EFFECT OF DONOR STRAIN AND MATURATION STAGE OF RABBIT OOCYTES ON RESULTS OF PENETRATION TEST OF RABBIT SEMEN

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ABSTRACT: The objective of this work was to develop a homologous in vitro penetration test to evaluate the behaviour of rabbit sperm. Three treatments were applied to sperm (fresh, treated with heparin and frozen with a DMSO-sucrose extender), and two types of oocytes (immature or in vitro matured) belonging to four different rabbit lines were used. The test was performed in TCM-199, under the following conditions: 37°C, 5% CO_2 and saturated humidity for 6 hours. After incubation, oocytes were denuded and they were observed under microscope to record the number of spermatozoa/oocyte. Results obtained showed significant differences between all the treatments of sperm in the percentage of oocytes with adhered spermatozoa (80%, 57%, 28% oocytes with adhered spermatozoa for fresh, treated with heparin and frozen semen, respectively, P<0.05). At the same time, larger number of sperm/penetrated oocyte were observed for fresh semen than for the other treatments (21.3± 3.7, 3.9 ± 0.6 and 2.3 ± 0.2 sperm/oocyte for fresh, treated with heparin and frozen semen, respectively, P<0.01). The resistance of fresh semen to the incubation conditions was higher than that of frozen or treated with heparin semen, which could determine the differences observed between them in the results of the test. From the results obtained, it could be concluded that immature oocytes could be used to evaluate the behaviour of rabbit sperm, since even fresh semen were capable to adhere to the surface of these oocytes; on the other hand, further studies are necessary to improve the resistance at the incubation conditions of frozen semen, which will probably permit to predict its fertility *in vivo*.

Key words: rabbit, semen, oocyte, penetration test.

RÉSUMÉ: Effet de la souche donneuse et du stade de maturation des oocytes de lapin sur les resultats du test de pénétration de la semence de lapins.
L'objectif de ce travail était de développer un test *in vitro* homologue de pénétration des oocytes, pour évaluer le comportement des spermatozoïdes de lapins. Trois traitements ont été appliqués sur la semence (fraîche, traitée avec de l'héparine, ou congelée avec un dilueur contenant du DMSO et du sucrose) et 2 types d'oocytes (immatures ou maturés *in vitro*) appartenaient à 4 lignées différentes. Le test a été réalisé dans du TCM-199 dans les conditions suivantes : 37°C, 5% de CO2, atmosphère saturée en humidité pendant 6 heures). Après incubation, les oocytes ont été dénudés et observés sous un microscope pour enregistrer le nombre de spermatozoïdes par oocyte. Les résultats obtenus mettent en évidence des différences significatives entre tous les traitements de la semence sur le pourcentage d'oocytes présentant des spermatozoïdes accolés (80%, 57% e t 28 %, respectivement pour la semence fraîche, traitée avec l'héparine ou congelée, p<0.05). En même temps, un plus grand nombre de spermatozoïdes/ oocyte pénétré est observé pour la semence fraîche comparé aux autres traitements (21.3± 3.7 *vs* 3.9 ± 0.6 et 2.3 ± 0.2 spermatozoïdes/oocytes respectivement pour la semence fraîche, traitée avec l'héparine ou congelé p<0.01). Une meilleure résistance de la semence fraîche aux conditions d'incubation pourrait expliquer les différences obtenues. On peut conclure que les oocytes immatures peuvent être utilisés pour évaluer le comportement des spermatozoïdes de lapins, puisque même les spermatozoïdes éjaculés sont capables d'adhérer à la surface des oocytes. Cependant, de nouvelles études seront nécessaires pour améliorer la résistance des spermatozoïdes aux conditions d'incubation de la semence préalablement congelée, afin de prédire la fertilité *in vivo*.

Mots clé : lapin, semence, oocyte, test de pénétration.

INTRODUCTION

After insemination, fertility and prolificacy depend on both, the physiological status of the doe and the quality and the number of the inseminated dose. Different tests have been developed to determine *in vitro* the sperm quality in order to predict its behaviour after insemination. Motility evaluation (subjective or computer-assisted analysis), morphological evaluations such as percentage of abnormal spermatozoa or percentage of intact acrosomes, or evaluation of the membrane integrity by means of dye exclusion (eosin-nigrosin staining or fluorescent probes) or percentage of swollen sperm after incubation in hypo-osmotic solutions (hypo-osmotic swelling test, where the functional integrity of sperm membrane is evaluated) have been an excellent tool to eliminate infertile or sub fertile ejaculates, but for fertile samples, they do not allow to distinguish between fertility levels. Biochemical probes such as lactate deshydrogenase activity,

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glutamic oxalo-acetic transaminase, Ca^{2+} flux or different fluorescent staining like CTC (Chlortetracycline staining) or FITC-PSA or FITC-PNA staining can be useful to show alterations or progression in capacitating process and a relationship between the values that are obtained and the storage period of the sperm could exist. There are biological probes to evaluate the quality of semen *in vitro*, for example cervical mucus penetration, sperm chromatin decondensation, egg yolk membrane binding assays, oocyte binding assays or IVF.

