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First Steps in the Development of a Functional Assay for Human Sperm Using Pig Oocytes

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Abstract

The use of mammalian oocytes to assess human sperm functionality could be a helpful tool with potential applications in clinical and research programs. In an attempt to develop the pig model, the aim of the present work was to study the interaction between human spermatozoa and pig oocytes at the zona pellucida (ZP), the oolemma, and the ooplasm levels. In vitro matured pig oocytes and human spermatozoa from fertile and low-fertility donors were employed. The induction of the acrosome reaction by the ZP, the ability of the sperm to penetrate the oocyte after cocincubation, and the male pronuclear formation after ICSI were evaluated. Human spermatozoa can bind to pig ZP and undergo the acrosome reaction (15% to 58%, depending on the individual); they are not able to fuse with the oolemma but they can decondense and form a male pronucleus (40%–100%) when injected into pig oocytes. In conclusion, this study shows that pig oocytes can be a useful model to assess human sperm functionality.

Key words: Sperm functionality assay, species specificity, sperm-oocyte interaction, heterologous ICSI

Mammalian fertilization is comprised of several ordered steps. It begins in the oviduct with the

interaction between the spermatozoa and the cumulus oophorus cells and binding of spermatozoa to the zona pellucida, continues with the fusion of egg and sperm plasma membranes, and ends with pronuclear formation (Yanagimachi, 1994; Wassarman, 1999).

The zona pellucida (ZP) is the first barrier in the sperm-oocyte interaction. It provides receptors for the attachment and binding of capacitated sperm and is involved in the subsequent induction of the sperm acrosome reaction (AR) (Florman and Storey, 1982; Berger et al, 1989; Urch and Patel, 1991). The ability of the sperm to bind to the ZP and undergo the acrosome reaction has demonstrated a high predictive value for fertilization outcome (Oehninger et al, 2000). The oolemma plasma membrane represents the second hurdle in the spermatozoon-oocyte interaction; it involves a firm cell-cell adhesion that culminates in fusion of the 2 membranes (Miller et al, 2002). The third component is the egg cytoplasm wherein the sperm, after fusion with plasma membrane, will normally form a male pronucleus.

The fertilizing capacity of human spermatozoa is the result of their ability to interact successfully at these 3 levels. Ideally, it could be assessed by depositing spermatozoa in the female genital tract or by in vitro fertilization (IVF) (Yanagimachi et al, 1976), but both approaches seem to be rather difficult in the routine of human infertility centers. The first approach consists of many complicating factors inherent with in vivo techniques, whereas the second one still needs the use of human oocytes, which is ethically and practically limited. Alternatively, mammalian eggs could be used for assessment of human sperm function.

The first useful assay employing hamster eggs was developed by Yanagimachi et al (1976) and used extensively (Rodgers et al, 1979; Liu and Baker, 1992). This assay evaluated the ability of human sperm to cross the plasma membrane of zona-free hamster eggs and develop a male pronucleus; only the information about the ability of the sperm to bind the ZP and undergo the AR was missing.

In spite of some undoubted advantages, the use of rodents presents different problems for the study of the human sperm functionality because rodent fertilization relies on a maternal centrosome (Schatten et al, 1991), whereas the centrosome is introduced by the sperm in humans (Simerly et al, 1995); consequently, human sperm do not undergo microtubule assembly when injected into hamster eggs (Hewitson et al, 1997) and the intracytoplasmic sperm injection (ICSI) is nowadays a principal tool in the clinical routine demanding accurate analysis of sperm function. Moreover, the difficulties in conducting assays with rodent oocytes include the need to breed the animals in specific facilities and also a precise control of the estrous cycle followed by sacrifice of the animals for recovery of oocytes.

In contrast, other mammals such as pig or cow show paternally inherited centrosomes (Szöllösi and Hunter, 1973; Navara et al, 1994; Kim et al, 1996) and offer the great advantage of unlimited availability of their female gametes at slaughterhouses coupled with a very well developed in vitro maturation systems (Mermillod et al, 1999; Coy et al, 2005). This advantage can be exploited, and new assays, including the evaluation of the ability of the sperm to bind the ZP and undergo the AR, could be available.

