

Factors influencing sperm–zona pellucida binding *in vitro* using the intact zona model

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The zona pellucida binding assay assesses the ability of spermatozoa to bind to the zona pellucida. The present study investigated the influence of: (i) prior oocyte exposure to spermatozoa on subsequent sperm–zona pellucida binding *in vitro*; and (ii) cryopreservation of oocytes. Only oocytes obtained from fertile donors were used and the binding capacity of non-inseminated, cryopreserved oocytes was compared with both inseminated/unfertilized, cryopreserved oocytes and inseminated/unfertilized, non-cryopreserved oocytes recovered from in-vitro fertilization cultures on sperm–zona pellucida binding using an intact zona model. There was no statistically significant difference in sperm–zona binding between non-inseminated, cryopreserved oocytes (range 9.6–23.2), inseminated/unfertilized, cryopreserved oocytes (range 15.0–16.0) and inseminated/unfertilized, non-cryopreserved oocytes (range 3.3–23.0). The coefficient of variation for sperm binding to all oocyte groups was very large (range 37–121%). We conclude that neither prior exposure of human oocytes to human spermatozoa nor cryopreservation of human oocytes influences the subsequent binding of a different population of spermatozoa to the zona pellucida. However, large oocyte-to-oocyte variation of sperm–zona binding may diminish the usefulness of this assay in clinical practice and as a research tool.

Key words: hemizona assay/human/oocyte cryopreservation/sperm–oocyte interaction/sperm–zona pellucida binding

Introduction

Tight binding of human spermatozoa to the human zona pellucida is a prerequisite for fertilization (Bedford, 1977). Sperm receptors recognize specific glycoproteins on the zona pellucida. In response to zona pellucida binding, redistribution of surface sperm receptors occurs and the acrosome reaction is initiated (Hershlag *et al.*, 1995). Because the human spermatozoa will tightly bind only to a human zona pellucida (Bedford, 1977), an assay assessing zona binding was proposed as a test of sperm fertilization potential (Overstreet and Hembree, 1976).

Initially, sperm binding and zona penetration were tested using oocytes recovered from post-mortem ovarian tissue and surgically removed ovaries (Overstreet *et al.*, 1980). However, the usefulness of these initial assays was diminished by the limited availability of human ovaries as a source of oocytes. With the advent of in-vitro fertilization (IVF) treatment programmes, a potential source of inseminated/unfertilized oocytes is now available for use in laboratory studies (Burkman *et al.*, 1988; Liu *et al.*, 1988; Oehninger *et al.*, 1989a; Franken *et al.*, 1991; Hammitt *et al.*, 1993). The inseminated/unfertilized oocytes from IVF cultures are typically discarded and offer the most abundant source of available oocytes for the sperm–zona binding assay.

Two methodologies using IVF harvested oocytes are published. One method, called the hemizona assay, utilizes a bisected zona pellucida (Burkman *et al.*, 1988). The zona of each oocyte is cut precisely in half under a microscope and the cytoplasm discarded. One-half of the zona is then cultured with the patient's spermatozoa and the other half with the spermatozoa of a fertile donor in a separate culture dish. The number of spermatozoa tightly bound to both halves of the zona is recorded and expressed as a ratio. A less labour-intensive technique utilizes an intact oocyte incubated with two sperm populations, one from the patient and one from a fertile control. Each sperm population is labelled with a distinctive fluorescent label and results are reported as the ratio of the number of fluorescent spermatozoa bound to the oocyte (Liu *et al.*, 1988).

The sperm–zona binding ratio obtained using both the intact zona and the hemizona model is reported to be well correlated with the IVF outcome (Liu *et al.*, 1989; Franken *et al.*, 1989b) and with sperm morphology assessment using strict morphology criteria (Franken *et al.*, 1993). However, due to high oocyte-to-oocyte variability in sperm–zona binding, the intra-assay and interassay coefficients of variation are reported to exceed 30% (Liu *et al.*, 1989).