Several authors have found significant relationships between fertility and the results obtained with some of these tests, but the reports have usually been contradictory, as Graham *et al.*, (1980) reported in an extensive review. However, it seems that biological probes such as oocyte binding assays or IVF evaluate fertility *in vitro* better than the other assays, because in these tests several steps which occur during fertilization process (sperm capacitation, binding to the zona pellucida and penetration of the zona pellucida, membrane fusion, sperm head decondensation, male pronucleus formation and even embryo development) are analysed. Nevertheless, some aspects of these biological tests difficult their application for example, an abundant supply of oocytes is required, the type of oocyte (homologous or heterologous, immature or mature) and environmental conditions of tests (treatment of spermatozoa, chemical composition of media, temperature and period) have to be determined. The first tests were developed with zona free hamster oocytes for semen of different species: guinea pig (Yanagimachi, 1972), human (Yanagimachi, 1976), bovine (Eaglesome and Miller, 1988), goat and pig (Berger *et al.*, 1994 and 1996). Although a few studies on breeding species have reported results predicting the fertility of semen, zona-free hamster oocytes do not seem to be a good model since they do not evaluate the initial steps of the fertilization process. Since 1990, several studies have been done using homologous oocytes to asses the fertilising ability of semen of breeding species. The advances in recovery and maturation of oocytes have allowed doing some works using both immature or mature oocytes, obtaining good results (Ivanova and Mollova, 1993; Graule *et al.*, 1995; Codde and Berger, 1995, Martinez *et al*, 1993 and 1996 in pig).

The aim of this study was to develop a homologous oocyte penetration test comparing the behaviour of fresh and frozen semen using immature and *in vitro* matured oocytes.

MATERIAL AND METHODS

Oocyte recovery

Nine hundred and ninety-seven immature oocytes were recovered from slaughtered does belonging to 3 maternal rabbit strains (V, H, T) and one selected for high growth rate (R). Recovery was carried out by antral follicular punction (size>1mm) with a needle 25GA5/8 connected to a 5 ml syringe with DPBS. The oocytes were scored and only those with compact cumulus complex (COC's) and dark and homogenous ooplasme were used in this study. COC's were washed twice (first with Dulbecco's Phosphate Buffered Saline- DPBS, and later with Medium 199 with Earle's salts and $_{L}$-glutamine- TCM199, Sigma), and after that, they were transferred to 100 μl droplets of maturation media or penetration media under mineral oil. The donor strains were recorded.

Semen treatment and evaluation

Semen was collected from 7 bucks by artificial vagina; all of them belonged to a maternal line (line V). Motility and normal apical ridge (NAR) were evaluated under a microscope with phase and interferential contrast, and concentration was measured using a Thoma-Zeiss counting cell chamber. A solution of glutaraldehyde (concentration 0.25%) was used to fix the spermatozoa in order to determine

the concentration and for the morphological evaluations; for morphology evaluation one hundred cells were counted in each sample, and the evaluation was performed as described by PURSEL and JOHNSON (1974). Only ejaculates with motility higher than 70% were used. Semen from each male was used in each of the three treatments (fresh, treated with heparin or frozen).

The sperm treated with heparin was incubated for 3 hours in TCM199 with heparin (50µg/ml) under the following conditions: 37°C, 5% CO_2 and saturated humidity; the concentration of heparin was the same which is used in other species (in goat sperm, IZQUIERDO *et al.*, 1998). The sperm that was going to be used as fresh semen, was incubated under the same conditions that were used for sperm treated with heparin.

