of Lake extender. On average, 2.00 mL of UVJS was collected in a Falcon tube and stored at 5.00 ˚C.[2]

***In vitro* spermatic conservation.** Five rooster seminal pool aliquots with a total of 100 × $10^6$ sperm in 500 µL of Lake extender were preserved at 5.00 ˚C under the following conditions: In the control sample, semen was preserved in Lake extender to preserve sperm viability. In the treatment samples, semen was preserved with 10.00%, 30.00%, 60.00%, and 90.00% UVJS to induce sperm decapacitation conditions. The ability to undergo the AR was determined by comparing the parameters before and after co-incubation of each aliquot with the perivitelline layer (PVL) as a natural inducer of this process.[3] Twenty-five evaluations were performed for each aliquot with fresh semen and this was performed after 40 min (capacitation time) and later at 6 and 24 hr which are acceptable times for fresh semen to be used in artificial insemination.[12,13]

**Basic sperm evaluation.** This was performed on all aliquots under different conditions and time points. The percentage of sperm with straight progressive motility in 10.00 µL semen aliquots was estimated by microscopy (BX51; Olympus, Tokyo, Japan) with a 40× objective.[12,14] Additionally, the sperm in 10.00 µL aliquots were stained with Eosin-Nigrosin and 100 sperm from each sample were analyzed using the optical microscope with a 100× objective to determine the percentage of live sperm.[14]

**Capacitated, decapacitated, and acrosome reaction sperm.** The pattern of capacitation, decapacitation, and AR in sperm was determined for all the aliquots,[14] using 5×$10^6$ sperm incubated with 0.90 M chlortetracycline (CTC) in the dark at 38. 00 ˚C.[15, 16] For all conditions, aliquots were adjusted to 50.00 µL with Lake extender with or without PVL (20.00 µg) obtained from a fresh hen egg after removing the vitello by washing with Lake extender and incubated for 30 min. Later, 25.00 µL of CTC was added and the mixture was incubated for 10 min.[17] After the incubation period, in all experiments, slides were prepared to observe the samples under the fluorescence microscope with a 100× objective (488 nm excitation and >560 nm emission). Image analysis was performed using Image-Pro Plus software (version 6.2.1; Media Cybernetics, Silver Spring, USA). Two hundred sperm per preparation were counted to determine the proportion of sperm that underwent capacitation, decapacitation, and AR.[16]

**Statistical analysis.** A Shapiro-Wilk test was done to assess data normality. In each evaluation, percentages were expressed as mean ± SD. ANOVA with the Tukey post-hoc test was done to determine the differences between means. When data were not normally distributed, Kruskal Wallis test was done. All statistical tests were done with a significance level of $p < 0.05$. All tests were performed with PAST Paleontological Statistics (version 3.18, National University of Ireland, Galway, Ireland).

## Results

**Basic sperm evaluation.** Sperm motility was different when comparing between preservation time points or the UVJS supplementation percentage used (Table 1). When evaluating sperm motility for each preservation time point (0 min, 40 min, and 6, or 24 hr), no statistically significant differences were observed ($p > 0.05$) between the UVJS supplementation percentages. When comparing different preservation time points, it was found that at 6 hr the sperm motility percentage was decreased ($p < 0.05$) in every aliquot regardless of the UVJS percentage applied. The sperm motility percentage was decreased ($p < 0.05$) in aliquots preserved for 24 hr with 30.00%, 60.00% and 90.00% UVJS. Regarding sperm viability similar behavior was observed among aliquots preserved without supplementation and those with 30.00% and 60.00% UVJS as well as between those with 10.00% and 90.00% UVJS (Table 2). The live sperm percentage was not different ($p > 0.05$) between aliquots with different UVJS percentages at each preservation time point (0 min, 40 min, 6 hr, and 24 hr). Nevertheless, when comparing different preservation time points, it was observed that in aliquots without supplementation and those with 30.00% and 60.00% UVJS, the live sperm percentage was lower at 40 min and higher at 24 hr. In aliquots with 10.00% and 90.00% UVJS, the live sperm percentages were similar ($p > 0.05$) at all preservation time points.

