BIOCELL
2005, 29(3): 243-251

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Seasonal variations in the heterologous binding of viscacha spermatozoa. A scanning electron microscope study

Claudia Aguilera Merlo, Estela Muñoz, Susana Dominguez, Mabel Fóscolo*, Luis Scardapane, and Juan Carlos de Rosas*

Cátedra de Histología y Embriología, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, 5700 San Luis, Argentina.
*Instituto de Histología y Embriología (IHEM), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, CONICET, 5500 Mendoza, Argentina.

Key words: viscacha (*Lagostomus maximus maximus*), spermatozoa, heterologous binding, seasonal variations.

ABSTRACT: Seasonal changes in the reproductive activity of the adult male viscacha (*Lagostomus maximus maximus*) were investigated during the annual reproductive cycle. Assays of heterologous *in vitro* binding between compatible gametes were used to evaluate the ability of viscacha spermatozoa to achieve primary binding during its annual reproductive cycle. Sperm were collected by mincing cauda epididymis in HECM-3 medium and the sperm concentration and motility were evaluated. Cumulus-free and zona-free oocytes obtained from superovulated hamsters were inseminated *in vitro* with capacitated sperm suspensions, incubated at 37ºC, 5% CO2 for 3 h, and then processed for studies by scanning electronic microscopy. Statistical analysis was used to compare the quantitative differences. The number of spermatozoa significantly decreases during the regression period, while sperm motility was progressive speed in both periods. During the active period elevated sperm binding to cumulus-free and zona-free oocytes was observed, while the binding during the regression period decreased drastically. In both periods, oocyte microvilli covered sperm heads and tails. These results suggest that the ability of viscacha spermatozoa to participate in gamete recognition is profoundly affected. This would likely be related to different functional stages of the spermatozoa and their epididymal microenvironment during the annual reproductive cycle of viscacha.

Introduction

Fusion between gametes is a key event in the fertilization process, involving the interactions of specific domains of the sperm and egg plasma membranes. Although considerable information has been obtained concerning the structural aspects of mammalian sperm-egg fusion, only recently progress has been made towards the identification of specific molecular components that mediate this event (Yanagimachi, 1988; Myles, 1993).

One of the major sources of the specificity of fertilization in mammals is the zona pellucida, a glycoprotein coat surrounding the egg proper, where species-specific gamete recognition and signaling occurs. When the zona is removed, the vitelline surface of the eggs of most mammalian species (e.g., hamster and rabbits) permits the penetration of sperm form heterologous species (Hanada and Chang, 1976).

Mammalian spermatozoa produced in the testis must mature in the epididymis to acquire their fertilizing capability. During fertilization, only the capacitated spermatozoa are capable of recognizing and binding to the zona pellucida or zona free-eggs and undergoing the acrosome reaction (Cross and Meizel, 1989).

Address correspondence to: Bioq. Claudia Aguilera Merlo. Cátedra de Histología y Embriología, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis. Av. Ejército de los Andes 950 – 2º Piso, 5700 San Luis. ARGENTINA. Fax: (+54-2652) 422644 / 426756. E-mail: cleram@unsl.edu.ar
Received on September 10, 2004. Accepted on May 12, 2005.

In regions with important differences in environmental factors, mammalian reproduction is entrained principally by photoperiod and temperature, and natural selection has produced different adaptations in order to subsist in adversity (Darwin, 1858). Species such as the *Dzungarian* or *Mesocricetys auratus* hamsters need to plan the birth of its progeny during an ideal period of temperature and diet. Thus, the reproductive tract is one of the systems most sensitive to these environmental adaptations (Badura *et al*., 1992; Ferkin and Gorman, 1992; Begay *et al*., 1993; O' Brien *et al*., 1993; Gutierrez *et al*., 1995).

Lagostomus maximus maximus (viscacha) is a seasonal rodent (Llanos and Crespo, 1952). Under natural conditions the adult male shows testicular involution during the shorts days of winter (July-August) and maximum gonadal activity during the long days of summer and autumn (December-March). In the regressed testes, the seminiferous tubules are reduced into cords (Fuentes *et al*., 1991). These changes are accompanied by a decrease in serum levels of testosterone and testicular concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), and prolactin (Prl) receptors, and a reduced capacity of Leydig cells to synthesize and secrete androgen (Fuentes *et al*., 1993; Muñoz *et al*., 1997). The viscacha epididymis accompanies the testes in their profound morphological and biochemical changes, and rhythmic fluctuations of testosterone (Aguilera Merlo *et al.*, 2000).

Biochemical, neural, and endocrine factors and behavioral rhythms play a central role in many different aspects of the reproductive process in animals. These rhythms provide the basis for the temporal organization of reproductive function in individual animals (Turek and Van Cauter, 1994).

