Spanish Journal of Agricultural Research (2004) 2 (1), 73-77

Ability of frozen-thawed ram spermatozoa to bind to vitrified homologous oocytes

F. Marco-Jiménez* and J. Vicente

Laboratorio de Biotecnología de la Reproducción. Departamento de Ciencia Animal. Universidad Politécnica de Valencia. Camino de Vera, s/n. 46071 Valencia. Spain

Abstract

This study was designed to evaluate the ability of ram spermatozoa (thawed or thawed *swim-up* selected) to bind to homologous oocytes subjected to different treatments (*in vitro* matured or immature, fresh or vitrified). Frozen semen from Guirra breed males was tested on 1,267 oocytes harvested from pre-pubertal ewes at an abattoir. Highest proportions of oocytes with bound spermatozoa were recorded when *in vitro* matured oocytes, whether fresh or vitrified, were coincubated with thawed spermatozoa separated by *swim-up* (60.8 and 56.8% for fresh and vitrified matured oocytes, *versus* 9.4 and 11.1% for fresh and vitrified immature oocytes, respectively, $p < 0.05$). The number of bound spermatozoa per oocyte was also significantly higher for the *swim-up* selected spermatozoa than the thawed untreated spermatozoa (3.2 ± 0.5 *vs* 1.6 ± 0.7, $p < 0.05$, respectively), and higher still when fresh *in vitro* matured oocytes were used (8.3 ± 0.5).

Key words: vitrification, sperm evaluation, ram, local breed.

Resumen

Adherencia del semen congelado de moruecos sobre óvulos vitrificados

Se estudió la capacidad de adherencia de los espermatozoides (descongelados o tras *swim-up*) dependiendo del tipo de ovocito utilizado (frescos o vitrificados, y madurados *in vitro* o inmaduros). Para ello se utilizó semen congelado de moruecos de la raza Guirra sobre 1.267 ovocitos de corderas recuperados de matadero. El porcentaje de ovocitos con espermatozoides adheridos fue mayor cuando los ovocitos, vitrificados o no, fueron previamente madurados *in vitro* y co-incubados con espermatozoides descongelados y seleccionados por *swim-up:* 60,8% en el caso de ovocitos frescos, y 56,8% en el caso de vitrificados, frente a un 9,4% y 11,1% con ovocitos inmaduros frescos y vitrificados, respectivamente ($P < 0,05$). El número de espermatozoides adheridos por ovocito fue también significativamente mayor en el caso del semen sometido a la técnica de *swim-up* ($3,2 \pm 0,5$ *vs* $1,6 \pm 0,7$, $P < 0,05$) y significativamente mayor cuando los ovocitos no fueron vitrificados, pero sí madurados *in vitro* ($8,3 \pm 0,5$).

Palabras clave: vitrificación, evaluación de semen, ovino autóctono.

Introduction

The Guirra (Sudat or Roja Levantina) sheep is the only ovine breed autochthonous to the *Comunidad Valenciana*, its current census being 3,640 females and 174 males. According to the FAO catalogue and Royal Decree 1682/97 (BOE, 1997), this breed is in danger of extinction and its recent decline has prompted a conservation program based on genetic resources which, among other measures, includes the creation of a semen bank.

The natural resistance of spermatozoa to cryopreservation varies among species, breeds and even among individual animals of the same breed. Detecting reduced-fertility males can be simple since these animals often show signs of altered semen quality that can be detected by routine analyses. However, not all the ejaculates showing adequate quality indicators are of normal fertility. The factors most commonly used to evaluate sperm quality in semen samples are sperm motility and morphology. Recently developed image analysis programs (computer assisted sperm analyser, CASA) provide an objective estimate of the number of motile spermatozoa in the ejaculate, their quality of movement and can even be used to correlate morpho-

* Corresponding author: framarj1@etsia.upv.es
Received: 09-05-03; Accepted: 30-10-03.

metric determinations with semen fertility (Verstegen *et al.*, 2002).

In the sheep, it has not been possible to significantly relate sperm motility characteristics with *in vivo* fertility (Sanchez-Partida *et al.*, 1999), and contradictory results have been observed in cattle. Thus, while Budworth *et al.* (1988) and Bailey *et al.* (1994) found no significant correlations with *in vivo* fertility, Farrell *et al.* (1998) were able to detect some associations by including several of the variables obtained using the CASA system in the analysis ($r^2 = 0.95$). Other studies have been able to relate sperm abnormalities in bull ejaculates with both *in vivo* and *in vitro* fertility.

