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PRELIMINARY RESULTS OF INTRA CYTOPLASMIC SPERM INJECTION (ICSI) APPLIED ON RABBIT : A TECHNICAL NOTE.

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ABSTRACT: In a foundation process of a maternal rabbit line using high selection intensities, from some hyper prolific females, selected as embryo donors, the subjects were very old and in an unhealthy condition (24%). An *in vitro* procedure to obtain embryos from this kind of females would be desirable. Some technical improvements used for human intracytoplasmic sperm injection (ICSI) have resulted in an enhancement of cleavage rates. This method may be applied to rabbits. Spermatozoa from ejaculated washed semen samples were immobilised in 5% PVP droplet by touching the tail and aspirated tail first into the microinjection pipette. The positioned oocyte was punctured. Some ooplasm was aspirated. After rupturing the plasmalema by gentle aspiration, the withdrawn ooplasm and the sperm were inserted into the oocyte. The cleavage rate of intact (86/107; 80%) oocytes was checked at 24 hours after ICSI (39/107; 36%). *In vitro* development up to the morulae (22/39; 56%) and blastocyst stage (5/39; 13%) were obtained.

RESUME : Premiers résultats d'injection intracytoplasmique de sperme (ICSI) chez le lapin : note technique.
Chez le lapin, dans la procédure permettant d'obtenir une lignée maternelle en utilisant la sélection intensive à partir de femelles très prolifiques, sélectionnées comme donneuses d'embryons, les animaux deviennent trop âgés et sont dans de mauvaises conditions sanitaires (24%). Il paraît préférable d'utiliser un procédé in vitro *pour obtenir les embryons de telles femelles. Des améliorations techniques utilisées pour l'injection intracytoplasmique du sperme chez l'homme (ICSI) ont amélioré le taux de division. Cette méthode peut être appliquée chez le lapin. Les spermatozoïdes provenant d'échantillons d'éjaculats lavés sont immobilisés, dans une goutte d'une solution contenant 5% de PVP, en touchant la queue et en les aspirant par la queue dans une micro pipette à injection. L'ovocyte repéré est ponctionné. Un peu d'ovoplasme est aspiré. Après avoir rompu le plasmalema par une légère aspiration, l'ovoplasme et le sperme sont injectés dans l'ovocyte. Le taux de division d'ovocytes intacts (86/107 ; 80%) est contrôle 24 heures après l'ICSI (39/107 ; 36%). Le développement* in vitro *jusqu'au stade morula (22/39 ; 56%) et blastocyste (5/39 ; 13%) a été atteint.*

INTRODUCTION

A new maternal line (called HH) has recently been founded, applying hyper prolific selection principles and embryo cryopreservation techniques (CIFRE *et al.*, 1997 a, b). Although the foundation procedure was satisfactory, some detected hyper prolific females were very old and in a very unhealthy condition. In fact, no normal embryos could have been obtained from these kind of females (24% ; 33/136 females; GARCIA-XIMENEZ *et al.*, 1996). This fact has contributed to decreasing the global efficiency of the foundation process.

In previous works, it has been described that every female contains preovulatory follicles in their ovaries, capable of being ovulated after hCG treatment, regardless of reproductive condition (with or without corpora lutea : ARMERO *et al.*, 1991; GARCIA-XIMENEZ *et al.*, 1996); furthermore, these follicles are susceptible to ovulation after hCG treatment. Moreover, these follicles would contain fertilizable oocytes (THIBAULT and GÉRARD, 1973). Thus, in order to avoid similarly losing genetic origins, in subsequent foundation-processes using similar genetic selection methodology, complementary *in vitro* embryo production and embryo transfer-related techniques would be considered.