To freeze the sperm, concentration was measured with a Thoma-Zeiss counting cell chamber, and concentration was adjusted to 120 million sperm/ml with a Tris-citric acid-glucose extender (VIUDES DE CASTRO *et al.*, 1999). After that, sperm was diluted (dilution 1:1) with a freezing extender composed by Tris-citric acid-glucose, and 3.5M of DMSO and 0.1M of sucrose were added as cryoprotectants (VICENTE and VIUDES-DE-CASTRO, 1996). Sperm was packaged in 0.5 ml plastic straws (IMV, France) and they were sealed with modelling paste. The freezing protocol consisted of two phases: 45 minutes at 5°C followed by a stage in a freezer at –30°C for 30 minutes and later they were plunged into liquid nitrogen. Straws were thawed in a water bath at 50°C for 15 seconds. To remove freezing media semen was centrifuged at 600g for 5 minutes and pellet was resuspended in 0.1 ml of TCM-199. Then the motility and the acrosomal integrity were evaluated under a microscope with phase and interferential contrast at 400 and 750x respectively.

Culture of oocytes

Oocytes were matured in a medium consisting of TCM-199 with Earle's salts supplemented with 20% FCS (Foetal Calf Serum) and 5 IU/ml hCG. Ten to twenty immature oocytes were placed in each droplet (50 µl) under mineral oil and incubated at 37°C, 5% CO_2 and saturated humidity for 24 hours.

Six to ten immature and *in vitro* matured oocytes were placed in droplets (100µl) and were co-incubated with 1 million of spermatozoa (fresh, fresh treated with heparin or frozen) at 37°C, 5% CO_2 and saturated humidity for 6 hours.

Evaluation of zona penetration test

Motility and acrosomal integrity were evaluated at the end of the assays, taking a sample from the droplet of the test. To evaluate the number of spermatozoa per oocyte, cumulus-oocyte-complex was desegregated by vortex and denuded oocytes were placed in slides to observe them under phase microscopy at 750x.

Artificial insemination

Multiparous receptive does belonging to three different rabbit lines selected for maternal characteristics (lines A, V and H) were induced to ovulate with 0.8 µg of busereline acetate (Hoechst) and were inseminated with fresh or frozen spermatozoa. Fertility and prolificacy were recorded per buck.

Statistical analysis

To analyse the percentage of penetrated oocytes, a Logistic Model was used, and the effects of buck, donor strain, type of oocyte (immature and *in vitro* matured) and treatment of semen (fresh, treated with heparin and frozen) were evaluated.

To analyse the number of spermatozoa per oocyte a General Lineal Model (GLM, SAS Institute, 1997) was used. Only those oocytes which presented bound sperm were included in the analysis. In a preliminary analysis, type of oocyte (*in vitro* matured or immature)

and the interaction between type of oocyte and treatment of sperm was not significant, so they were removed from the analysis; NAR and motility before the incubation period were included as covariates in the model, and they were not significant, so they were removed from the analysis. Donor strain and treatment of semen (fresh, treated with heparin and frozen) were used as fixed effects, buck into treatment of sperm was used as random effect and the interaction between treatment of semen and donor strain was included in the model. NAR and motility after incubation were used as quantitative factors. The Tukey test was used to compare means.

RESULTS

The percentage of penetrated oocytes after 6 hours of incubation was 43.6% (434/997). The analysis showed that this penetration rate was significantly affected by the fixed factors semen and oocyte type (P<0.001, Table 1) and slightly affected by donor strain (P<0.10, Table 1). The seminal parameters acrosomal integrity and motility rates before the incubation and motility rate after incubation had an estimated coefficient statistically significant (-0.005±0.001, P<0.001, 0.003±0.001, P<0.01 and 0.008±0.002, P<0.001, respectively). It seems that a high motility at the end of the test and a low damage of acrosomes at the beginning of the assay increased the number of penetratred oocytes.

The highest percentage of penetrated oocytes was obtained with fresh semen *versus* sperm treated with heparin and frozen sperm (Table 2); this difference could be explained by the higher motility rate before and after incubation of the fresh semen compared to the motility of treated with heparin and frozen sperm (83% and 45% *vs* 70% motility at the beginning and 44%, 15% and 1% motility at the end of the test for fresh, treated with heparin and frozen, respectively, Table 5). In addition to this, frozen semen had the minor percentage of acrosomal integrity before incubation (48% *vs* 95 and 93%, fresh and capacitated, Table 5).

Other important factor to define the condition of a fast penetration test is the status of oocyte, in this work a higher percentage of oocytes with adhering spermatozoa was observed for *in vitro* matured oocytes than immature ones (51.4 *vs* 42.3, P<0.05). Nevertheless, this difference was due to the frozen sperm, since only in this group the percentage of oocytes with bound sperm was higher for the mature oocytes than for the immature ones. The ability of oocytes to favour the adherence of frozen spermatozoa seems to be developed by *in vitro* maturation.