While the cow model has been tried in human ICSI to assess human sperm functionality (Terada et al, 2004), there are no references using the pig model, even although the polytocous characteristic of this species makes it especially suitable as a source of oocytes. Pigs share many physiological similarities with humans (Larsen et al, 2004) and are used frequently as an animal model in biomedical research (Vodicka et al, 2005), offering advantages over rodents, as was demonstrated by Pinton et al (2005). However, studies of any model related to the sperm-ZP binding and induction of

the AR or the sperm-oolemma fusion are, to our knowledge, nonexistent.

In an attempt to develop the pig model, the aim of the present work was to investigate if human sperm functionality could be assessed by studying the interaction between human spermatozoa and pig oocytes at the 3 mentioned levels: the ZP, the oolemma, and the ooplasm. With this purpose, our specific objectives consisted of 1) the ability of human spermatozoa to bind to pig ZP and undergo the AR (sperm-ZP interaction), 2) the ability of the human spermatozoa to fuse with in vitro matured pig zona-free oocytes (sperm-oolemma interaction), and 3) to assess in different patients and healthy donors the ability of their spermatozoa to decondense and transform into a male pronucleus when injected into in vitro matured pig oocytes (sperm-ooplasm interaction).

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Culture Media

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química SA (Madrid, Spain). The medium used for oocyte maturation was NCSU-37 (Petters and Wells, 1993) supplemented with 0.57 mmol cysteine l^{-1}, 1 mmol dibutyryl cAMP l^{-1}, 5 µg insulin mL^{-1}, 50 µmol ß-mercaptoethanol l^{-1}, 10 IU eCG mL^{-1} (Foligon, Intervet International BV, Boxmeer, Holland), 10 IU hCG mL^{-1} (Chorulon; Intervet International BV, Boxmeer, Holland), and 10% porcine follicular fluid (v/v).

The IVF media were Medi-Cult Universal IVF medium and modified Tyrode albumin-lactate-pyruvate (TALP) medium, the latter consisting of 114.06 mmol NaCl l^{-1}, 3.2 mmol KCl l^{-1}, 8 mmol Ca-Lactate.$5H_2O$ l^{-1}, 0.5 mmol $MgCl_2.6H_2O$ l^{-1}, 0.35 mmol NaH_2PO_4 l^{-1}, 25.07 mmol $NaHCO_3$ l^{-1}, 10 mL Na lactate l^{-1}, 1.1 mmol Na-pyruvate l^{-1}, 5 mmol glucose l^{-1}, 2 mmol caffeine l^{-1}, 3 mg BSA (A-9647) mL^{-1}, 1 mg polivynilalcohol (PVA) mL^{-1}, and 0.17 mmol kanamycin sulfate l^{-1}.

Oocyte Collection, In Vitro Maturation, and Zona Pellucida Digestion

Within 30 minutes of slaughter, ovaries from prepubertal gilts were transported to the laboratory in saline containing 100 µg kanamycin sulfate mL^{-1} at 38 °C and washed once in 0.04% cetrimide solution and twice in saline. Oocyte-cumulus cell complexes (COCs) collected from antral follicles (3–6 mm diameter) by slicing were washed twice with Dulbecco phosphate buffered saline (DPBS) supplemented with 1 mg PVA mL^{-1}. Only COCs with a complete and dense cumulus oophorus were selected for use as immature oocytes for the experiments (Coy et al., 2002).

For obtaining in vitro matured oocytes, groups of 50 immature COCs were cultured in 500 µL maturation medium for 22 hours at 38.5 °C under 5% CO_2 in air. After culture, oocytes were washed twice in fresh maturation medium without dibutyryl cAMP, eCG, and hCG and cultured for an additional 20–22 hours (Funahashi and Day, 1993). This in vitro maturation system has been demonstrated to produce viable oocytes reaching full-term development after IVF and embryo transfer (Coy et al., 2005).