Oocytes recovered at 9-12 h post-hCG (whether from intrafollicular and/or ovarian surface) have been fertilised by standard *in vitro* procedures, using either *in vivo* or *in vitro* capacitated spermatozoa. The *in vitro* fertilisation rates with in vivo capacitated spermatozoon were 43.7-81% (BRACKETT *et al.*, 1972; MILLS *et al.*, 1973; OH *et al.*, 1975; TROTNOW *et al.*, 1981); however, high variability has been obtained amongst experiments (7% to 96%; BRACKETT *et al.*, 1972). One additional objection against using in vivo capacitated spermatozoa is the number of required capacitator-does (three or more), due to the variability in number and quality of recovered spermatozoa. Moreover, the *in vitro* embryo development up to the blastocyst stage was poor and also variable (5% to 26% referred to oocytes sperm co-incubated; TROTNOW *et al.*, 1981). So, *in vitro*-fertilisation failure could be a reflection either of variability in oocyte maturation (BRACKETT *et al.*, 1972) and/or at worst, reflection of sperm quality (ROUDEBUSH *et al.*, 1993).

In vitro oocyte maturation could exert detrimental effects on ovum coats and/or nuclear and cytoplasmic response, affecting, in turn, both penetration as male pronuclear formation and subsequent embryo development. Recent technology to solve human sterility (male-factor related) is based on bypassing the zona pellucida and also the oolema. This fertilisation

technique, referring as Intracytoplasmic Sperm Injection (ICSI), was first documented in rabbits by HOSOI (1988) ; and later, it was extended to other productive species (cattle: GOTO *et al.*, 1990 ; KEEFER *et al.*, 1990 ; pig : IRITANI *et al.*, 1992). Monospermic-fertilisation, regardless of Fertilising spermatozoa condition, is the main application of ICSI. After injection of a single spermatozoa into the ooplasm of rabbit ova, incubated rabbit sperm was more likely to form a male pronucleus than non-incubated sperm (45% vs. 30% respectively ; KEEFER, 1989). Alternatively, mechanical manipulation of each human spermatozoa before injection could enhance their ability to undergo pronuclear formation (CATT and O'NEILL, 1995 ; TESARIK and SOUSA, 1995).

On the other hand, the usual cause of cleavage failure and subsequent poor embryo development, would be attributed to insufficient oocyte activation. It has recently been suggested that some aspiration of cytoplasm until rupture of the ooplasm may exert a beneficial effect on human oocyte-responsiveness (e.g. oocyte activation : TESARIK and SOUSA, 1995). Moreover, this added manipulation ensures a correct injection and gamete mixing, compared to the mere sperm placing (HOSOI *et al.*, 1988; KEEFER , 1989). In the same sense, the presence of calcium ions in both spermatozoa suspension and microinjection medium is needed to sustain spermatozoa motility and to induce oocyte activation (PALERMO *et al.*, 1995; TESARIK and SOUSA, 1995).

The aim of this work was to evaluate in rabbits the improvements in ICSI procedure derived from human technology, using high quality oocytes (i.e. metaphase II, MII). Preliminary rabbit-ICSI results obtained during the period of training are summarised in this technical note.

MATERIAL AND METHODS

Oocyte Recovery

New Zealand adult virgin does were used as oocyte donors. Ova were collected 14 hours after an endovenous hCG injection (20 IU, Coriogan, Ovejero, Spain) by flushing oviducts with phosphate buffered saline (DPBS ; Sigma, Spain, Cat. N° :D-5773) and 0.3% antibiotic solution (Biomicina 1 :1, Ovejero Lab., Spain). The recovered oocytes were washed twice in DPBS.

Oocytes with corona-cumulus-complex were rinsed and kept in DPBS plus 20% (v/v) ovulated-doe serum at 37°C in air until injection time (maximum for 2-3 hours after recovery).

At the injection time, 5-8 oocytes per batch were denuded of their surrounding cumulus cells utilising 0.2% (w/v) hyaluronidase (Type IV, Sigma, Spain, Cat. N°. : H-4272) in DPBS without Ca^{2+}. Then, denuded oocytes were washed twice in DPBS. Only normal metaphase II oocytes (MII) containing first polar body and normal appearance were included in the experiment.

Semen Processing

Ejaculates were collected using an artificial vagina. Semen samples (0.5-1.0 ml) were washed twice with 3 ml defined-medium (DM ; BRACKETT and OLIPHANT, 1975) without bovine serum albumin (BSA) by centrifugation at 360 g for 5 min at 30°C. The final pellet was re-suspended in 1 ml DM without BSA and spermatozoa concentration was adjusted at 10^6 cells per ml. Spermatozoa were incubated until injection time at culture condition (39°C, 7% CO_2 in humidified atmosphere). The total incubation period was not higher than three hours.