In the study reported here, we have shown results obtained through heterologous primary binding between hamster oocytes and viscacha spermatozoa during the seasonal reproductive cycle.

Materials and Methods

Animals

Twelve adult viscachas (*Lagostomus maximus maximus*) weighing 4-8 kg were captured in their habitat near San Luis, Argentina (33º 20' south latitude, 760 m altitude) during the periods of maximum gonadal activity (summer-autumn) and gonadal regression (winter). Viscachas were anaesthetized with Nembutal (pentobarbital) and quickly decapitated. The epididymides were exposed through abdominal incision and a ligature was placed at the limit between corpus and cauda. The ligated cauda was removed and immersed in HECM-3 medium (Ogura and Yanagimachi, 1993).

Semen processing

Sperm collected from viscacha were handled essentially as described previously (Bleil and Wassarman, 1983). Sperm were collected by mincing cauda epididymis in 2.0 ml of HECM-3 medium and epididymal tissue was removed by centrifugation. Aliquots of 0.5 ml to 1.0 ml were put in plastic culture tubes and 2 volumes of HECM-3 medium were added. The sperm suspension was incubated at 37ºC in air supplemented with 5% CO_2 during 1 h. Then, the supernatant containing motile spermatozoa (swim-up fraction) was transferred to new culture tubes and incubated at 37ºC in air supplemented with 5% CO_2 for 4-5 hr. The swim-up fraction was centrifuged 3 times at 500 g for 5 min in conical tubes (Falcon 2063). The resulting pellets were resuspended in fresh medium to obtain a final concentration of $1x10^6$ sperm/ml and $0.5x10^6$ sperm/ml (Who Protocol, 1999).

Oocyte preparation

Oocytes were collected from the oviducts of superovulated golden hamsters between 15 and 17 h after injection of HCG. The oocytes were freed from cumulus cells by treatment for 5-10 min in HECM-3, containing 0.1% bovine testicular hyaluronidase (Sigma Chem. Co, St Louis MO). After being rinsed four times in fresh medium, zona-free oocytes were obtained for treatment with 1% bovine pancreas trypsin type III (Sigma Chem. Co, St Louis MO) for a few minutes. The oocytes were rinsed thoroughly in fresh medium five times, transferred into 100 µl HECM-3 medium and placed under mineral oil in a plastic Petri dish at 37°C to be used within 30 min. Abnormal oocytes were discarded (WHO Protocol, 1999).

Sperm-oocyte interaction

Drops of 100 µl of the capacitated viscacha sperm suspension (from both regressive and high activity periods of the reproductive cycle), diluted to a concentration of $0.5x10^6$ sperm/ml and $1x10^6$ sperm/ml in HECM-3 medium were placed under mineral oil in Falcon 3001dishes (35x10mm). Approximately, fifteen cumu-