The number of sperm per oocyte (Table 3) was significant affected by treatment of sperm, and donor

Table 1: Evaluation of qualitative and quantitative factors from logistic regression in percentage of penetrated oocytes.

Source	Significance
Semen type	***
Donor strain	+
Oocyte type	**
Acrosomal integrity percentage pre-incubation	***
Motility pre-incubation	**
Motility at the end of the test.	***

*** P<0.001; **P<0.01; *P<0.05; +P<0.1

strain and their interaction. The random effect of buck into treatment of semen was not significant in this experiment. From quantitative parameters only the motility and normal acrosome rate at the end of the assay had a significant estimated coefficient (1.08± 0.20 and −1.12± 0.38 respectively). These parameters were relatively high in fresh semen (44% and 83%, respectively, Table 5), moderated in semen treated with heparin (15% and 87% respectively, Table 5) and very low in frozen semen (1% and 1%, respectively, Table 5). The low viability of frozen semen after the incubation at 37°C seemed to be determinant in the low penetration rate they presented.

Fresh semen showed more spermatozoa adhered per oocyte than semen treated with heparin and frozen semen (Table 4). When COC's were incubated with frozen semen, 1 to 4 spermatozoa began to penetrate the zona pellucida in 27.6% of COC's. The behaviour of frozen semen and sperm treated with heparin were not affected by the donor strain probably being the effect which led to a low number of bound sperm their very low resistance to the incubation conditions. With respect to fresh sperm, a significant interaction between donor strain of the oocytes and the sperm was observed (Table 4), obtaining for the strain T the highest number of bound spermatozoa/ oocyte.

The sperm used in this work had been used to inseminate females from different experiments. For fresh semen high fertility and prolificacy rate were obtained (79% and 8.3 alive kits) meanwhile results obtained using frozen semen were, in general, lower. Results observed for frozen sperm from the males are summarized in Table 6; differences between males were observed for fertility and kindling rate as well as for total number and born and alive kits.

With respect to the type of oocyte (immature or *in vitro* matured), no significant differences were observed between them in the number of sperm/oocyte for any of the treatments of sperm. However, a larger number of bound sperm were observed in all the groups for immature oocytes (22.5±3.9, 4.2±0.7,

Table 2: Effect of semen and oocyte type and donor strain on percentage of penetrated oocytes. Number of oocytes between brackets.

Treatment sperm	Donor strain				Oocyte type		TOTAL
	V	H	R	T	Immature	*In vitro* matured	
Fresh	84.4 (45)	78.3 (115)	80.0 (10)	80.5 (41)	82.0 (194)	58.8 (17)	80.1^{a} (211)
Heparin	78.0 (50)	45.7 (46)	50.0 (12)	44.4 (36)	58.5 (128)	43.8 (16)	56.9^{b} (144)
Frozen	28.0 (150)	29.9 (278)	22.3 (179)	34.3 (35)	26.0 (601)	51.2 (41)	27.6^{c} (642)
Total	48.6 (245)	44.2 (439)	26.9 (201)	54.5 (112)	42.3^{A} (923)	51.4^{B} (74)	43.6 (997)

a, b, c: values in the same column with different superscript are statistically different (P<0.05).
A, B: values in the same row with different superscript are statistically different (P<0.05).

Table 3: Evaluation of qualitative and quantitative effects on the number of sperm per oocyte.

Source	Significance
Semen type	**
Donor strain	***
Semen type x donor strain	***
Acrosomal integrity percentage at the end of the test.	**
Motility at the end of the test.	*

*** P<0.001; **P<0.01; *P<0.05

2.4±0.2 spermatozoa/oocyte for fresh, heparinized and frozen sperm, respectively, data not shown in tables) than for *in vitro* matured oocytes (2.4±0.7, 1.1±0.1, 1.6±0.3 spermatozoa/oocyte for fresh, heparinized and frozen sperm, respectively, data not shown in tables).