**Sperm physiological status.** The decapacitated sperm percentage in aliquots preserved with Lake extender showed a 30.00% decrease compared to those preserved and supplemented with different UVJS percentages at 0 and 24 hr, showing statistically significant differences ($p < 0.05$). On the contrary, when semen was co-incubated with PVL the decapacitation percentages were ranged between 11.00% and 17.00% without statistically significant differences ($p > 0.05$), except at 0 hr when the decapacitation percentage was 32.00%.

The capacitated sperm percentage in semen preserved with Lake extender was ranged between 30.00% - 40.00% showing no statistically significant differences ($p > 0.05$) among the aliquots. The capacitated sperm percentage was slightly lower in semen co-incubated with PVL with a range between 25.00% - 35.00% showing no statistically significant differences ($p > 0.05$) among the aliquots.

**Table 1.** Percentages of motility in sperm preserved in Lake extender supplemented with uterus vaginal junction secretions (UVJS).

| UVJS (%) | Preserving time | | | |
|---|---|---|---|---|
| | 0 min | 40 min | 6 hr | 24 hr |
| **0** | 72.00 ± 1.20[a] | 67.00 ± 4.60[a] | 41.00 ± 7.10[b] | 31.00 ± 5.50[b] |
| **10.00** | 71.00 ± 1.87[a] | 55.00 ± 7.07[a] | 46.00 ± 8.27[ab] | 26.00 ± 7.31[b] |
| **30.00** | 69.00 ± 1.00[a] | 55.00 ± 5.24[ab] | 44.00 ± 5.09[b] | 23.00 ± 5.14[c] |
| **60.00** | 72.00 ± 2.00[a] | 67.00 ± 3.39[a] | 50.00 ± 5.47[b] | 26.00 ± 4.00[c] |
| **90.00** | 69.00 ± 3.31[a] | 56.00 ± 4.00[ab] | 41.00 ± 6.40[b] | 20.00 ± 3.16[c] |

[abc] Different letters indicate statistical differences in each row at $p < 0.05$.

**Table 2.** Percentages of alive sperm preserved in Lake extender supplemented with uterus vaginal junction secretions (UVJS).

| UVJS (%) | Preserving time | | | |
|---|---|---|---|---|
| | 0 min | 40 min | 6 hr | 24 hr |
| **0** | 98.60 ± 0.74[a] | 96.00 ± 0.94[ab] | 94.20 ± 1.35[b] | 94.40 ± 0.67[b] |
| **10.00** | 97.40 ± 0.67[a] | 96.00 ± 0.63[a] | 93.80 ± 1.31[a] | 94.80 ± 1.24[a] |
| **30.00** | 98.20 ± 0.66[a] | 97.00 ± 0.70[ab] | 94.00 ± 0.70[bc] | 91.60 ± 1.20[c] |
| **60.00** | 97.60 ± 0.81[a] | 95.80 ± 0.73[ab] | 89.40 ± 4.62[b] | 92.00 ± 1.70[b] |
| **90.00** | 97.40 ± 1.20[a] | 91.0 0± 3.46[a] | 93.40 ± 1.72[a] | 94.60 ± 1.43[a] |

[abc] Different letters indicate statistical differences in each row at $p < 0.05$.

In sperm preserved with Lake extender, an obvious trend of increased sperm with AR ability was observed as a function of time and regardless of UVJS supplementation. The percentages were lower than 7.00% in fresh semen and after 24 hr the percentages were higher than 37.00%, showing statistically significant differences between them ($p < 0.05$).

On the contrary, semen co-incubated with PVL showed approximately 55.00% of sperm with AR in similar percentages ($p > 0.05$) at 40 min, 6 hr, and 24 hr. Semen without UVJS supplementation showed 37.00% of sperm with AR at 0 hr, while aliquots co-incubated with PVL were at 50.00% (Fig. 1), with statistically significant differences ($p < 0.05$).