ZP-free oocytes were prepared by incubating the mature oocytes (denuded by pipetting) in 50 µL droplets of prewarmed 0.5% (w/v) pronase in DPBS. The oocytes were removed from pronase solution as soon as the ZP was no longer visible with a stereomicroscope (Coy et al., 2002) and washed several times with DPBS.

Sperm Processing

Samples of human semen were obtained, with previous informed permission, from healthy donors and patients of Clínica IVI-Murcia. This study was approved by the National Committee of Human Assisted Reproduction from the Spanish Ministry of Health and Consumer Affairs. A total of 60 human spermatozoa samples were obtained. Fresh ejaculates were allowed to liquefy at 37 °C for 30–45 minutes. Motile spermatozoa were obtained using a 2-step density gradient (Pure Sperm). The sperm were then resuspended in Medi-Cult Universal IVF or TALP medium, centrifuged again at 600 x *g* for 5 minutes and then resuspended in Medi-Cult Universal IVF or TALP medium. Finally, the sperm concentration was adjusted according to the experiment.

Induction of the Acrosome Reaction and Evaluation of the Acrosomal Status of the Sperm

For the induction of the AR, the capacitated human spermatozoa were incubated with 5 µmol calcium ionophore (A23187 Fluka) at 38.5 °C for 3 hours in IVF medium (TALP or Medi-Cult Universal IVF). Finally, the sperm samples were centrifuged and washed twice in culture medium.

The acrosomal status of the zona-bound sperm was evaluated according to the procedures described by Liu and Baker (1996). This was performed on a glass slide with a small droplet of DPBS, and the zona-bound sperm were smeared over a limited area. The sperm droplets were then allowed to air–dry, fixed in 95% ethanol for 30 minutes, and staining with fluorescein isothiocyanate labeled pisum sativum (FITC–PSA; Sigma) 40 µg/mL for 20 minutes at 38 °C. Thereafter, they were mounted with 1,4–diazobicyclo-(2,2,2)-octane (DABCO) and evaluated for acrosomal status with an epifluorescence microscope. Two hundred cells were counted in a blind fashion in each well of the spotted slide, and results were expressed as percentage of acrosome-reacted sperm. The following staining patterns were evaluated as acrosome-reacted sperm: 1) distinct staining in the equatorial region occurring as an equatorial bar, 2) no staining observed over entire sperm surface, and 3) patchy staining on acrosomal region (Cross et al, 1988; Cummins et al, 1991; Franken et al, 1996, 2000).

Coincubation of Porcine Oocytes With Human Spermatozoa

After denuding by pipetting, the in vitro matured pig oocytes were cultured with human spermatozoa at 38.5 °C in 5% CO2 in air for 2.5 hours in Medi-Cult Universal IVF or TALP media. Then, the oocytes with attached and bound sperm were transferred to a 500-µL droplet of warm DPBS. Attachment is considered to be a loose, nonspecific interaction between sperm and egg ZP, whereas binding is a tight and more specific association leading to AR and penetration through the zona. Consequently, removing attached spermatozoa from the ZP could be done by gentle rinsing several times with DPBS using a narrow-bore Pasteur pipette (Fazeli et al, 1997). The zona-bound sperm, on the other hand, were removed by vigorous aspiration in and out of a narrow-gauge micropipette with and inner diameter (<120 µm) slightly smaller than that of the oocyte.

Coincubation of Porcine Zona-Free Oocytes With Human Spermatozoa and Evaluation of the Penetration

Groups of 15 zona-free matured porcine oocytes were transferred into pre-equilibrated 40-µL drops of IVF medium under paraffin oil. Semen was prepared as described above, and 10 µL of this suspension was added to the fertilization drop containing the oocytes to give a final concentration according to each experiment. At 18–20 hours postinsemination, oocytes were washed 3 times, fixed for 30 minutes in 0.5% v/v glutaraldehyde in DPBS, stained for 15 minutes in 1% w/v Hoechst 33342 in DPBS, and finally washed in DPBS and mounted on glass slides. The meiotic stages and sperm penetration were assessed at 400x magnification under an epifluorescence microscope.