DISCUSSION

A higher percentage of oocyte with bound sperm were observed for fresh semen than for sperm treated with heparin or frozen sperm (Table 2). At the same time, larger number of bound spermatozoa/oocyte were observed for the fresh semen than for sperm treated with heparin and frozen sperm (Table 5). OVERSTREET and BEDFORD (1974a), working with ovulated oocytes and non-capacitated or *in vivo* capacitated sperm observed more capacitated sperm bound to the zona pellucida than non-capacitated semen (33.7 capacitated *vs* 3.8 non-capacitated sperm/oocyte). They suggested that the capacitation of the sperm could facilitate their passage trough the granulosa cell investment, but capacitation would not change the adhesive properties of the sperm head, since both types of sperm adhered at the same level when denuded oocytes were used. Our observations differ from their reports, probably due to differences in the capacitation of the spermatozoa (*in vivo vs* treated with heparin), in the type of oocytes used (*in vivo* matured *vs* immature or *in vitro* matured), or in the incubation conditions (*in vivo*, 3 hours *vs in vitro*, 6 hours). Fresh semen presented more sperm bound/oocyte than frozen sperm; this has been seen before by other authors in different species (in pigs, CLARKE and JOHNSON, 1987 and in horses, DOBRINSKI *et al.*, 1995).

What remains clear is that the behaviour of frozen and sperm treated with heparin is similar (Table 4). The longevity of frozen and sperm treated with heparin was lower than that of fresh semen, and this could be the reason for their lower adherence to the oocytes. Fertility of cryopreserved sperm is usually lower than that of fresh semen, being necessary to increase the number of frozen sperm inseminated to achieve the fertility levels obtained with fresh semen (SHANNON and VISHWANATH, 1995). Several causes for the reduced fertility of frozen sperm have been suggested (impaired transport and poor survival in the female reproductive tract, YOSHIDA, 2000). This is due to sub-lethal cryodamage produced by the freeze-thaw procedure, which leads to a premature capacitation of the sperm, reducing its functional life span (for a review, see BAILEY *et al.*, 2000). The generation of

reactive oxygen species (ROS) during cryopreservation process of ram and bull sperm reduces the motility and viability of the remaining living sperm (UPRETI *et al.*, 1998); this could have been the reason for the reduced longevity of frozen sperm in the present experiment.

Although acrosome reaction is a pre-requisite to penetrate the zona pellucida by sperm in mammalian species, spermatozoa with intact acrosomes adhering to the zona pellucida have been seen to indent it deeply sometimes in rabbit and human (data not published and BEDFORD, 1998). This study demonstrated, at least, that immature or *in vitro* matured rabbit oocytes allowed the adherence of fresh semen which had the highest value of morphologically intact acrosomes (95%), but zona pellucida did not seem to have been penetrated, since no heads of spermatozoa were observed in the ooplasm. Working with boar sperm and ovulated or immature pig oocytes, MARTÍNEZ *et al.*, (1993) observed that immature oocytes presented decondensed sperm into the ooplasm (so immature pig oocytes allow spermatozoa penetration), but there was no male pronucleus formation (since the oocyte ooplasm was immature).

In our study, less sperm was adhered to oocytes which had been matured previously *in vitro*. OVERSTREET and BEDFORD (1974b) observed that the penetrability of the granullosa cell investment or the zona pellucida did not change for immature or ovulated oocytes when working with capacitated (*in vivo*) rabbit sperm (59% mature *vs* 66% immature oocytes penetrated), however, none of the immature oocytes presented spermatozoa in the ooplasm. The difference between ovulated and immature oocytes seems then to be in the vitelline surface and in the ability of the ooplasm of ovulated oocytes to induce decondensation of the fertilizing sperm nucleus and male pronucleus formation (OVERSTREET and BEDFORD, 1974b). It has been observed that oocytes matured under inadequate culture conditions are less penetrable by spermatozoa (MARTINEZ *et al.*, 1993); this could be the reason why in our study, less sperm adhered/*in vitro* matured oocyte were observed. The high percentage of intact acrosomes at the end of the test in the fresh semen and the sperm treated with heparin could have been due to inadequate culture conditions when *in vitro* matured oocytes were used or to the lack of maturation of the zona pellucida in the immature oocytes; in the case of fresh semen, capacitating agents were not included in the co-incubation medium, so this could be the reason why very few spermatozoa were acrosome reacted at the end of the test. However, for sperm treated with heparin, the high percentage of spermatozoa with intact acrosomes could have been

Table 4: Effect of semen and donor strain and their interaction on the number of spermatozoa/oocyte (mean ± standard error). Number of oocytes between brackets.