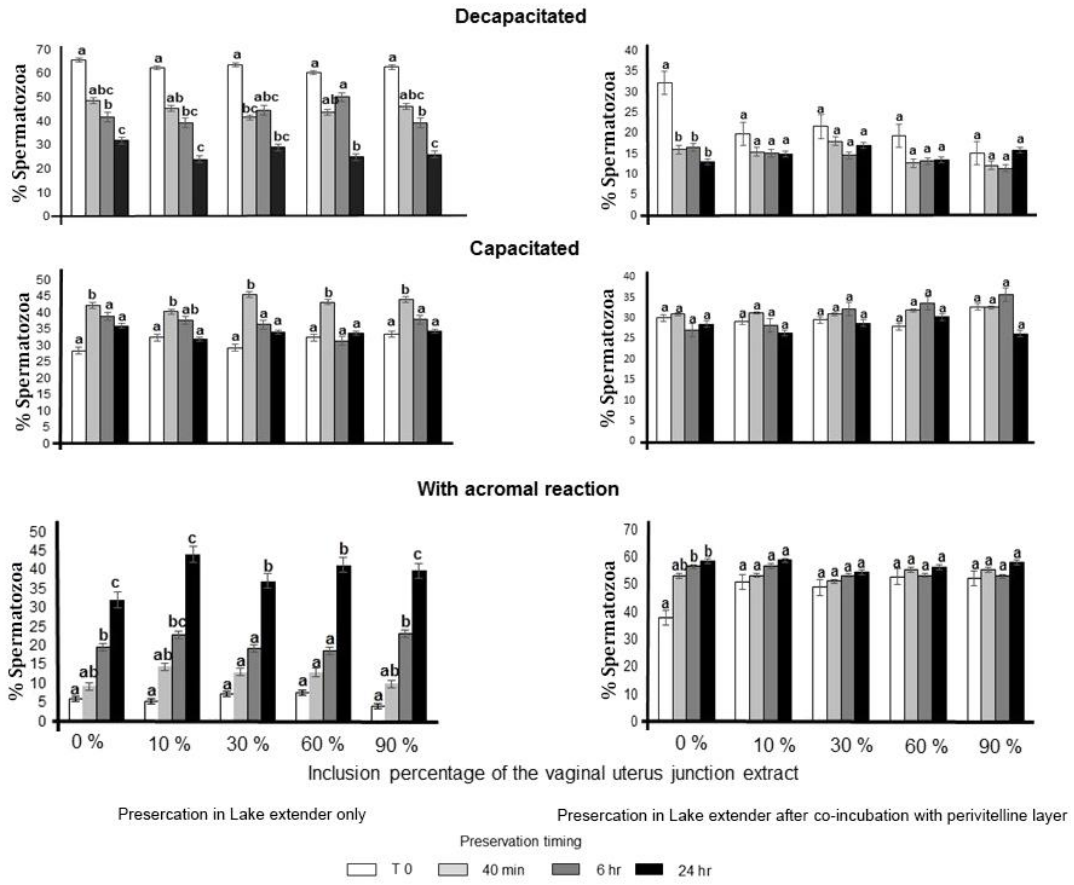


**Fig. 1.** Physiological status of sperm preserved in different conditions. [abc] Different letters indicate statistical differences at $p < 0.05$.

## Discussion

Semen preserved with UVJS showed a lower sperm motility percentage compared to semen preserved with Lake extender without UVJS. This demonstrated the *in vitro* effect of UVJS in terms of decreasing sperm motility as was observed to happen *in vivo* in sperm storage tubules of uterus vaginal junction.[2,4] It is known that sperm stored in SST cannot move, thus, it is believed that UVJS induces quiescence, i.e. sperm are inactive as a result of a decrease in ATP consumption. The mechanism by which sperm reach the SST is unknown, however, it likely requires vigorous sperm motility. Nevertheless, once sperm entered the SST, they become inactive due to unknown mechanisms and are stored until fertilisation.[18] This agrees with the present study as sperm preserved in Lake extender demonstrated decreased motility (from 72.00% to 20.00%), likely influenced by the lipid supply from SST. Endogenous large chain fatty acids stimulate sperm motility inside the vagina, while exogenous large chain fatty acids support it inside SST lumen. The released fatty acids have been identified as oleic and linoleic acids which are absorbed and metabolized by the sperm to increase their viability.[19] The effect of eggshell proteins has been demonstrated during storage to maintain sperm viability in SST.[20]