At present, there is little information as to which factors are responsible for the variation in sperm–zona binding. One explanation is that the zona pellucida's ability to bind spermatozoa may be influenced by the maturational stage of the oocyte (Mahadevan *et al.*, 1987; Tesarik *et al.*, 1988; Oehninger *et al.*, 1989b). Hypothetically, other factors may also influence the zona pellucida's ability to bind spermatozoa. Poor quality oocytes with defective zona pellucida obtained from infertile women, oocyte ageing during IVF culture with spermatozoa, sperm-induced changes in the zona as a result of the exposure of the zona to spermatozoa in the IVF culture, zona pellucida hardening subsequent to oocyte penetration by a spermatozoon which failed to form a pronucleus (clinically unfertilized

oocyte), and oocyte preservation and storage prior to the zona binding assay may diminish the sperm binding capacity (Hammit *et al.*, 1993).

The present study was designed to investigate the influence of the following on sperm–zona pellucida binding using an intact zona model: (i) prior oocyte exposure to spermatozoa on subsequent sperm–zona pellucida binding *in vitro*; and (ii) cryopreservation of oocytes. Only oocytes obtained from fertile donors were used and the binding capacity of non-inseminated, cryopreserved oocytes was compared with both inseminated/unfertilized, cryopreserved oocytes and inseminated/unfertilized, non-cryopreserved oocytes recovered from IVF cultures.

Materials and methods

The study protocol was approved by the Institutional Review Board of the University of Southern California School of Medicine, Los Angeles, CA, USA.

Overall study design

The first set of experiments (experiments 1, 2 and 3) was designed to determine if prior exposure of oocytes to spermatozoa altered the ability of those oocytes to bind with a second population of spermatozoa using the intact zona assay. Two populations of cryopreserved donor oocytes were utilized for these experiments: one group (non-inseminated, cryopreserved oocytes) that had not been exposed to spermatozoa prior to cryopreservation and a second group (inseminated/unfertilized, cryopreserved oocytes) that had been inseminated with spermatozoa prior to cryopreservation. Oocytes cryopreserved by the method used in these experiments were, in our experience, non-fertilizable; however, the zona pellucida remained intact. For these experiments, semen was obtained from two donors.

The second set of experiments (experiments 4, 5 and 6) was designed to determine: (i) if prior cryopreservation of oocytes interfered with the ability of those oocytes to bind spermatozoa in the intact-zona assay; and (ii) if the variability of sperm–zona binding can be reduced by using spermatozoa obtained from one ejaculate. For these experiments, three populations of donor oocytes were utilized: (i) non-inseminated, cryopreserved oocytes; (ii) inseminated/unfertilized, cryopreserved oocytes; and (iii) inseminated/unfertilized, non-cryopreserved (fresh) oocytes. Semen was obtained from one donor.

Semen processing

For experiments 1, 2 and 3, two healthy volunteers donated semen by masturbation (donors A and B). After liquefaction and assessment of sperm concentration and motility, semen was diluted with Ham's F-10 culture medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% plasmanate (Irvine Scientific), a synthetic serum substitute, and centrifuged at 400 *g* for 8 min. The supernatant was then discarded and the pellet was resuspended with fresh medium and washed again. After the second wash, 0.5 ml of fresh culture medium was gently layered over the pellet and the test tube was incubated at a 45° angle at 37°C in air supplemented with 5% CO₂. Following a 60 min incubation, the supernatant containing motile spermatozoa (swim-up fraction) was transferred to a new culture tube and incubated overnight (37°C, 5% CO₂). The next morning, the swim-up fraction was centrifuged and the sperm pellet from donor A was resuspended with a fluorescent label solution, fluorescein isothiocyanate (FITC, isomer I; Sigma Chemical Co., St Louis, MO, USA) at a concentration of 200 µg/ml. The sperm pellet of donor B was stained with tetramethylrhodamine isothiocyanate (TRITC; Sigma) at

a concentration of 100 µg/ml as previously described by Parrish and Foote (1985). Following a 15 min incubation with the fluorescent label, each sperm suspension was diluted with 5 ml fresh culture medium and centrifuged again in order to remove the excess of the label. The resulting pellets were resuspended with fresh medium to a motile sperm concentration of 2×10^6 spermatozoa/ml.