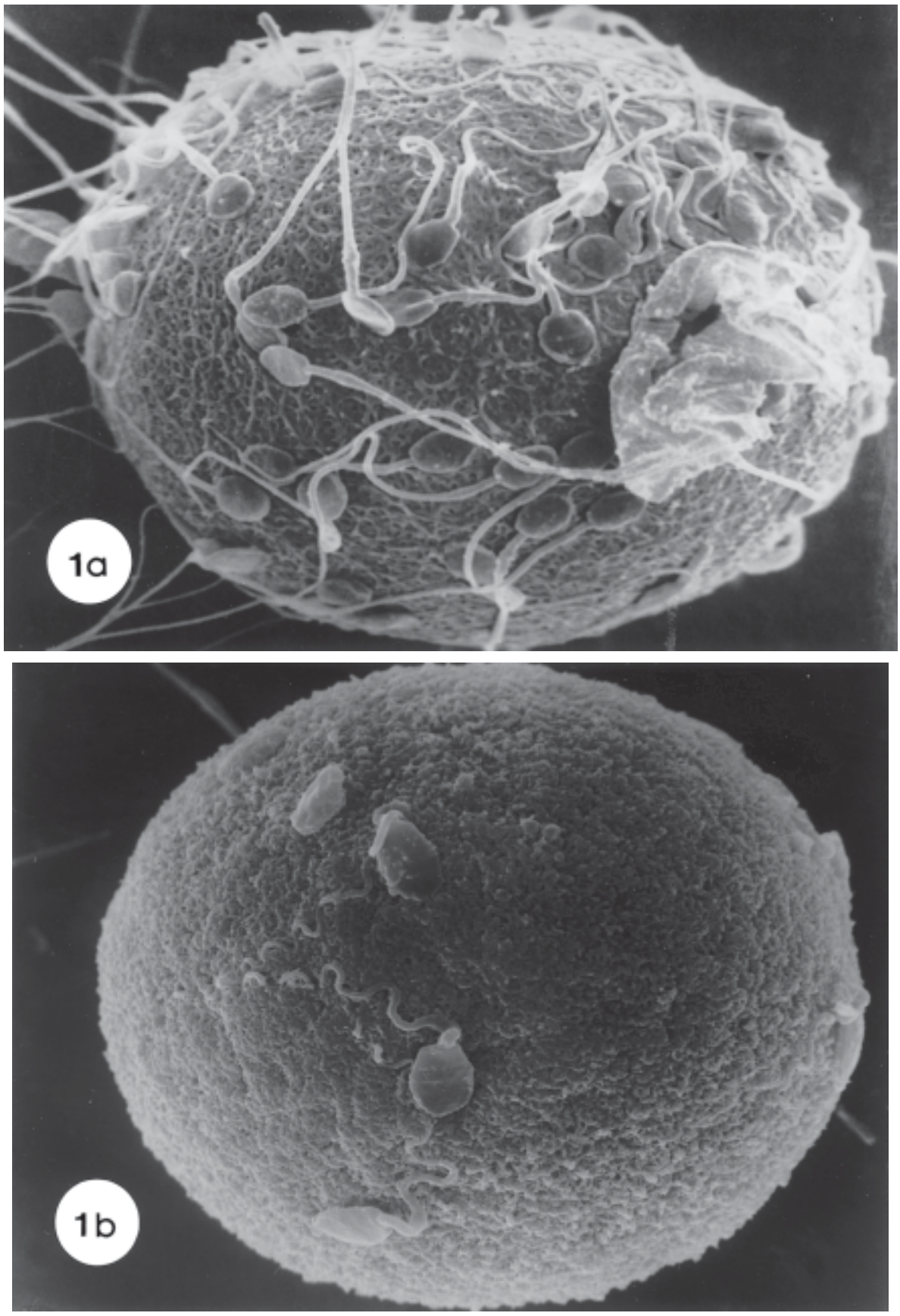

FIGURE 1. Interaction between hamster oocyte zona pellucida and preincubated viscacha spermatozoa. **a:** Period of maximum gonadal activity. X 2,500. **b:** Period of gonadal regression. X 2,500

lus-free and ten zona-free oocytes were introduced into each sperm suspension drop and incubated for 3 h at 37ºC in air supplemented with 5% $CO_{2.}$ (WHO Protocol, 1999). The sperm-oocytes complexes were washed 3 times in fresh medium to eliminate weakly adhered spermatozoa.

Scanning procedure

Bound sperm-oocytes were fixed with 1% glutaraldehyde in PBS buffer (pH 7.4) for 2 h at 4°C, and then were washed repeatedly to eliminate excess glutaraldehyde. The bound gametes were placed on metal grills (covered with 1% gelatin), processed by dehydration through graded alcohol-acetone concentrations, critical point drying and gold evaporation in a sputter device for scanning electron microscopy (SEM), and observed in an Autoscan Siemens ETEC.

Statistical Analysis

All the data were expressed as means ± SEM. Differences between groups were evaluated using Kruskal-Wallis test (nonparametric ANOVA) followed by pairwise comparisons using a Dunn test. A p-value < 0.05 was accepted as statistically significant.

Results

Sperm Analysis

Spermatozoa from cauda epididymides were able to develop progressive motility. These patterns of movement were observed both in active and regressive gonadal periods. The sperm quantification was $470x10^6$ ($\pm 12.5x\ 10^6$) during the active period and $70.8x10^6$ ($\pm 6.27x\ 10^6$) in the gonadal regressive period.

Binding of viscacha sperm to cumulus-free hamster oocytes

Active Period

During the *period of maximum gonadal activity* (summer-autumn), viscacha sperm bound avidly to zona pellucida (ZP) oocytes forming the sperm-oocyte complexes that rotate in the medium after initiating the incubation period (Fig. 1a). The number of viscacha sperm binding to each ZP of hamster oocyte was elevated at a concentration of $1x10^6$ sperm/ml, while a significant decrease of viscacha sperm binding to ZP was observed at concentration of $0.5x10^6$ sperm/ml (Table 1). Most sperm remained tightly bound after washing the vis-

TABLE 1.

Number of spermatozoa strongly bound to hamster oocytes (range, mean ± SEM) during the seasonal reproductive cycle of the viscacha. Cumulus free and zona-free oocytes of hamster were co-incubated with two concentrations of viscacha spermatozoa

Period	Activity		Regression	
	n= 3	n= 3	n= 3	n= 3
Concentration (sperm/ml)	**$1x10^6$**	**$0.5x10^6$**	**$1x10^6$**	**$0.5x10^6$**
Spermatozoa per oocyte				
Cumulus-free	75.25 ± 5.84	36.37 ± 3.11	3.37 ± 0.59	0.87 ± 0.29
Zona-free	236.75 ± 14.43	88.37 ± 3.06	7.50 ± 0.31	2.01 ± 0.75

Values between columns with significant difference ($p< 0.001$).