The live sperm percentages were decreased as a function of time, nevertheless, this was maintained above 90.00% in sperm preserved in Lake extender and supplemented with different levels of UVJS. This agrees with a study performed on the sperm storage mechanism, in which UVJS was prepared and sperm were incubated with and without UVJS. When UVJS extracts were added to sperm, the *in vitro* life span was extended.[21]

The parameters assessed in this study showed that sperm could inhibit the movement of hyperactivation by adding decapacitating substances *in vitro* (present in UVJS) or on the contrary by inducing movement or hyper-activation as a consequence of *in vitro* capacitation. It has been reported that movement inhibition or hyper-activation in other species occur during sperm maturation in the male reproductive tract. Storage structures such as the epididymis have been identified in which sperm decapacitation has been found to be regulated by physiological substrates (lipids, glucose, lactate and glycerol) and autoregulatory mechanisms as low oxygen tension, low osmotic pressure and low extracellular concentrations of $H^+$, $Ca^{2+}$, $Na^+$ and $HCO^{3-}$.[22] In birds, it is known[4] that SST microvilli provide physiological substrates and exogenous macromolecules to inhibit sperm functions in a reversible way associated with fertilization that this process may involve decapacitation.

It has been suggested that sperm in SST is under the reversible suppression of physiological activity, motility, and AR capability. Our study demonstrated that UVJS could inhibit sperm capacitation *in vitro* in response to a natural inductor like PVL. These decapacitated sperm can capacitate and reach acceptable AR parameters. According to *in vitro* AR parameters, it was demonstrated that AR parameters in Lake extender supplemented with UVJS were inversely proportional to decapacitated sperm parameters and that viability was shown after co-incubation with PVL. This increased AR percentages, superior to those observed in sperm preserved in Lake extender and homogeneously in every aliquot preserved with UVJS and co-incubated with PVL. This suggested that sperm inside SST were decapacitated and could achieve capacitated status once they left the SST and entered the uterus, associated with a high cholesterol content on the membrane.[3]

Oligosaccharides isolated from hen PVL glycoproteins can induce AR in rooster sperm. It has been suggested that the induction factor for AR is probably an oligosaccharide bound to nitrogen in N-acetyl-glucosamine, sialic acid, mannose, and glucose as part of the gamete recognition glycoproteins.[18] Additionally, it has been reported that progesterone and other substances as simple saline containing $Ca^{2+}$ can induce AR.[15]

Based on our results, we demonstrated that it was possible to induce sperm decapacitation *in vitro* using UVJS, thereby maintaining sperm viability. This could be demonstrated through AR after *in vitro* induction with PVL. This study provided information on sperm physiological status during *in vitro* preservation, which could be associated with uterus vaginal junction processes. Further research on avian sperm cellular mechanisms might provide vital information and contribute to more efficient conservation during sperm preservation.

## Acknowledgments

The authors thank Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico City, Mexico for scholarships 793369 and 842028 granted to A.K. Vargas and S.A. Cárcoba, respectively.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## References

1. Bakst MR. Role of the oviduct in maintaining sustained fertility in hens. J Anim Sci 2010; 89(5): 1323-1329.
2. Sasanami T, Matsuzaki M, Mizushima S, et al. Sperm storage in the female reproductive tract in birds. J Reprod Dev 2013; 59(4): 334-338.
3. Bakst MR, Akuffo V. Alkaline phosphatase reactivity in the vagina and uterovaginal junction sperm-storage tubules of turkeys in egg production: Implications for sperm storage. Br Poult Sci 2007; 48(4): 515-518.