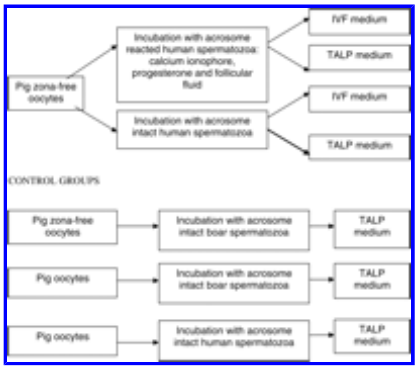
ICSI

Porcine oocytes cultured for 44 hours in maturation medium were mechanically denuded by gentle aspiration with a pipette. Denuded oocytes were washed twice in supplemented DPBS medium and transferred to ICSI drops. ICSI was conducted on a heated microscope at 200x magnification using a Nikon Diaphot 300 inverted microscope with attached micromanipulators. Only fully matured MII oocytes were microinjected. The ICSI medium used was DPBS supplemented with 10% fetal calf serum (FCS). Prior to ICSI, oocytes were loaded on 4-µL microinjection drops placed on a Petri dish lid (1 oocyte per drop). In total, 10–15 microdrops were placed in each lid surrounding central sperm drops which resulted from mixture of 4 µL of DPBS-FCS and 1 µL of the sperm suspension. Microdrops were covered with mineral oil (Sigma M-8410). ICSI was performed as described by Garcia et al (2006). Briefly, a single sperm was immobilized by crushing the midpiece with the tip of the injection pipette. The immobilized sperm was aspirated with the tail first. Thereafter, the injection pipette was moved into the drop containing the oocytes to be injected. A single oocyte was fixed by the holding pipette, positioning the polar body at 6-o'clock or 12-o'clock position. The injection pipette was pushed through the ZP and subsequently through the oolemma into the cytoplasm at the 3-o'clock position. A small amount of ooplasm was aspirated into the injection pipette in order to ensure oocyte membrane penetration. Subsequently, the immobilized spermatozoon was released into the cytoplasm. The temperature was maintained at 38.5 °C throughout the procedure using a heated microscopical stage. Injected oocytes were placed in TALP medium.

Experimental Design

Experiment 1 was performed to study the ability of human spermatozoa to undergo the AR after binding to the pig ZP (sperm-ZP interaction). In vitro matured pig oocytes were used. Fifteen human sperm samples were coincubated in presence (experimental groups) or absence (control groups) of porcine oocytes (15 oocytes/50 µL). The percentage of induced AR in the experimental groups was calculated from the difference with the spontaneous AR in the control group at 2.5 hours. After a first series of experiments with 3 semen samples in 3 replicates using 2 different culture media (TALP or Medi-Cult Universal IVF), a second assay was performed using only Medi-Cult Universal IVF medium. This was due to the absence of differences between both media in the first assay and to the convenience of continuing for the rest of the trials with a commercial medium usually employed for human IVF, facilitating the development of a future test for sperm functionality. An additional control group was performed in which the human spermatozoa were incubated with porcine zona-free oocytes in the same conditions. Incubation was performed at 2.5 x 10^6 spz/mL for 2.5 hours. The acrosomal status of the spermatozoa bound to the oocyte (ZP or oolemma) was evaluated.

In experiment 2, the ability of the human spermatozoa to fuse with in vitro matured pig zona-free oocytes was studied. Pig zona-free oocytes were coincubated with 33 human spermatozoa either treated or not with 5 µM calcium ionophore to induce the AR. The addition of progesterone (10 µM) and porcine follicular fluid (2%) to the culture medium as natural agonists for the induction of the AR was also investigated. Different control groups were used: pig zona-free oocytes inseminated with boar spermatozoa, pig oocytes inseminated with boar spermatozoa, and pig oocytes inseminated with human spermatozoa. Before coincubation, an aliquot of the semen samples was stained with PSA-FITC and the acrosomal status of the spermatozoa was determined according to Liu and Baker (1996). Finally, oocytes were fixed and stained with Hoechst 3342 to assess binding, penetration, spermatozoa swollen inside the ooplasm, and nuclear stage. The diagrammatical representation of this experiment can be seen in the Figure.