Besides morphological and motility tests, the recently developed *in vitro* techniques evaluate the functionality of the plasma membrane (endosmosis and acrosome exocytosis induction tests) or gamete interaction. Hence, in cattle, several authors have found significant relationships between fertility (rate of non-return to oestrous) and the percentage of endosmosis positive thawed spermatozoa, and between fertility and *in vitro* fertilisation or embryo development.

The aim of the present study was to evaluate the capacity of spermatozoa (thawed or *swim-up* prepared) to bind to fresh or vitrified oocytes that had been matured *in vitro* or left immature. Our ultimate goal is to include data related to sperm adhesion capacity, along with the data usually used, in a multiple regression analysis as a predictor of fertility.

Material and Methods

The ovaries used were obtained from ewes slaughtered at a commercial abattoir that were mainly of the Manchega breed and approximately 3 months of age. The ovaries were transported to the laboratory in a vacuum flask within 2 h of animal sacrifice. Cumulus-oocyte complexes (COC) were recovered from the ovaries by aspirating follicles larger than 1 mm. Optimal quality oocytes were defined according to the criteria described by Martino *et al.* (1994, 1995) for the goat. The COCs were washed three times in PBS modified (PBSm) by supplementing with 0.5 g l^{-1} BSA, 36 mg l^{-1} sodium pyruvate, 1 g l^{-1} glucose and 50 µg ml^{-1} gentamicin. In total, 1,267 COCs were recovered and assigned to the following experimental groups: (1) fresh, immature; (2) fresh, *in vitro* matured; (3) vitrified, immature and (4) vitrified, *in vitro* matured.

Cumulus-oocyte complexes were vitrified according to the method described by Vajta *et al.* (1998). Exposure to the cryoprotectant solution was performed in two steps: in the first, the COCs were immersed in 10% ethylene glycol (EG), 10% dimethylsulfoxide (DMSO) in PBS for 45 s, and in the second step, in a solution containing 20% EG, 20% DMSO plus 0.5 M sucrose in PBS for 25 s. The COCs were then loaded in 0.25 ml straws and plunged into liquid nitrogen. Thawing was performed in a water bath at 20ºC after removing the cryoprotectant in three stages in Nunc® plates containing 5 ml of medium: first in PBSm, 20% foetal bovine serum (FBS) and 0.25 M sucrose for 1 min; second in PBSm, 20% FBS and 0.15 M sucrose for 5 min and, in a final step, the COCs were washed in TCM199 medium supplemented with 10% FBS.

The COCs allotted to groups 2 and 4 were placed in culture for *in vitro* maturation in Nunc® 4-well plates containing 500 µl of TCM199 medium (Tissue Culture Medium, Sigma M-4530) supplemented with 10% FBS, 10 ng ml^{-1} epithelial growth factor and 50 mg ml^{-1} gentamicin for 26 h at 38.5ºC in a 5%CO_2 water saturated atmosphere. For the sperm adhesion tests, the COCs were transferred to Nunc® 4-well plates containing TALP (Tyrode-albumin-sodium lactate-sodium piruvate) supplemented with 10 µg ml^{-1} heparin.

The semen used for the tests was a pooled sample from 5 Guirra breed rams from a herd belonging to the *Universidad Politécnica de Valencia*. The ejaculates were obtained by the artificial vagina method, mixed (to avoid inter-individual differences) and cryopreserved by directly adding the diluent (Biociphos plus®, IMV Technologies) to the ejaculate fraction at room temperature to obtain a 1:5 dilution. Next, the samples were loaded into 0.25 ml straws, during approximately 10 min. The straws were kept at 5ºC for 2 h. After this period, the semen was frozen at –120ºC in a nitrogen gas column (5 cm liquid nitrogen surface) for 10 min and finally stored in liquid nitrogen. The semen was thawed in a water bath at 42ºC for 12 s.

Once thawed, the semen was washed in STL medium (HEPES buffered TALP medium) and divided into two portions: one subjected to *swim-up* (Parrish *et al.*, 1984) and the other preserved in an incubator at 37ºC. The concentration of semen used was 2×10^6 ml^{-1} and cocultures were maintained for 6 h at 38.5ºC in a 5% CO_2 water saturated atmosphere.

After coculture, the COCs were transferred to an Eppendorf tube containing sodium citrate and intensely vortexed for 3 min. Once denuded, the oocytes we-

re stained for 5 min with Hoechst 33342 (Sigma, B-2261). Adhered spermatozoa were then counted under a fluorescence microscope (Nikon Eclipse E400, magnification 100×-400×).