4. Bakst MR, Bauchan G. Apical blebs on sperm storage tubule epithelial cell microvilli: Their release and interaction with resident sperm in the turkey hen oviduct. Theriogenology 2015; 83(9): 1438-1444.
5. Long JA, Conn TL. Use of phosphosphatidylcholine to improve the function of turkey semen stored at 4 ˚C for 24 hr. Poul Sci J 2012; 91(8): 1990-1996.
6. Freedman SL, Akuffo VG, Bakst MR. Evidence for the innervation of sperm storage tubules in the oviduct of the turkey (*Meleagris gallopavo*). Reproduction 2001; 121: 809-814.
7. Marzesco AM, Wilsch-Bräuninger M, Dubreuil V, et al. Release of extracellular membrane vesicles from microvilli of epithelial cells is enhanced by depleting membrane cholesterol. FEBS Lett 2009; 583: 897-902.
8. National Research Council. Nutrient Requirements of Poultry. 8th revised Ed. Washington DC, USA: National Academy Press 1984.
9. Official Mexican Standard NOM-062-ZOO-1999, Technical specifications for the production, care and use of laboratory animals. 1999; 1-58.
10. Burrows WH, Quinn JP. The collection of spermatozoa from the domestic fowl and turkey. Poul Sci J 1937; 16(1): 19-24.
11. Lake PE, Ravie O. An exploration of cryoprotective compounds for fowl spermatozoa. Br Poult Sci 1984; 25(1): 145-150.
12. Herrera JA, Quintana JA, López MA, et al. Individual cryopreservation with dimethyl sulfoxide and poly-vinylpyrrolidone of ejaculates and pooled semen of three avian species. Arch Androl 2005; 51(5): 353-360.
13. Santiago-Moreno J, Castaño C, Toledano-Díaz A, et al. Semen cryopreservation for the creation of a Spanish poultry breeds cryobank: Optimization of freezing rate and equilibration time. Poult Sci 2011; 90(9): 2047-2053.
14. Ricart MC, Breininger E, Rodriguez PC, et al. Participation of membrane adenylyl cyclase in heparin-induced capacitation in cryopreserved bovine spermatozoa. Andrologia 2015; 47(1):30-36.
15. Lemoine M, Mignon-Grasteau S, Grasseau I, et al. Ability of chicken spermatozoa to undergo acrosome reaction after liquid storage or cryopreservation. Theriogenology 2011; 75(1): 122-130.
16. Herrera JA., Calderón G, Guzmán A, et al. Evaluation of two diluents for the storage of fresh and cryopreserved semen of Harris hawk (*Parabuteo unicinctus*). Austral J Vet Sci 2017; 49(1), 39-43.
17. Lemoine M, Grasseau I, Brillard JP, et al. A reappraisal of the factors involved in *in vitro* initiation of the acrosome reaction in chicken spermatozoa. Reproduction 2008; 136(4):391-399.
18. Matsuzaki M, Mizushima S, Ichikawa Y, et al. Effects of a protein kinase inhibitor on sperm motility in the Japanese quail. J Poult Sci 2017; 54(1): 73-79.
19. Huang A. Isobe N. Obitsu T, et al. Expression of lipases and lipid receptors in sperm storage tubules and possible role of fatty acids in sperm survival in the hen oviduct. Theriogenology 2016; 85(7): 1334-1342.
20. Riou C, Cordeiro L, Gérard N. Eggshell matrix proteins OC-116, OC-17 and OCX36 in hen's sperm storage tubules. Anim Reprod Sci 2017; 185: 28-41.
21. Froman DP, Rhoads DD. Breeding and genetics symposium: A systems biology definition for chicken semen quality. J Anim Sci 2013; 91(2): 523-529.
22. Papa FO, Melo CM, Fioratti EG, et al. Freezing of stallion epididymal sperm. Anim Rep Sci 2008; 107(3-4): 293-301.