Approximately 100µl from this sperm suspension was added to 100 µl DPBS droplet containing 10% (w/v) polyvinilpyrrolidone (PVP-360; MW 360 000; Sigma, Spain). This 200 µl 5% PVP solution containing the spermatozoa was used to slow sperm motility, to remove debris and to facilitate aspiration control.

Microinjection Procedure

The cover of disposable Petri dishes (9 cm diameter and 9 mm elevation border) were used as the injection dishes. A 200 µl PVP droplet containing-sperm was put in the middle of the dish and a 0.3 ml elongated DPBS droplet (oocyte droplet) below. All droplets were covered with light weight mineral oil (Sigma, Spain, Cat. N°: M-8410).

Five to eight oocytes from each batch were put in oocyte droplet.

Manipulation was carried out under an inverted phase microscope (Leitz, Germany) at X320 magnification and equipped with ICT optics and micromanipulator sets (Leitz, Germany).

Intra cytoplasmic sperm injection pipettes were made from thin-walled glass capillary tubes (Model G-1, Narishige Co. LTD., Japan) using a micropipette puller (Model PB-7, Narishige Co. LTD., Japan) and a Narishige MF-90 microforge (Narishige Co. LTD., Japan). The tips of micropipettes were opened and bevelled 35° using a Narishige EG-4 microgrinder (Narishige Co. Ltd., Japan). The outer diameter of pipettes was adjusted to 10 µm. The tips were refined by repeated aspirations in 15% fluorhydric acid. Micropipettes were washed six times in bidistillate water and three times in acetone before being heat-dried. The microinjection pipettes were slightly larger than spermatozoa. Using a microforge, a distal spike was formed on the injection pipette. A 45° bent was also formed 5 mm from the tip, to enable micromanipulation.

Holding pipettes were prepared in a similar manner, but their inner and outer diameter was 50 and 200 µm respectively. These pipettes were fire-polished to produce a smooth blunt surface.

For the spermatozoa immobilising process, the injection pipette was first lowered onto the spermatozoon-PVP droplet on the bottom of the injection dish. One spermatozoon was chosen (the most available motile and morphologically well shaped) and immobilised by touching its tail near the mid-piece with the micro pipette. The immobilised spermatozoon was aspirated tail first into the injection pipette. If the tail was dissected or became kinked during the immobilisation process, the procedure was repeated with another spermatozoon (PALERMO *et al.*, 1995).

Oocyte was held in place by suction pressure through a holding pipette. Placing and securing the oocyte in position (polar body at 12 or 6 o'clock position) was defined to produce minimal damage to meiotic spindle (ASADA *et al.*, 1995).

The injection pipette was introduced at 3 o'clock position through the zona pellucida and oolema up to the centre of the oocyte. To ensure cytoplasmic injection, back pressure was used to aspirate the oocyte cytoplasm into the injection pipette until the cytoplasm passed the sperm more freely. When rupturing the plasmalema, an additional aspiration of cytoplasm was necessary to activate the eggs and to optimise the interaction between sperm and cytoplasm (PALERMO *et al.*, 1995; TESARIK and SOUSA, 1995).

The immobilised sperm was inserted together with the withdrawn cytoplasm and the smallest volume of PVP solution possible. After injection, the oocyte was released from holding pipette on the opposite side of the droplet. Up to 5 oocytes were injected with a single, living, immobilised spermatozoon for 30 minutes. A group of oocytes were also micromanipulated as described above, but no spermatozoa were injected. These oocytes served as a control group (sham-ICSI oocytes).

The sham-ICSI and ICSI oocytes were rinsed and incubated in 3 ml culture medium (Ham's F-10 medium plus 20% (v/v) homologous serum) in 35x10 mm tissue culture dishes (Nunclon, Delta, Denmark) at 39°C, in 7% CO_2 in humidified atmosphere.

Table 1.: Damage and cleavage rates of microinjected rabbit oocytes evaluated at 24 hours of culture.