Statistical analysis

The proportions of oocytes with adhered spermatozoa (thawed or thawed/*swim-up* prepared) according to the type of oocyte (fresh immature/*in vitro* matured or vitrified immature/*in vitro* matured) were compared by the chi-squared test, while the effect of the type of semen or oocyte and their interaction on the number of spermatozoa bound to each oocyte was evaluated using a general linear model (GLM, Statgraphics 4.1).

Results

Tables 1 and 2 show the results obtained in terms of the proportion of oocytes with bound spermatozoa and the number of bound spermatozoa per oocyte. Significant differences in the percentage of oocytes with adhered spermatozoa were observed between vitrified or fresh *in vitro* matured *versus* the vitrified or fresh immature oocytes (38.4% or 44.2% *vs* 8.5% or 16.3%, $P < 0.05$ respectively, Table 1). The two types of semen also yielded different results; a higher percentage of oocytes with adhered spermatozoa being recorded when the semen was *swim-up* selected after thawing (36.3% *vs* 13.6%, $P < 0.05$, Table 1). Best results in terms of percentage binding were obtained when matured vitrified or non-vitrified oocytes were coincubated with thawed *swim-up* prepared spermatozoa (56.8% or 60.8% respectively, Table 1).

Table 1. Percentage of oocytes with bound spermatozoa

Oocyte type	Semen type		Total (N)
	Thawed/ *Swim-up* (N)	Thawed (N)	
Fresh-non IVM	11.1^{b} (135)	21.5^{a} (135)	16.3^{b} (270)
Fresh-IVM	60.8^{a} (153)	23.1^{a} (121)	44.2^{a} (274)
Non-IVM-vitrified	9.4^{b} (202)	7.1^{b} (141)	8.5^{b} (343)
IVM-vitrified	56.8^{a} (248)	3.8^{c} (132)	38.4^{a} (380)
Total	36.3^{A} (738)	13.6^{B} (529)	

^{a,b,c}: significant differences ($P < 0.05$) within a column. ^{A,B}: significant differences ($P < 0.05$) within a row. IVM: *in vitro* matured.

Table 2. Number of bound spermatozoa per oocyte (LSM ± SEM)

Oocyte type	Semen type		Total (N)
	Swim-up/ thawed (N)	Thawed (N)	
Fresh-non IVM	1.2 ± 1.3^{b} (15)	$2.62 \pm 0,9$ (29)	1.9 ± 0.8^{b} (44)
Fresh-IVM	8.3 ± 0.5^{a} (93)	1.7 ± 0.9 (28)	5.0 ± 0.5^{a} (121)
Non-IVM-vitrified	1.1 ± 1.2^{b} (19)	1.1 ± 1.6 (10)	1.1 ± 0.9^{b} (29)
IVM-vitrified	2.2 ± 0.4^{b} (141)	1.2 ± 2.2 (5)	1.68 ± 1.6^{b} (146)
Total	3.2 ± 0.5^{A} (268)	1.6 ± 0.7^{B} (72)	

^{a,b,c}: significant differences ($P < 0.05$) within a column. ^{A,B}: significant differences ($P < 0.05$) within a row. IVM: *in vitro* matured. LSM ± SEM: least square means ± standar error of the means.

Table 2 provides the numbers of spermatozoa adhered per oocyte. Both the type of semen and oocyte and interactions between them affected the sperm binding capacity. The spermatozoa selected by the *swim-up* procedure after thawing showed a greater binding capacity than spermatozoa that had been simply thawed (3.2 ± 0.5 *vs* 1.6 ± 0.7, respectively, $P < 0.05$). The highest number of adhered prepared spermatozoa was recorded for the non vitrified matured oocytes compared to the other oocyte types (5.0 ± 0.5 *vs* 1.1 ± 0.9 to 1.9 ± 0.8, Table 2).

Discussion

Routine semen quality tests used to evaluate males only assess the spermatozoon. The importance of including tests in which spermatozoa and live oocytes interact is the information provided on fertilising capacity (Bavister, 1990). These tests also more closely mimic the steps occurring during fertilisation. The sperm binding test used by Zhang *et al.* (1998) enabled them to predict the fertilising capacity of semen from young high fertility bulls using three frozen ejaculates. This capacity was subsequently correlated (Zhang *et al.*, 1999) with post- artificial insemination fertility ($r = 0.88$, $p < 0.001$).

The use of intact homologous oocytes is a simple method and is less tedious and exact than the use of oocytes subjected to microbisection (hemizona assay, Pérez-Pe *et al.*, 2000) or oocytes devoid of the zona pellucida (Graule *et al.*, 1995), provided a high number of oocytes is used to minimise variation among

oocytes (Zhang *et al.*, 1995). Variation in the binding capacity of spermatozoa to individual oocytes is the most significant limiting factor for evaluating sperm binding capacity to the zona pellucida, since the characteristics of the zona pellucida are related to oocyte quality (Fazeli *et al.*, 1996). To reduce inter-oocyte variability and conduct the semen evaluation test irrespective of the time of semen or oocyte availability requires the use of simple, cryopreservation protocols.