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Diagrammatical representation of experiment 2.

In experiment 3, the aim was to investigate the ability of human spermatozoa from different patients and healthy donors to decondense and transform into male pronucleus when injected into in vitro matured pig oocytes. Twelve human sperm samples were used. Ten in vitro matured porcine oocytes per sample were injected with a single human spermatozoon. The injected oocytes were transferred to TALP medium for 18 hours. Then they were fixed in 0.5% glutaraldehyde and stained with bisbenzimide (Hoechst 33342) to check the sperm head stage and nuclear stage of the oocyte.

Statistical Analysis

Comparisons of the percentages of acrosome-reacted sperm under the different experimental conditions were performed using 1-way or repeated measures ANOVA as appropriate. The Tukey multiple comparisons test was used to assess individual differences among conditions tested. *P* values less than .05 were considered statistically significant. Results are expressed as mean ± SEM.

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Experiment 1

After 2.5 hours of incubation, binding between human sperm and porcine ZP was observed. When the AR was investigated on bound sperm, according to the procedures described by Liu and Baker (1996), the percentage of reaction was significantly higher for those bound spermatozoa than for those from the control groups (Table 1). There were no significant differences between spermatozoa cultured in TALP or Medi-Cult Universal IVF media.

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Table 1. *Percentages of acrosome reacted human spermatozoa in samples coincubated with or without (control) pig oocytes in 2 different culture media*

When the Medi-Cult Universal IVF medium was employed, differences with control groups were again observed, including the control from the pig zona–free oocytes (Table 2).

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Table 2. *Percentages of acrosome reacted human spermatozoa in samples coincubated with pig oocytes (zona-free oocytes or matured complete oocytes) or without pig oocytes (control group) in Medi-Cult Universal IVF medium*

The percentages of AR in the last group (samples of human spermatozoa coincubated in Medi-Cult Universal IVF medium for 2.5 hours with pig in vitro matured oocytes) were individually analyzed and the results showed significant differences among samples, varying from 15% to 58% of induced AR.

Experiment 2

When heterologous fertilization among zona-free pig oocytes and human spermatozoa was performed under different conditions (Figure), no fusion of gametes was observed. In fact, none of the zona-free oocytes was penetrated in any group by human sperm. In some cases, some spermatozoa (<15/oocyte) were attached to the oolemma, but penetration was never demonstrated. Neither the addition of calcium ionophore, progesterone, nor porcine follicular fluid showed any influence on penetration, even though the induction of AR was higher (75%) for the spermatozoa treated with progesterone and calcium ionophore and 45% for those treated with follicular fluid (Table 3). By contrast, all pig zona-free oocytes inseminated with boar spermatozoa were penetrated, and high numbers of spermatozoa per oocyte were frequently observed in this group. Similarly, conventional IVF among pig oocytes and spermatozoa was successful, and penetration and pronuclear formation were observed.

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Table 3. *Results of penetration, pronuclear formation and monospermy after in vitro coincubation of boar or human spermatozoa with pig zona-free or intact oocytes**

Experiment 3

Male and female pronuclear formation for all the human semen samples could be observed (Table 4) when ICSI of pig oocytes and human spermatozoa was performed and evaluated 18 hours later. Moreover, some cleavages (2-cell stages) were observed at this time in samples from healthy donors.

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Table 4. *Results of heterologous ICSI with different samples of human spermatozoa and pig oocytes, assessed after 18 hours of coincubation**

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Human Sperm Are Able to Bind to Pig ZP and Undergo the AR

The results shown in this work indicate that human spermatozoa can bind tightly to the ZP from in vitro matured pig oocytes. Besides, the induction of the human AR when human sperm were incubated with pig oocytes for 2.5 hours indicated that the binding was active and it led to AR. The possibility of an AR induced by progesterone from the cumulus cells cannot be an explanation in the present work because all the oocytes were denuded before incubation with the sperm. Thus, although still preliminary, this is a new finding that makes possible the future use of pig oocytes in assays of human sperm functionality if a correlation with fertility can be demonstrated.