For experiments 4, 5 and 6, semen was obtained from one donor by masturbation and was allowed to liquefy. Because these experiments evaluated sperm binding to non-cryopreserved oocytes, the sperm sample was processed by Percoll separation and used on the same day when the oocytes were diagnosed as unfertilized (72 h after harvesting). Only one semen sample was used in order to control for sperm variability. After sperm concentration and motility were assessed, two 1 ml aliquots of whole semen were then layered over a discontinuous Percoll gradient (3 ml of 55% Percoll over 2.5 ml of 80% Percoll) in two separate test tubes. After a 20 min centrifugation at 400 *g*, each pellet concentrated at the bottom of the 80% Percoll layer was transferred to a new test tube and diluted with 5 ml of Ham's F-10 culture medium with 10% plasmanate. The pellets resulting from the second centrifugation were resuspended in a fluorescent label solution: one pellet in FITC solution (spermatozoa C) and the other in TRITC solution (spermatozoa C'). Following a 15 min incubation, both sperm suspensions were diluted with 5 ml of fresh culture medium and centrifuged again to remove the excess of the label. Each final pellet was resuspended with the culture medium to a motile sperm concentration adjusted to 2×10^6 spermatozoa/ml.

Oocyte preparation

Oocytes were obtained by an ultrasound-guided needle aspiration from fertile donors undergoing ovarian stimulation with gonadotrophin releasing hormone agonist, human menopausal gonadotrophin and human chorionic gonadotrophin for IVF. Three different categories of metaphase I oocytes were used: (i) non-inseminated, cryopreserved oocytes ($n = 17$); (ii) inseminated/unfertilized, cryopreserved oocytes ($n = 34$); and (iii) inseminated/unfertilized, non-cryopreserved (fresh) oocytes ($n = 15$).

The non-inseminated, cryopreserved oocytes were retrieved from a single donor and were not incubated with spermatozoa prior to cryopreservation. The inseminated/unfertilized, cryopreserved oocytes were pooled from five different donors. Oocytes which failed to develop two pronuclei and did not cleave were cryopreserved. The inseminated/unfertilized, non-cryopreserved oocytes were obtained from an additional two donors and used for the experiment 'fresh', on the day when the 72 h incubation with IVF spermatozoa was completed and the oocytes failed to develop two pronuclei.

Prior to freezing, oocytes were placed in a phosphate-buffer solution containing 2.5 M propanediol, 0.2 M sucrose and bovine serum albumin (5 mg/ml). After a 10 min incubation, the oocytes were placed in straws, then submerged in liquid nitrogen and stored.

Sperm–zona pellucida binding assay: experiments 1, 2 and 3

Experiments 1, 2 and 3 were performed simultaneously. In experiment 1, 12 non-inseminated cryopreserved oocytes were thawed and divided into two culture dishes. In dish A→B, six of these oocytes were inseminated with spermatozoa A (FITC-stained) and in dish B→A, another six oocytes were inseminated with TRITC-stained spermatozoa B. The final motile sperm concentration for all sperm suspensions was 1×10^6 spermatozoa/ml. After a 4 h incubation, all oocytes were vigorously washed three times to remove all loosely attached spermatozoa using a narrow glass pipette which had been fire-polished and pulled to obtain a diameter just larger than the diameter of an oocyte. Three oocytes from each dish were placed on a slide under a supported cover slip and examined using a fluorescent microscope.

The number of spermatozoa bound tightly to the zona was recorded. The remaining three washed oocytes in each dish were transferred into a fresh drop of medium and re-inseminated with cross-over specimens (dish A→B with spermatozoa B and dish B→A with spermatozoa A). After another 4 h incubation and washing, sperm binding to the zona was assessed.

In experiment 2, the same protocol as in experiment 1 was repeated using 12 inseminated/unfertilized, cryopreserved oocytes. In experiment 3, another five inseminated/unfertilized, cryopreserved oocytes were inseminated with a 1:1 mixture of spermatozoa A + B and incubated for 4 h and the number of spermatozoa tightly bound to the zonae was recorded.

In addition, the average number of unstained spermatozoa tightly bound to the zona pellucida of the same inseminated/unfertilized oocytes was recorded. The unstained spermatozoa represented the spermatozoa bound to the zona during the original IVF culture and remained tightly bound.