In our study, once the frozen semen had been thawed, the *swim-up* procedure served to select the most motile and viable sperm in the sample, explaining the improved results observed both in terms of the proportions of oocytes with bound spermatozoa and the number of spermatozoa per oocyte, compared to the directly thawed sperm. This selection technique has been previously related to an increased fertilisation rate (Parrish *et al*., 1984).

The effects of cryopreservation on oocytes include chromosome alterations (cell picnosis), reduced fertility rates and a high incidence of polyspermia. Cryopreservation causes damage to several organelles (microvilli, cortical granules, mitochondrial membranes) and the zona pellucida (Fuku *et al.*, 1995). Here, vitrification was not found to be a determining factor for the percentage of oocytes with adhered spermatozoa, and this role was, instead, taken on by the factor *in vitro* maturation. The percentage of oocytes with adhered spermatozoa, irrespective of whether the oocytes had been vitrified or not, showed a drop of around 30% if the oocytes had not been matured *in vitro*. Maturation systems are not without their defects, and the overall efficiency of the process is still low for most species. It is thus common to compare the use of immature and mature oocytes. Our findings contradict those reported by other authors for different species (rabbit: Mocé and Vicente, 2001; pig: Martínez *et al.*, 1993; cow: Niwa *et al.*, 1991), who noted no binding differences between immature and *in vitro* matured oocytes. Low immature oocyte penetration rates have been attributed to quantitative and qualitative differences in the thickness and composition of the immature zona pellucida (Lucas *et al.*, 2003). The zona pellucida forms during the maturation process (Braw-Tal and Yossefi, 1997) and it seems that immature oocytes differ from mature oocytes in the glycoproteins (Zp1, Zp2 and Zp3) of the zona pellucida, along with a lower number of spermatozoon receptors (Lucas *et al.*, 2003).

The decreased number of spermatozoa adhered to vitrified *in vitro* matured oocytes compared to oocytes subjected to *in vitro* maturation immediately after their recovery could be explained by alterations to the zona pellucida induced by rupture of the cortical granules during cryopreservation, leading to the early permeabilization of the zona pellucida (Van Blerkom, 1991; Vincent and Johnson, 1992). Enzymes released in the cortical region modify sperm receptors and components of the vitelline membrane, preventing sperm binding (Otoi *et al.*, 1993; Fuku *et al.*, 1995; Martino *et al.*, 1996).

In summary, our findings suggest the suitability of using the protocol described by Vajta *et al.* (1998) to vitrify immature oocytes obtained from abattoir sheep. These vitrified oocytes could be used to create a bank for sperm binding tests after subjecting the oocytes to 24 h of *in vitro* maturation.

Acknowledgements

This study was funded by the INIA (Project RZOO-014-C3-01) and a special grant from the Universidad Politécnica de Valencia. The authors thank Dr. Eva Mocé for her useful discussions and improvements to the manuscript.

References

BAILEY J.L., ROBERTSON L., BUHR M.M., 1994. Relationships among *in vivo* fertility, computer-analysed motility and *in vitro* Ca^{2+} flux in bovine spermatozoa. Can J Anim Sci 74, 53-58.

BAVISTER B.,D., 1990. Test of sperm fertilizing ability. In: Gamete Physiology (Asch R.H., Balmaceda J.P. and Johnston I., eds). Norwell, Massachusetts: Serono Symposia, USA, pp. 77-105.

BOE (Boletín Oficial del Estado), 1997. Royal Decree 1682/97, of 24 January, that the Official Catalogue of Races of Cattle of Spain is updated. BOE no. 279, 21/11/1997.

BRAW-TAL R., YOSSEFI S., 1997. Studies *in vivo* and *in vitro* on the initation of follicle growth in the bovine ovary. J Reprod Fertil 47, 99-104.

BUDWORTH P.R., AMANN R.P., CHAPMAN P.L., 1988. Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. J Androl 9, 41-54.

FARRELL P.B., PRESICCE G.A., BROCKETT C.C., FOOTE R.H., 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (C.A.S.A.) and the relationship to fertility. Theriogenology 49, 871-879.

FAZELI A.R., STEENWEG W., BEVERS M.M., COLEBRANDER B., 1996. The hemizona assay: evaluation of sperm zona pellucida interaction. Theriogenology 44, 751-760.