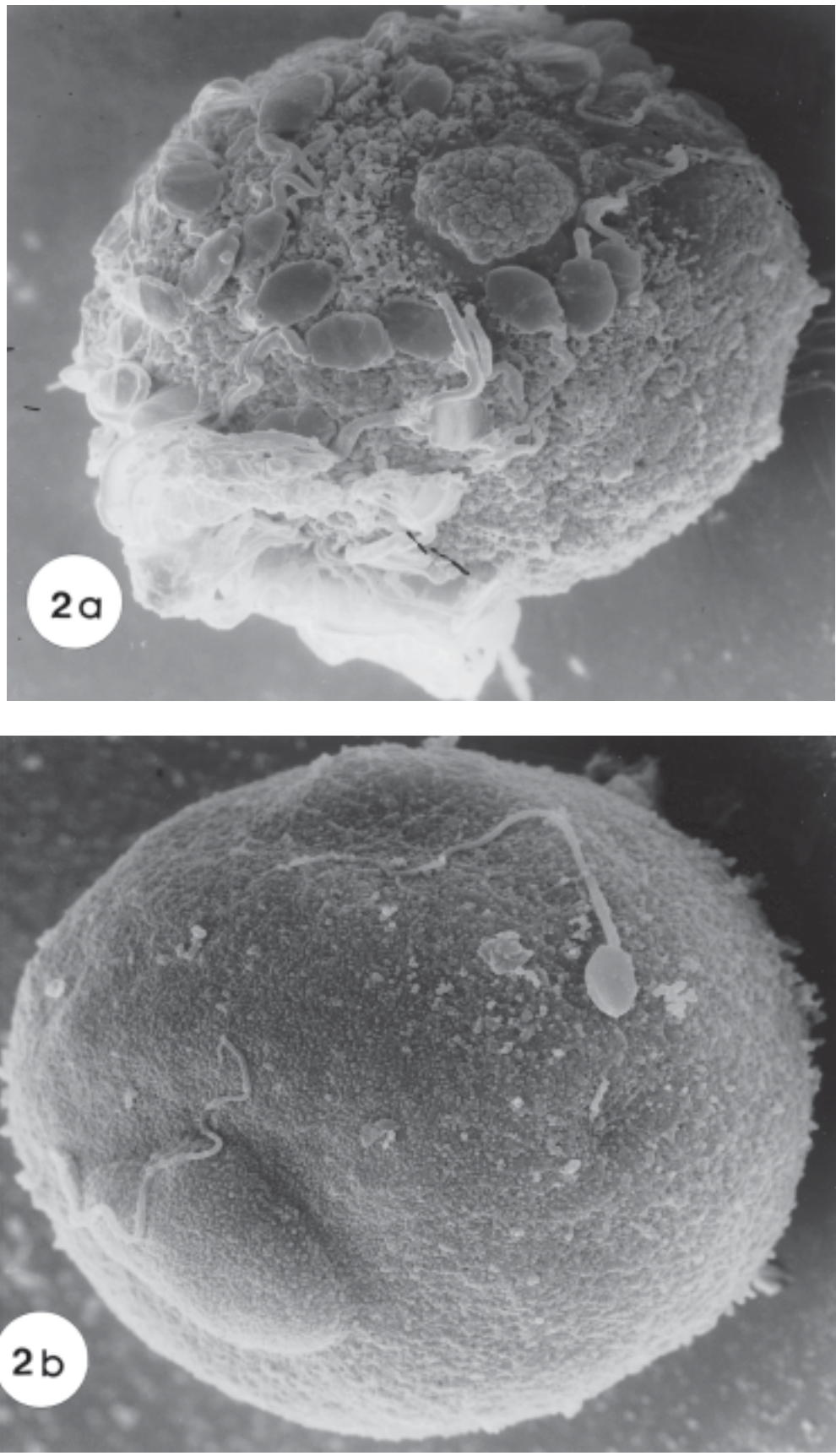

FIGURE 2. Image of zona-free hamster oocyte with viscacha spermatozoa. **a:** Period of maximum gonadal activity. X 2,500. **b:** Period of gonadal regression. X 2,500

cacha sperm-oocyte complex. There was no penetration of the ZP by sperm during the incubation time. All the ZP expressed affinity for gametes of the heterologous specie. Nonetheless, some areas of the ZP remained free of sperm (Fig. 3).