Sperm–zona pellucida binding assay: experiments 4, 5 and 6

In experiment 4, five non-inseminated, cryopreserved oocytes were inseminated with a 1:1 mixture of spermatozoa C and C' (C + C') and the sperm–zona binding was assessed after a 4 h co-incubation. Due to the small number of these oocytes, one insemination treatment was tested. In experiment 5, six inseminated/unfertilized, cryopreserved oocytes were thawed and inseminated with spermatozoa C first (C→C') and then, after sperm–zona binding was assessed in three oocytes at the end of the 4 h incubation, an aliquot of spermatozoa C' was added to the remaining three oocytes for another 4 h incubation. In a separate dish, six additional inseminated/unfertilized, cryopreserved oocytes were co-incubated for 4 h with spermatozoa C' first (C'→C) and then with spermatozoa C second. Another five inseminated/unfertilized, cryopreserved oocytes were placed into a drop of spermatozoa C and C' mixed 1:1 suspension (C + C') for 4 h. The same experimental design was used in experiment 6, except that inseminated/unfertilized, non-cryopreserved (fresh) oocytes were incubated with the labelled population of spermatozoa and only three of these oocytes were available for test with the mixed sperm suspension.

Statistical analysis

Data were analysed using a repeated measures analysis of variance and linear correlation.

Results

Experiments 1, 2 and 3

Table I shows the mean \pm SEM number of spermatozoa bound to the zona pellucida of oocytes incubated in experiments 1 and 2. There was no significant statistical difference between the number of spermatozoa bound to the zona pellucida regardless of which semen sample was incubated with the oocytes first. Preincubation of the oocytes with spermatozoa did not alter the ability of a subsequent sperm population to bind to the zona pellucida. This was true whether the oocytes tested had been incubated with spermatozoa at the time of IVF and prior to cryopreservation or had not been incubated with spermatozoa prior to cryopreservation. The mean number of unstained spermatozoa tightly bound to the zona pellucida of the inseminated/unfertilized oocytes was statistically similar in all three treatments.

The oocyte-to-oocyte variation in sperm–zona binding was

large within each treatment. The coefficient of variation for spermatozoa binding to the zonae of non-inseminated, cryopreserved oocytes was 37% for spermatozoa labelled with FITC (spermatozoa A) and 71% for the spermatozoa labelled with TRITC (spermatozoa B). The coefficient of variation of sperm–zona binding in the experiment using the inseminated/unfertilized, cryopreserved oocytes (experiments 2 and 3) was 73% for sperm population A and 121% for sperm population B.

Experiments 4, 5 and 6

The mean \pm SEM number of spermatozoa bound to the zona pellucida of oocytes in these three experiments is shown in Table II.

No significant differences were noted in the ability of labelled spermatozoa to bind to the zona pellucida of previously cryopreserved oocytes as compared to fresh oocytes (experiments 5 and 6). Once again, there was no significant difference in the zona pellucida's binding ability if the oocytes had been pre-exposed to spermatozoa prior to incubation with the labelled spermatozoa (experiments 4 and 5).

Both cryopreserved and non-cryopreserved inseminated/unfertilized oocytes with more unstained (IVF) spermatozoa attached, tended to bind more fluorescent-labelled spermatozoa; however, this correlation did not reach statistical significance ($r = 0.09$, $P = 0.7$). The mean number of spermatozoa bound to the non-inseminated oocytes appeared slightly larger when compared to the number of spermatozoa bound to the inseminated/unfertilized oocytes, but this difference was not statistically significant.

The coefficient of variation for sperm binding was 66% for non-inseminated, cryopreserved oocytes, 89% for inseminated/unfertilized, cryopreserved oocytes and 107% for inseminated/unfertilized, non-cryopreserved oocytes. The coefficient of variation was 64% for the binding of the unstained spermatozoa to the zonae of all oocytes studied.

Discussion

The results of this study indicate that: (i) long incubations for the intact zona assay are not necessary; (ii) cryopreserved oocytes are a suitable source of oocytes; and (iii) the variation in the sperm–zona binding documented requires the use of a large number of oocytes per assay. The zona's ability to bind to the same sperm population after a 4 h incubation was statistically similar to the binding observed after an 8 h incubation. The non-inseminated, cryopreserved oocytes as well as the inseminated/unfertilized, cryopreserved oocytes did not have their sperm binding capacity diminished after exposure to a different population of spermatozoa during the initial 4 h incubation. The binding during the secondary sperm exposure was not statistically different from the binding during the initial exposure. There was no difference in sperm–zona binding whether the non-inseminated, cryopreserved oocytes were exposed to spermatozoa A first and spermatozoa B second or vice versa.