FUKU E., KOJIMA T., SHIOYA Y., MARCUS G.J., DOWNEY B.R., 1995. *In vitro* fertilization and development of frozen-thawed bovine oocytes. Cryobiology 29, 485-92.

GRAULE B., BRAUN J., STOLLA R., 1995. Binding of spermatozoa to bovine oocytes and their penetration rates in a homologous IVF system. Theriogenology 53, 224, Abstr.

LUCAS X., MARTÍNEZ E.A., ROCA J., VÁZQUEZ J.M., GIL M.A., PASTOR L.M., ALABART J.L., 2003. Influence of follicle size on the penetrability of immature pig oocytes for homologous *in vitro* penetration assay. Theriogenology 60, 659-670.

MARTÍNEZ E., VÁZQUEZ J.M., MATÁS C., ROCA J., COY P., GADEA J., 1993. Evaluation of boar spermatozoa penetrating capacity using pig oocytes at the germinal vesicle stage. Theriogenology 40, 547-557.

MARTINO A., MOGAS T., PALOMO M., PARAMIO M., 1994. Influence of the collection technique of prepubertal goat oocytes on *in vitro* maturation and fertilization. Theriogenology 42, 859-873.

MARTINO A., MOGAS T., PALOMO M., PARAMIO M., 1995. *In vitro* maturation and fertilization of prepubertal goat oocytes. Theriogenology 43, 473-485.

MARTINO A., SONGSASEN N., LEIBO S.P., 1996. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 54, 1095-1069.

MOCÉ E., VICENTE J.S., 2001. Test homólogo de penetración zonal para evaluar semen fresco y congelado de conejo. III Congreso Ibérico de Reproducción Animal, Oporto, Portugal, pp. 415-420.

NIWA K., PARK C.K., OKUDA K., 1991. Penetration *in vitro* of bovine oocytes during maturation by frozen-thawed spermatozoa. J Reprod Fertil 91, 329-336.

OTOI T., TACHIKAWA S., KONDO S., TAKAGI M., SUZUKI T., 1993. Developmental capacity of bovine oocytes frozen in different cryoprotectants. Theriogenology 40, 801-807.

PARRISH J.J., SUSKO-PARRISH J.L., FIRST N.L., 1984. Effect of *swim-up* separation and heparin pretreatment of frozen-thawed spermatozoa on *in vitro* fertilization of bovine oocytes. Biol Reprod 32(1), 112.

PÉREZ-PE R., STROM HOLST B., MUIÑO-BLANCO T., SÖDERSQUIST L., RODRÍGUEZ-MARTÍNEZ H., 2000. Development of a sperm zona pellucida binding assay for ram semen: Preliminary results. Proc of the 14th Int. Congr. Anim. Reprod. Stockholm (Sweden), pp 2-14.

SANCHEZ-PARTIDA L.G., WINDSOR D.P., EPPLESTON J., SETCHELL B.P., MAXWELL W.M., 1999. Fertility and its relationship to motility characteristics of spermatozoa in ewes after cervical, transcervical, and intrauterine insemination with frozen-thawed ram semen. J Androl 20, 280-288.

VAJTA G., HOLM P., KUWAYAMA M., BOOTH P.J., JACOBSEN H., GREVE T., CALLESEN H., 1998. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev 51, 53-58.

VAN BLERKOM J., 1991. Cryopreservation of the mammalian oocyte. In: Animal applications of research in mammalian development (Pederson R.A., McLaren A., First N.L., eds). Cold Spring Harbor: Lab Press, pp. 83-120.

VERSTEGEN J., IGUER-OUADA M., ONCLIN K., 2002. Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology 57, 149-179.

VINCENT C., JOHNSON M.H., 1992. Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. In: Oxford reviews of reproductive biology (Milligan S.R., ed). Oxford Univ Press, pp. 73-100.

ZHANG B.R., LARSSON B., LUNDEHEIM N., HÄÄRD M.G.H., RODRÍGUEZ-MARTÍNEZ H., 1999. Prediction of bull fertility by combined *in vitro* assessments of frozen-thawed semen from dairy bulls entering an AI-program. Int J Androl 22, 253-260.

ZHANG B.R., LARSSON B., LUNDEHEIM N., RODRÍGUEZ-MARTÍNEZ H., 1998. Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. Int J Androl 21, 206-217.

ZHANG BR., LARSSON B., RODRÍGUEZ-MARTÍNEZ H., 1995. Influence of batches of bovine oocytes on the outcome of an intact zona pellucida binding assay and *in vitro* fertilization. Int J Androl. 18, 213-220.