Regressive Period

Viscacha sperm obtained during the *period of gonadal regression* (winter) showed elevated motility, but little recognition by male gametes to ZP of hamster oocytes was observed in this period compared to the active period (Table 1). Areas completely free of sperm were observed (Fig. 1b). The flagellar activity of the sperm produced displacement and rotation of the oocytes as in the active period. There were no signs of fertilization at time of incubation.

Binding of viscacha sperm to zona-free hamster oocytes

Active Period

During the *active period* of the seasonal viscacha reproductive cycle, sperm showed a high binding capacity for zona-free hamster oocytes. The intense flagellar activity of male gametes displaced and rotated zona-free oocytes in the medium during many minutes after initiating incubation. An elevated number of male gametes were bound to plasma membrane oocytes at concentrations of $1x10^6$ sperm/ml, while a significant decrease of male gamete binding to zona-free oocyte was observed at $0.5x10^6$ sperm/ml (Table 1). The number of viscacha sperm binding to each zona-free oocyte of unfertilized hamster was so high that it was difficult to count all of the bound sperm in one plane of focus (Fig.

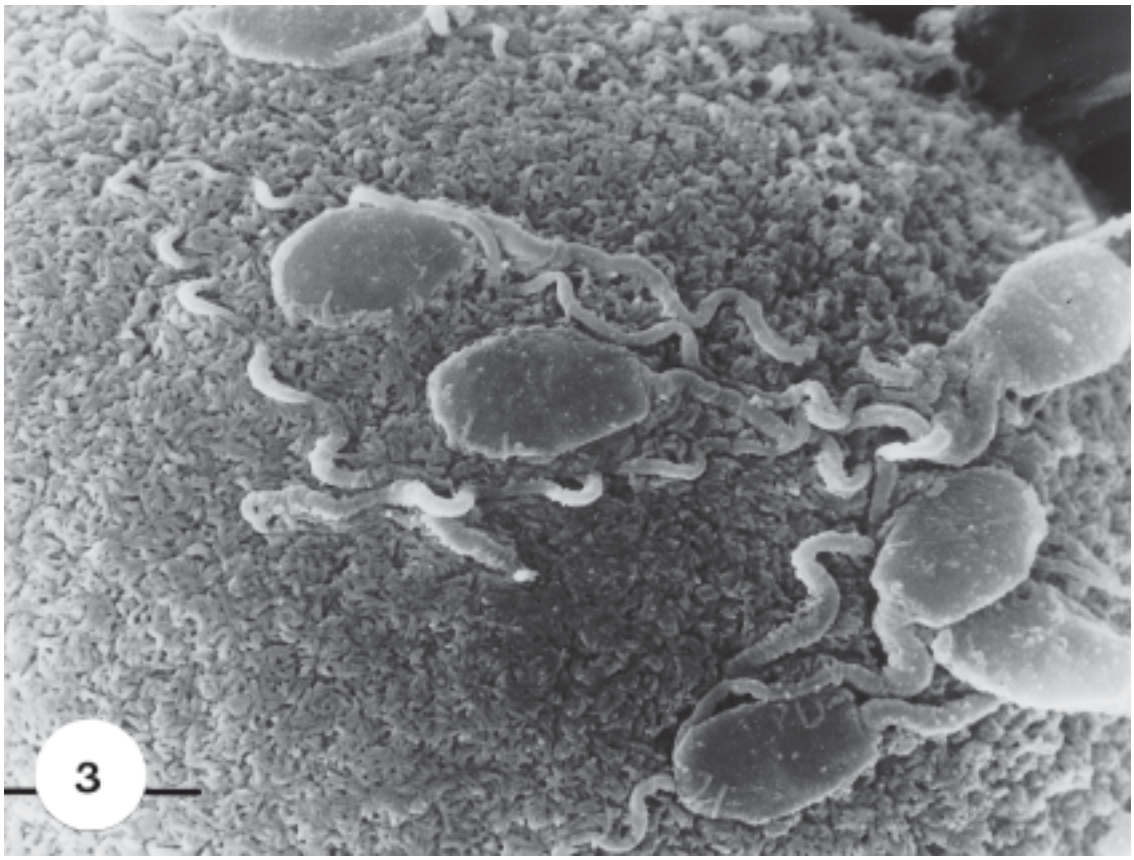

FIGURE 3. Detail at greater magnification of the interaction between viscacha spermatozoa and hamster oocyte zona pellucida. X 7,000

2a). There were no changes in the amount of sperm binding to zona-free oocytes after washing the viscacha sperm-oocyte complex. The oocyte microvilli showed a strong relation with the heads and sperm tails (Fig. 4).

Regressive Period

During the *gonadal regression period* of viscacha, a reduced binding of sperm to zona-free oocytes was observed (Fig. 2b). Different concentrations of sperm showed significant differences in the number of bound spermatozoa to zona-free oocytes. On the other hand, the oocyte microvilli showed strong adhesion to the heads and tails of bound spermatozoa, regardless of the repeated washed in fresh medium to eliminate weakly adhered spermatozoa.