Group	N° Oocytes	Intact (%)	Cleavage *
ICSI	107	86 (80)	39 (36; 45)
SHAM-ICSI	19	18 (95)	3 (16; 16)
	126	104 (83)	

*Values in parentheses are percentages : eggs cleaved per injected oocytes and eggs cleaved per intact oocytes, respectively

The success of fertilisation (number of normal-appearing cleavage) was checked the following morning.

In vitro embryo development to the 2-, 4-cell, morula-early blastocyst and blastocyst stage was recorded at 24, 72 and 96 hours of culture, respectively. Due to the preliminary character of these performed assays, the in vivo viability of *in vitro* produced embryos was not tested.

RESULTS

A total of 126 metaphase II (MII) oocytes was injected (ICSI and sham-ICSI). Twenty-two of the injected oocytes were lysed or degenerated either during the microinjection procedure or in the first 24 hours of culture (17% ; Table 1).

Procedure efficiency was evaluated by the cleavage rate (normal-appearing cleavage division at 24 hours after microinjection). Sperm-injected group showed 45% (39/86) cleavage. In sham-injected oocytes, the cleavage rate was much less (3/18 ; 16%).

The injected-cleaved oocytes were cultured. The development reached up to the 4-cell, morula, compacted morula-early blastocyst and blastocyst stage was 56, 41 and 13% respectively. In contrast, in the sham-operated group, no further development beyond the 8- to 16-cell stage was observed (Table 2).

DISCUSSION

Mechanical damage resulting from the injection procedure (17%) was found to be slightly higher than that obtained in human oocytes (3.6 to 10% : PALERMO

Table 2 : Development reached by the cleaved ICSI or Sham-ICSI oocytes during the culture period.

Group	2-4-cell	Morulae (%)	Compacted morulae - early blastocyst (%)	Hatched blastocyst (%)
ICSI	39	22 (56)	16 (41)	5 (13)
SHAM-ICSI	3	0 (0)	0 (0)	0 (0)

et al., 1996) and lower when compared to pioneer works on rabbits (HOSOI *et al.*, 1988 and KEEFER, 1989 : 37% and 66% respectively). Less damage occurred as practice was acquired throughout this training period.

Independently of application of a sperm capacitation procedure, all authors are in agreement to microinject viable spermatozoa whenever possible, although a whole single motile, immobile (dead) or immobilised sperm would be injected into the oocyte.

HOSOI *et al.* (1988) and KEEFER (1989), injected an incubated-immobile (but not necessarily dead) rabbit sperm into oocytes. Spermatozoa appeared to be immobile when added to 10% PVP (in Ca^{2+}-free medium) solution and manipulated. These authors achieved cleavage rates of 14 and 25% respectively, whereas in our initial study, a forty-five per cent of intact-injected oocytes cleaved. In humans, higher cleavage rates were obtained when a non-incubated motile or immobile sperm was injected (46% HOSHI *et al.*, 1995 ; PALERMO *et al.*, 1995).

Our first results show that ICSI applied to rabbit allows cleavage and even *in vitro* embryo development to the blastocyst stage. Further work has to be done to improve the *in vitro* fertilisation and development rates before incorporating this complementary technique into the subsequent foundation process for new hyper prolific strains.

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REFERENCES

ARMERO E., PERIS-FAJARNES F., GARCIA-XIMENEZ F., VICENTE J.S., 1991. Respuesta ovulatoria a la hCG en presencia o no de cuerpos luteos previos en conejas de eliminación. *ITEA, Vol. 87A (I), 17-22.*

ASADA Y., BAKA S.G. HODGEN G.D. LANZENDORF S.E., 1995. Evaluation of the meiotic spindle apparatus in oocytes undergoing intracytoplasmatic sperm injection. *Fertil. Steril., 64 (2), 376-381.*

BRACKETT B.G., MILLS J.A., JEITLES G.G., 1972. *In vitro* fertilisation of rabbit ova recovered from ovarian follicles. *Fertil. Steril., 23 (12), 898-909.*

BRACKETT B.G., OLIPHANT G., 1975. Capacitation of rabbit spermatozoa *in vitro*. *Biol. Reprod., 12, 260-274.*