ZP has been classically recognized as the place where the species-specificity resides during mammalian fertilization (Hanada and Chang, 1972; Yanagimachi, 1994). In most species, certain exposed oligosaccharide chains of the ZP and corresponding complementary carbohydrate-binding proteins on the spermatozoa plasma membrane mediate the specific binding and recognition between sperm and ZP (Bedford, 1977; Töpfer-Petersen and Calvete, 1995; Hoshiba and Sinowatz, 1998; Sinowatz et al, 1998). However, different authors have considered that the binding of sperm to the ZP is not strictly species-specific, although is a significant barrier to many crosses in vitro (Wassarman et al, 2005). The non–species-specific binding shown in this work is not the only reference to heterologous interaction between spermatozoa and ZP, although it has not been used to study the functionality of the spermatozoon.

In humans, Lee et al (1987) reported heterologous sperm-stimulating factors when they observed that acid-solubilized mouse ZP induced the human AR. Oehninger et al (1993) observed a higher incidence of acrosome reacted sperm on the ZP in homologous (human oocyte and spermatozoa) compared to heterologous experiments (*cynomolgus* monkey and hamster oocyte with human spermatozoa), corroborating that a heterologous human AR is possible as well.

O'Rand et al (1985) and Naz et al (1991) reported that porcine zona protein can bind to proteins on human spermatozoa. However, Windt et al (1992) observed that human spermatozoa which had been exposed to porcine zona protein showed increased sperm-zona binding when compared to control spermatozoa; these results would be contradictory to previous studies. Delle Monache et al (2003) demonstrated that Gp273, the ligand molecule for sperm-egg interaction in a bivalve mollusk, binds to human sperm and induces AR. This finding, in agreement with our results, suggests that a strict species-specificity at primary binding does not exist (Focarelli et al, 2001). In other species, previous papers have demonstrated heterologous binding and induction of the AR between bovine ZP and porcine or equine spermatozoa (Sinowatz et al, 2003).

Taken together, these observations may indicate that, under in vitro conditions, species-specificity of spermzona interactions is less restrictive than thought previously (Hartmann, 1983). Sperm-zona binding reflects multiple sperm function events depicting spermatozoa-ZP interaction, that is, completion of capacitation as manifested by the ability to bind to the ZP and to undergo ligand-induced AR (Esterhuizen et al, 2001).

A diagnostic test underlining the ability of the spermatozoa to undergo the AR in response to homologous ZP would be a valuable additional tool in the male fertility workup schedule (Franken et al, 1990; Liu and Baker, 1992; Oehninger et al, 1992). Alternatively, the use of heterologous porcine ZP could be a more available option as diagnostic test.

Human Spermatozoa Do Not Penetrate Through Pig Oolemma

The plasma membrane represents the second barrier in the interaction between spermatozoon-oocyte; it involves a firm cell-cell adhesion that culminates in fusion of the 2 membranes. The mechanism of the fusion between sperm-egg is not completely known, but different molecules have been identified. Cysteine-rich secretory protein 1 (CRISP1) and ADAMs (fertilin alpha, fertilin beta, and cyritestin) on sperm, and integrins, CD9, and other integrin-associated proteins on oocytes, seem to be responsible of this mechanism (Evans, 2002). Whereas the first barrier (ZP) is recognized in some cases to be efficient for the species-specificity in the sperm-oocyte interaction (Hanada and Chang, 1972; Yanagimachi, 1994), such species-specificity for interactions between gamete membranes in mammals is not always evident.

Our results demonstrated that capacitated and acrosome reacted human spermatozoa can bind to the plasma membrane of in vitro matured pig oocytes, but they cannot fuse and penetrate inside the ooplasm.

In in vitro studies, zona-free mammalian eggs tend to fuse only with sperm from the same or a closely related species; however, in some cases, gamete incompatibility at the vitelline membrane was observed between closely related species (antelope sperm and cow oocytes; Kouba et al, 2001).