In both periods of seasonal reproductive cycle no signs of fertilization were observed.

Discussion

The data obtained in the present work clearly shows a high gamete interaction between viscacha sperm and hamster oocyte with and without zona pellucida during the period of maximum gonadal activity, while this capacity of primary binding between gametes diminishes significantly in the period of testicular and epididymal regression.

During the period of gonadal regression, the population of viscacha spermatozoa demonstrated a reduced binding to zona pellucida and denuded oocytes, although they did not show any significant difference in the parameters of morphology, motility and vitality between the periods studied (Aguilera Merlo *et al.*, 2000). Generally, this is a point of substantial controversy. Some authors propose a slight relation among these standardized parameters and the ability of the sperm to penetrate

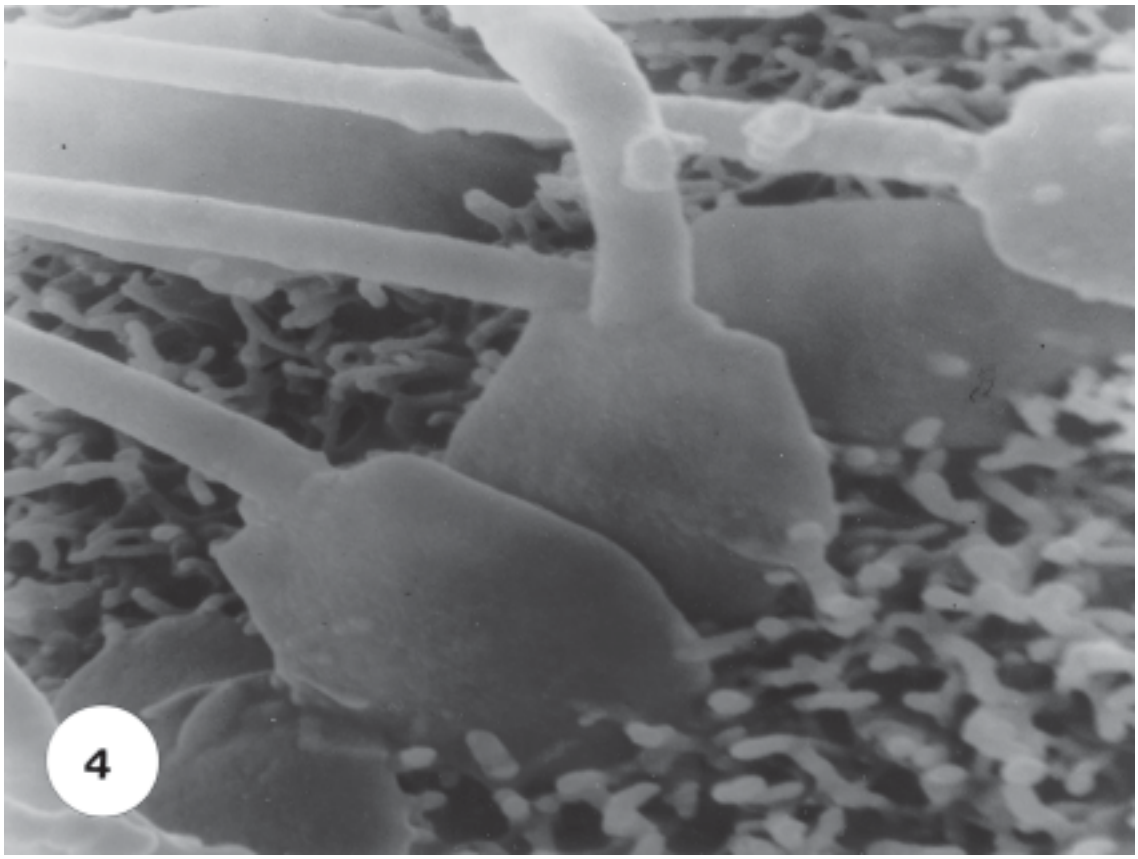

FIGURE 4. Detail at greater magnification of the surface of the zona-free hamster oocyte with heads of spermatozoa included among the oocyte microvilli. X 11,000

the ZP *in vitro* (Martinez *et al.*, 1996; Hammitt *et al.*, 1989). Moreover, it has been proposed that the lack of correlation among the conventional sperm parameters and assays of gamete binding suggest that these assays measure different aspects of the viability and fertilizing capacity of the spermatozoa (Jeyendran *et al.*, 1984).

Gamete recognition or primary binding between cells of the same species needs glycoproteins associated with the oocyte zona pellucida that recognize and establish a chemical link with complementary protein receptors of the spermatozoon (Bedford, 1991; Yanagimachi, 1994).