	Donor strain				
Treatment semen	V	H	R	T	TOTAL
Fresh	13.4 ± 2.11^{bc} (38)	16.1 ± 3.98^{b} (90)	2.8 ± 0.84^{bc} (8)	49.1 ± 14.55^{a} (33)	21.3 ± 3.71^{A} (169)
Capacitated	5.1 ± 0.87^{bc} (39)	1.8 ± 0.27^{bc} (21)	7.8 ± 6.04^{bc} (6)	2.4 ± 0.40^{bc} (16)	3.9 ± 0.62^{B} (82)
Frozen	3.6 ± 0.60^{bc} (42)	2.1 ± 0.21^{c} (83)	1.7 ± 0.25^{bc} (40)	1.8 ± 0.32^{bc} (12)	2.3 ± 0.19^{B} (177)
Total	7.2 ± 0.85^{d} (119)	8.5 ± 0.91^{d} (194)	2.5 ± 0.71^{d} (54)	27.6 ± 8.38^{e} (61)	10.1± 2.95 (428)

a, b, c: Values with different superscript are statistically different (P<0.001). A, B: Values in the same column with different superscript are statistically different (P<0.01). d, e: Values in the same row with different superscript are statistically different (P<0.001).

Table 5: Results obtained for motility and percentage of intact acrosomes before and after the test for fresh spermatozoa, sperm treated with heparin and frozen sperm.

	Motility (%)		NAR (%)	
	Before incubation	After incubation	Before incubation	After incubation
Fresh	83	44	95	83
Heparin	70	15	93	87
Frozen	45	1	48	1

NAR: Normal apical ridge

due to a failure in the detection of the state of the capacitation process (which is better evaluated by fluorescent staining such as the CTC staining, Fraser *et al.*, 1995); on the other hand, few works have been done to determine the optimal concentrations of different capacitating substances for rabbit sperm, and optimal concentration vary between species (since in bovine sperm, concentrations of 10 mg of heparin are habitually used, Parrish *et al.*, 1988). Further studies are necessary to determine the optimal conditions to capacitate rabbit spermatozoa.

With respect to the genetic origin of the oocytes, more spermatozoa adhered per oocyte were observed for fresh semen in the oocytes from line T. The reason is not known, maybe some differences in the composition of zona pellucida could exist; in boars, differences in follicle maturation have been observed between Meishan and Large-White hybrid pig which could lead to an improved ability of Meishan oocytes to support oocyte maturation and fertilization *in vitro* (Xu *et al.*, 1998).

Fresh and frozen semen from bucks used in this experiment were tested *in vivo* in different experiments; for fresh semen high fertility and prolificacy rate were obtained. In accordance to the low resistance at the incubation, the results obtained using frozen semen were lower (61% fertility at birth and 4.8 alive kits, Mocé *et al.*, 2002a). However, when frozen semen was used to inseminate, differences between the bucks in fertility rate and in prolificacy were observed in this study (Table 6) as well as in a preliminary work (Mocé *et al.*, 2002b); we observed two groups of males depending on their fertility rate: those which had more than 60% fertility rate (3 males) and 4 males which had less than 50% fertility rate. Differences between these groups were also observed *in vivo* in the total number born and in the alive born. However, in this study we did not observe differences in the behaviour of frozen sperm from different males after *in vitro* co-culture with oocytes.

In conclusion, the results for the percentage of penetrated oocytes were affected by the treatment of

Table 6: Fertility, kindling rate and ratios of total and live born by kindling female after artificial insemination with frozen sperm. Means ± standard errors.

Fertility group	Number of females inseminated	Fertility rate (number)	Kindling rate (Number)	Total born/ kindling female (LSM ±SE)	Live born/ kindling female (LSM ±SE)
>60% (3 males)	147	65%[a] (96)	63%[a] (93)	7.6 ± 0.4[a]	7.1 ± 0.4[a]
<50% (4 males)	359	46%[b] (164)	40%[b] (145)	6.2 ± 0.3[b]	5.8 ± 0.3[b]

a, b: values in the same column with different superscripts are statistically different. (P<0.01).

sperm used, and by the strain donor (in fresh semen). Taking into account the results observed in the present work, the use of immature oocytes to evaluate rabbit semen seems to give promissing results. To our knowledge, this is the first study carried out to try to develop a homologous oocyte penetration test to evaluate the quality of rabbit semen *in vitro*. However, further studies are necessary to improve the resistance at the incubation of frozen semen which will probably permit to predict their fertility *in vivo*, since these test offer additional information about the behaviour of the sperm (they evaluate gamete contact), moreover if it has been conserved.

Acknowledgements: This work was supported by CICYT project n° AGF98-0470-C02-01. The authors thank Mr. Neil Macowan for revising the English version.

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