In contrast, eggs of the golden hamster can fuse with the sperm from many mammalian orders when the zona is removed from the egg. Because of this freedom, the zona-free hamster egg has been used extensively in the sperm penetration assay to assess the fertilizing capacity of human spermatozoa (Yanagimachi et al, 1976; Rodgers et al, 1979; Liu and Baker, 1992). However, the vitelline membrane block to cross-fertilization by human spermatozoa has been observed using oocytes from closely related species with the golden hamster, such as rat and mouse (Hanada and Chang, 1978; Quinn, 1979; Pavlok, 1980) and unrelated species such as pig (Cánovas et al, 2005a).

Other examples of non-species-specific gamete binding and penetration have been observed previously using different combinations of mammalian sperm and oocytes (Bedford, 1977; Yoshimatsu et al, 1988; Liu et al, 1991; Cox, 1992; Lanzendorf et al, 1992; Oehninger et al, 1993).

From the data in the bibliography, it can be inferred that the fusion between gametes from different species is unpredictable (Yanagimachi, personal communication), and specific trials are necessary to know the results in each case. Experiments shown in this work demonstrate that human spermatozoa cannot fuse and penetrate zona-free pig oocytes, thus indicating the presence of a kind of vitelline membrane block to cross-fertilization between these species. In this sense, Bedford (1977) considered that the human spermatozoa display unusually limited affinities in their interaction with oocytes of other species, with the exception of the hamster. This author suggests that the evolution has been accompanied by a restrictive change in the nature of the sperm surface which has limited and made more specific the complementary surface to which human spermatozoa may adhere, while the surface of human oocytes is less specific and can bound spermatozoa from different species.

Human Spermatozoa Can Decondense and Form a Male Pronucleus Into Pig Oocytes

Numerous studies have assessed the fertilizability of human sperm, but most involve simply the sperm's ability to enter the egg cytoplasm. However, fertilization is concluded only with the union of the male and female genomes at metaphase of first mitosis, not merely sperm entry. Because of its importance to events after sperm entry, several scientists have used heterologous ICSI employing oocytes from different species, mainly rodents. Lee et al (1996) used ICSI of human spermatozoa with normal and aberrant head morphologies in mouse oocytes to analyze chromosome constitution. Ahmadi et al (1996) used hamster ICSI assay as a test for fertilizing capacity of the severe male-factor sperm. However, Terada et al (2004) indicated that centrosomal function during the entire first cell

cycle may be an important assay to monitor for testing the full ability of human sperm to support the fertilization process. Humans inherit their centrosomes from their fathers, while rodents are among the rare exceptions, since they inherit their centrosomes maternally. Consequently, heterologous assays involving rodents are inappropriate models to test human sperm centrosome activity (Hewitson et al, 1997).

Although the ability of human sperm to form a male pronucleus after injection into pig oocytes has been previously reported (Kim et al, 1999), the use of this approach as a novel tool to assess sperm functionality (Cánovas et al, 2005b) was not proposed. In this previous reference, Kim et al (1999) revealed that high percentages of oocyte activation and pronuclear formation could be achieved, which is in agreement with the results in the present paper. However, these authors did not observe syngamy or normal 2-cell division as reported in this work. Differences in the culture media could explain in part such discrepancy, since we have previously observed the positive effect on cleavage of TALP medium after ICSI compared to NCSU23 (García-Roselló et al, in press).

The results from this work, showing different percentages of male pronuclear formation after injection of different samples of human spermatozoa in pig oocytes, open a new window for future tests of sperm functionality in humans. This heterologous ICSI system can be appropriate for assessing human sperm oocyte activation ability and human sperm centrosomal function, and we propose the development of the pig model by future studies with high number of patients from infertility centers to demonstrate possible correlations with final fertility.

In conclusion, results from the present paper indicate that human spermatozoa can bind to pig ZP and undergo the AR; they are not able to cross the oolemma, but they can decondense and form a male pronucleus when injected into pig oocytes. Further studies will be needed regarding the efficacy of these assays as a potential human infertility test.

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Footnotes

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