Heterologous inter-gamete binding occurs only rarely. *In vitro* analysis of species specificity of fertilization indicate that sperm capacitation and the physiological affinity between the sperm of one species and the vitellus of another may be important limiting factors, but the zona pellucida appears to be a major block to interspecific fertilization (Hanada and Chang, 1976; Juneja *et al.*, 1998).

On the other hand, the molecular consolidation and capacitation of spermatozoa is carried out in the epididymis, whose epithelium establishes the particular environment necessary to confer on the spermatozoon their fertilizing ability (Serre and Robaire, 1998). In humans it has been observed that the epididymal spermatozoa of proven fertile men or patients with a normal epididymis acquire the ability to recognize, bind to and fuse with oocytes. Spermatozoa retrieved from the initial segment or caput region failed to bind or penetrate zona-free hamster oocytes while those from the cauda region were successful (Moore *et al.*, 1983).

This finding permits us to suggest that studies of binding amongst gametes is a good measure to evaluate the maturation of epididymal spermatozoa obtained during extreme periods of activity and gonadal (epididymal) regression in seasonally reproducing animals, such as our experimental model the *Lagostomus maximus maximus* (Fuentes *et al.*, 1991). Furthermore, heterologous *in vitro* fertilization (IVF) is an attractive method for evaluating the fertilizing capacity of sperm samples in rare or wild species because it does not require the use of valuable homologous gametes (Soler and Garde, 2003).

The variable capacity of heterologous binding observed in the viscacha spermatozoa might indicate that these spermatozoa present different functional stages depending on the epididymal epithelial cells. Observations obtained previously in our laboratory demonstrated that Leydig cells in the testes of viscacha are in the full process of synthesis during the active period, while in the period of gonadal regression the lowest levels of circulating testosterone coincide with the presence of hypotrophic Leydig cells with evident signs of nuclear and cytoplasmic degeneration, and a decreased testicular concentrations of LH, FSH and PRL receptors (Fuentes *et al*., 1993; Muñoz *et al*., 1997). In agreement with Orgebin-Crist *et al.* (1975), we also think that changes in the androgen levels can alter the capacity of the viscacha epididymis to store spermatozoa by affecting ion and protein profiles of the fluid found in the cauda epididymal lumen.

On the other hand, perhaps the epithelium of the epididymis does not totally lose the ability to stimulate the maturity of male gametes, or alternatively, the epididymis of viscacha maintains stored spermatozoa with morphological features and functional fertilizing capacities obtained during a previous active period (Aguilera Merlo *et al.*, 2005). With regards to this latter possibility, it has been estimated that sperm viability is retained in the cauda epididymidis for 2-3 weeks in animals or even longer (Turner, 1995; Moore, 1996).

In the future, more studies will be needed to establish the molecular and biochemical causes of the highly differential binding between viscacha spermatozoa and hamster oocytes throughout the annual reproductive cycle of viscacha.

Acknowledgements

The authors wish to thank Mrs. J. Arroyuelo and N. Perez for their technical participation. This work was supported by Project 22Q/303, CyT. U.N.S.L.

References

Aguilera Merlo C, de Rosas JC, Muñoz E, Perez Romera E, Fóscolo M, Domínguez S, Scardapane L, Piezzi RS (2000). *Viscacha epididymis and spermatozoa in the reproductive period*. Biocell 24(3), (Abstract 72/294).

Aguilera Merlo C, Muñoz E, Dominguez S, Scardapane L, Piezzi RS (2005). *Epididymis of viscacha (Lagostomus maximus maximus). Morphological changes during the annual reproductive cycle.* Anat Rec 282: 83-92.

Badura L, Sisk C, Nunez A (1992). *Photoperiodic regulation of prolactin release in male hamster with hipotalamic knife cuts*. Brain Res Bull. 29: 231-237.

Bedford JM (1991). *The coevolution of gametes*. In: A Comparative Overview of Mammalian Fertilization. B.S. Dunbar, M.G. O´Rand Eds. Plenum Press, New York, pp. 3-50.

Begay V, Falcon J, Thibault Ch, Ravault JP (1993). *Pineal photoreceptor cells: photoperiodic control of melatonin production after cell dissociation and culture*. J Neuroend 4: 337-345.

Bleil J, Wassarman PM (1983). *Sperm-eggs interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein*. Dev Biol 95: 317-324.

Cross N, Meizel S (1989). *Methods for evaluating the acrosomal status of mammalian sperm*. Biol Reprod. 41: 635-641.

Darwin Ch (1858). *The origen of species by means of natural selection, on the preservation of favoured races the struggle for life*. London J.M., Albermale street.