Prior exposure of human oocytes to human spermatozoa does not influence the subsequent sperm binding after the oocytes were re-inseminated with a different sperm population

Table I. Sperm–zona pellucida binding^a: experiments 1, 2 and 3

Sperm donor	Duration of incubation (h)	Oocytes				
		Experiment 1 Non-inseminated Cryopreserved		Experiment 2 Inseminated/unfertilized Cryopreserved		Experiment 3 Inseminated/unfertilized Cryopreserved
		(n = 6) A→B	(n = 6) B→A	(n = 6) A→B	(n = 6) B→A	(n = 5) A + B
A	4	14.0 ± 4.9	–	1.5 ± 0.5	–	7.4 ± 3.3
B	4	–	36.4 ± 18.2	–	7.8 ± 4.5	2.0 ± 1.0
A	8	19.0 ± 3.0	8.3 ± 0.3	6.6 ± 3.2	3.5 ± 1.5	–
B	8	22.7 ± 3.0	10.7 ± 2.5	3.8 ± 3.4	6.3 ± 2.6	–
Unstained spermatozoa	–	–	–	13.0 ± 6.2	4.3 ± 1.9	17.4 ± 7.8

^aAll values are mean ± SEM and represent the number of spermatozoa bound to the zona pellucida.

Table II. Sperm–zona pellucida binding^a: experiments 4, 5 and 6

Sperm donor	Duration of incubation (h)	Oocytes						
		Experiment 4 Non-inseminated Cryopreserved		Experiment 5 Inseminated/unfertilized Cryopreserved		Experiment 6 Inseminated/unfertilized Non-cryopreserved (fresh)		
		(n = 5) C + C'	(n = 5) C + C'	(n = 6) C → C'	(n = 6) C' → C	(n = 3) C + C'	(n = 6) C→C'	(n = 6) C'→C
C	4	23.2 ± 9.4	21.8 ± 9.1	29.3 ± 21.5	–	12.0 ± 11.0	9.3 ± 4.6	–
C'	4	9.6 ± 1.8	15.6 ± 3.9	–	36.3 ± 31.9	18.0 ± 14.0	–	12.7 ± 4.4
C	8	–	30.7 ± 10.5	31.7 ± 23.2	–	3.3 ± 1.7	23.3 ± 21.8	–
C'	8	–	–	72.3 ± 22.9	16.7 ± 4.4	–	11.0 ± 7.0	14.0 ± 9.1
Unstained spermatozoa	–	44.6 ± 7.91	53.5 ± 18.6	20.8 ± 6.2	63.5 ± 11.5	54.3 ± 15.7	17.3 ± 6.0	–

^aAll values are mean ± SEM and represent the number of spermatozoa bound to the zona pellucida.

and cannot explain the large variation in sperm–zona binding. Therefore, inseminated/unfertilized oocytes salvaged from IVF cultures can be used for testing of the sperm–zona pellucida interaction. However, the variability of sperm binding by different donor oocytes under the same culture conditions and using the same sperm population was so large that it makes the clinical relevance of the zona-binding assay at least questionable.

The mean number of sperm bound to the zonae never previously exposed to spermatozoa appeared slightly higher than the number of spermatozoa bound to the zonae of re-inseminated (inseminated/unfertilized) oocytes but this difference did not reach statistical significance, probably because of the large variation in the number of spermatozoa bound by different oocytes. These observations are in agreement with the results reported by Franken *et al.* (1991) who used salt-stored, non-inseminated and inseminated/unfertilized oocytes and obtained similar sperm–zona binding for both kinds of oocytes. In contrast, Hammitt *et al.* (1993) noted a significant difference in the sperm binding by non-inseminated, cryopreserved oocytes which was higher compared with sperm–zona binding by re-inseminated, cryopreserved oocytes.

The observation that the zona pellucida's sperm binding ability of the re-inseminated oocytes was not altered by the previous interaction with the spermatozoa in the culture *in vitro* is in agreement with published evidence that the ZP1 receptor in the zonae remained unchanged after exposure of

previously inseminated/unfertilized oocytes to spermatozoa (Shabanowitz and O'Rand, 1988). The ZP1 receptor is one of the glycoproteins in the zona known to interact with the sperm membrane. Only oocyte activation after fertilization altered the structure of the sperm ZP1 receptor in the zonae of human oocytes (Shabanowitz and O'Rand, 1988). Whether the sperm binding capacity of zonae may be influenced by ageing in the IVF culture remains unknown.