CATT J., O'NEILL Ch., 1995. Manipulation of sperm before intracytoplasmic sperm injection improves fertilisation rates. *Fertil. Steril., 64 (6), 1210-1212.*

CIFRE J., BASELGA M., GARCIA-XIMENEZ F., VICENTE J.S., 1997a. Performance of a hyperprolific rabbit line I. Litter size traits. *J. Anim. Breed. Genet., 114 (in press).*

CIFRE J., BASELGA M., GARCIA-XIMENEZ F., VICENTE J.S., 1997b. Performance of a hyperprolific rabbit line II. Maternal and growth performances. *J. Anim. Breed. Genet., 114 (in press).*

GARCIA-XIMENEZ F., VICENTE J.S., CIFRE J., BASELGA M., 1996. Foundation of a maternal rabbit line using hysterectomy and embryo cryopreservation. in : *Proc. 6th World Rabbit Congr., Toulouse July 1996, vol. 2,* 285-288.

GOTO K., KINOSHITA A., TAKUMA Y., OGAWA K., 1990. Fertilisation of bovine oocytes by the injection of immobilised, killed spermatozoa. *Veterinary Record, 127, 517-520.*

HOSOI Y., MIYAKE M., UTSUMI K., IRITANI A., 1988. Development of rabbit oocytes after microinjection of spermatozoa. in : *Proc. of the 11th International Congr. on Anim. Reprod. and A.I., Dublin June 1988. Abstr. 331.*

HOSHI K., YANAGIDA K., YAZAWA H., KATAYOSE H., SATO A., 1995. Intracytoplasmatic sperm injection using immobilized or motile human sperm. *Fertil. Steril.* **63 (6)**, 1241-1245.

IRITANI A., UTSUMI K., HOSOI Y., 1992. Fertilisation by assisted micromanipulation of gametes. in : LAURIA A., GANDOLFI F. (ed). Embryonic development and micromanipulation in animal production. *Portland Press, London.51-58.*

KEEFER C.L., 1989. Fertilisation by sperm injection in the rabbit. *Gam. Res., 22, 59-69.*

KEEFER C.L., ABDEL MONEIM I.Y., BRACKETT B.G., 1990. Cleavage development of bovine oocytes fertilized by sperm injection. *Mol. Reprod.Dev.* **25**, 281-285.

MILLS J.A., JEITLES G.G., BRACKETT B.G., 1973. Embryo transfer following *in vitro* and in vivo fertilisation of rabbit ova. *Fertil. Steril.*, **24 (8)**, 602-608.

OH Y.K., BRACKETT B.G., 1975. Ultrastructure of rabbit ova recovered from ovarian follicles and inseminated *in vitro*. *Fertil. Steril.*, **26 (7)**, 665-685.

PALERMO G.D., COHEN J., ALIKANI M., ADLER A., ROSENWAKS Z., 1995. Intracytoplasmic sperm injection : a novel treatment for all forms of male factor infertility. *Fertil. Steril., 63 (6), 1231-1240.*

PALERMO G.D., COHEN J., ROSENWAKS Z., 1996. Intracytoplasmic sperm injection : a powerful tool to overcome fertilisation failure. *Fertil. Steril., 65 (5), 899-907.*

ROUDEBUSH W.E., FUKUDA A.I., MINHAS B.S., 1993. Enhanced embryo development of rabbit oocytes fertilized *in vitro* with platelet activating factor (PAF)-treated spermatozoa. *J. Assisted Reproduction and Genetics, 10 (1), 91-94.*

TESARIK J., SOUSA M., 1995. Key elements of a highly efficient intracytoplasmic sperm injection technique : Ca^{2+} fluxes and oocyte cytoplasmic dislocation. *Fertil. Steril. , 64 (4), 770-776.*

THIBAULT C., GÉRARD M., 1973. Cytoplasmic and nuclear maduration of rabbit oocytes *in vitro*. *Ann. Biologie Animale, Bioch. Bioph.*, **13** (extra edition), *145-156.*

TROTNOW S., AL-HASANI S., SADTLER C., 1981. Experience with *in vitro* fertilisation of follicular rabbit oocytes and embryo transfer. *Arch. Gynecol. 231, 41-50.*