Ferkin MH, Gorman MR (1992). *Photoperiod and gonadal hormones influence odor preferences of the male meadow vole, Microtus pennsylvanicus.* Physiology & Behavior 51: 1087-1091.

Fuentes L, Caravaca N, Pelzer L, Scardapane L, Piezzi RS, Guzman J (1991). *Seasonal variations in the testis and epididymis of viscacha (Lagostomus maximus maximus).* Biol Reprod. 45: 493-497.

Fuentes L, Calvo JC, Charreau E, Guzman J (1993). *Seasonal variations in testicular LH, FSH and Prl receptors; in vitro testosterone production and serum testosterone concentration in adult male viscacha (Lagostomus maximus maximus).* Gen Comp Endocrinol. 90: 133-141.

Gutierrez L, Burgos M, Brengio S (1995). *Cauda epididymis of Galea musteloides in periods of reproduction and gonadal regression. Ultrastructural and cytochemical studies*. Biocell 19: 141-151.

Hammitt DG, Martin PA, Callanam T (1989). *Correlation between heterospermic fertility and assays of porcine seminal quality before and after cryopreservation.* Theriogenology 32: 385-399.

Hanada A, Chang MC (1976). *Penetration of hamster and rabbit zona-free eggs by rat and mouse spermatozoa with special reference to sperm capacitation*. J Reprod Fertil. 46: 239-241.

Jeyendran R, Van der Ven H, Perez-Pelaez M, Crabo B, Zaneveld L (1984). *Development of an assay to asses the functional integrity of the human sperm membrane and its relationship to other semen characteristics.* J Reprod Fertil. 70: 219-228.

Juneja R, Agulnik S, Silver L (1998). *Sequence divergence within the sperm-specif polypeptide TCTE 1 is correlated with species-specific differences in sperm binding to zona-intact eggs*. J Androl. 19, 2:183-188.

Llanos AC, Crespo JA (1952). *Ecología de la vizcacha (Lagostomus maximus maximus) en el nordeste de la provincia de Entre Ríos.* Revista Invest Agric Bs As. 6: 289.

Martinez E, Vazquez JM, Matas C, Gadea J, Alonso MI, Roca J (1996). *Oocyte penetration by fresh or stored diluted boar spermatozoa before and after in vitro capacitation treatments.* Biol Reprod. 55:134-140.

Moore HDM (1996). *The influence of the epididymis on human and animal sperm maturation and storage*. Hum Reprod 11 (Nat Suppl): 103-110.

Moore HDM, Pryor JL, Harman TD (1983). *Development of the oocyte penetrating capacity of the human epididymis*. Internat J Androl 6: 310-318.

Muñoz E, Fogal T, Dominguez S, Scardapane L, Guzman J, Piezzi RS (1997). *Seasonal changes of the Leydig cells of viscacha (Lagostomus maximus maximus). A light and electron microscopy study*. Tissue & Cell 29: 119-128.

Myles DG (1993). *Molecular mechanisms of sperm-egg membrane binding and fusion in mammals*. Dev Biol. 158: 35-45.

O'Brien GM, Curlewis JD, Martin L (1993). *Effect of photoperiod on the annual cycle of testis growth in a tropical mammal, the little red flying fox, Pteropus scapulatus*. J Reprod Fertil. 98: 121-127.

Ogura A, Yanagimachi R (1993). *Round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy*. Biol Reprod. 48: 219-225.

Orgebin-Crist MC, Danzo BJ, Davies J (1975). *Endocrine control of the development and maintenance of sperm fertilizing ability in the epididymis.* In: Handbook of Physiology, sec. 7 vol. 5, edited by RO Greep and EB Astwood, pp.319-338. American Physiological Society, Washington, DC.

Serre V, Robaire B (1998). *Segment-specific morphological changes in aging Brown Norway rat epididymis*. Biol Reprod. 58: 497-513.

Soler AJ, Garde JJ (2003). *Relationship between the characteristics of epididymal red deer spermatozoa and penetrability into zona-free*. J Androl 24(3): 393-400.

Turner TT (1995). *On the epididymis and its role in the development of the fertile ejaculate*. J Androl 16: 292-298.

Turek FW, Van Cauter E (1994). *Rhythms in Reproduction*. In: The Physiology of Reproduction. E. Knobil and J. Neil, Eds. Raven Press, New York, pp 999-1080.

WHO Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. (1999). 4º Edition. Cambridge University Press.

Yanagimachi R (1988). *Sperm egg fusion*. In: Current Topics in Membranes and Transport. N. Duzgrines and F. Bronner, Eds. Academic Press, Orlando, 32 pp. 3-43.

Yanagimachi R (1994). *Mammalian fertilization*. In: The Physiology of Reproduction, 2nd Edition. E. Knobil and J.D. Neil, Eds. Raven Press, New York, pp. 189-317.