The influence of oocyte quality on sperm–zona binding is unclear. Oocyte maturity has also been considered a factor influencing sperm–zona binding ability. Metaphase I oocytes appear to bind significantly fewer spermatozoa than metaphase II oocytes (Oehninger *et al.*, 1989b; Oehninger *et al.*, 1991). In our study all oocytes were at metaphase I stage in order to eliminate this possible source of variation. Because most metaphase II oocytes become fertilized in the IVF procedure, it is difficult to obtain sufficient quantities of unfertilized metaphase II oocytes for testing. Hammitt *et al.* (1993) found that unfertilized oocytes obtained from IVF cycles with a <50% fertilization rate bound significantly more spermatozoa to the zona in an in-vitro assay than oocytes from cycles with a fertilization rate of >50%. They concluded that in these instances the poor IVF rate in the former group was probably due to a sperm factor, explaining why oocytes of high sperm binding ability were not fertilized. In contrast, Liu *et al.* (1988) reported that in their in-vitro assay significantly fewer spermatozoa bound to the zonae pellucidae of unfertilized

oocytes retrieved from IVF cultures where the fertilization rate was <50%. They argued that poor oocyte quality and low sperm binding capacity secondary to an oocyte abnormality were responsible for the low IVF score and subsequent low sperm–zona binding assay score. In the present study, oocytes from fertile donors were used in order to minimize the possibility of abnormal gametes.

The method of zona preservation, cryopreservation or salt storage prior to the sperm–zona assay may also be a potential factor influencing the variability of sperm binding to the zona pellucida. Yoshimatsu *et al.* (1988) reported that zona function was influenced by the age of oocytes in a salt solution. They found that salt-preserved human oocytes lost their species selectiveness and allowed binding and penetration of the zona pellucida by hamster spermatozoa. Carroll *et al.* (1990) investigated whether the statistically significant decrease in the fertilization rate of mouse oocytes resulting from freezing and thawing could be attributed to the changes in zona pellucida properties. In their experiment, the fertilization rate obtained using zona-drilled frozen–thawed mouse oocytes was not significantly different from the fertilization rate of non-cryopreserved oocytes. However, the structure of the zona before and after cryopreservation was not investigated. Karlsson *et al.* (1996) found no effect of oocyte cryopreservation on mouse fertilization. Others reported that aged oocytes can be effectively cryopreserved to establish a bank for use in sperm function testing (Morroll *et al.*, 1992); that fresh oocytes can be vitrified using only a brief exposure to cryoprotective agents and survive to undergo fertilization (Hunter *et al.*, 1995); and that there are no differences between the sperm binding ability of human oocytes stored either in salt or cryopreserved (Franken *et al.*, 1989a). Sperm binding capacity of intact zonae stored for 12 months in liquid nitrogen has also been reported (Hammit *et al.*, 1993).

In the present study, no significant difference was found in the number of spermatozoa bound to the inseminated/unfertilized, cryopreserved oocytes and inseminated/unfertilized, non-cryopreserved oocytes. The degree of sperm binding variability was not different between cryopreserved and non-cryopreserved, inseminated/unfertilized oocytes. However, sperm preincubation time has been shown to influence the number of spermatozoa bound to the zona pellucida (Singer *et al.*, 1985). The data obtained in our study did not show any significant difference between incubation times. Furthermore, the variability of sperm binding to oocytes was not influenced by the length of the preincubation time. No decrease in oocyte binding variability following a short sperm incubation period is consistent with other published studies (Lanzendorf *et al.*, 1992; Sueldo *et al.*, 1993).

The variation in the sperm–zona binding demonstrated in the present study is in agreement with the findings of others (Burkman *et al.*, 1988; Hammit *et al.*, 1993). Factors determining the sperm–zona binding capacity of an individual oocyte have not been identified. This capacity may be different for different oocytes obtained from the same woman in the same cycle. The capacity of the zona pellucida to bind spermatozoa is difficult to predict and control for at present. Incubating the spermatozoa with a greater number of oocytes may improve

the reliability of the assay, but may also limit the number of laboratories that can perform the assay. The development of sperm binding assays using recombinant ZP3 may overcome both the problem of oocyte scarcity and the variability in sperm–zona binding (van Duin *et al.*, 1994).

In summary, the intact zona assay's usefulness is enhanced by the fact that cryopreserved and previously inseminated oocytes can serve as a source of testing oocytes. However, the large oocyte-to-oocyte variation in the number of spermatozoa bound to the zona *in vitro* diminishes the usefulness of this assay.

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