Aging, Hyaluronidase Removal of the Cumulus, and Microinjection Do Not Affect the Sperm Binding Potential of Human Oocytes

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Purpose: We studied the influence of aging, hyaluronidase removal of the cumulus, and microinjection on the sperm binding potential of human oocytes under intact zona assay conditions to determine the safe use of unstored aged unfertilized ICSI oocytes on zona binding tests. Results were also compared with those for aged IVF oocytes under the same conditions.

Methods: To avoid the large variation in sperm binding scores, we compared the ratios of the number of sperm bound to nontreated versus to treated oocytes. Treated oocytes were those whose zonae were exposed to hyaluronidase, an agent that decreases sperm binding. Experimental groups were fresh oocytes, experimentally aged fresh oocytes, fresh oocytes whose cumulus was removed either mechanically or with hyaluronidase, aged ICSI oocytes, and aged IVF oocytes.

Results: Statistical analysis within and between groups showed that aging, previous insemination, cumulus removal by hyaluronidase, and microinjection did not affect the sperm binding potential of human oocytes.

Conclusions: As the zona binding ability of fresh and unfertilized aged ICSI oocytes is similar, they can be used safely in zona assays.

KEY WORDS: human; intracytoplasmic sperm injection; zona binding assay.

INTRODUCTION

The human sperm–zona pellucida binding test (ZBT) has been developed using a hemizona (1–3) as well as an intact zona (4–6) model. This test has been used either to investigate the molecular mechanisms of human gamete recognition (7–14) or as a clinical assay to determine the potential use of a patient sperm in in vitro fertilization (IVF) attempts (15–20). In these studies, ZBTs have been performed either with fresh immature or mature oocytes or with aged unfertilized IVF oocytes, which were pooled until use in a concentrated salt solution or cryopreserved. In these cases, immaturity, pooling, storage, aging, or previous exposure of the zonae to sperm seemed not to affect the results of the ZBTs adversely (1–20).

Due to the widespread availability of infertility treatments and the use of intracytoplasmic sperm injection (ICSI) (21–24), some assisted reproduction laboratories now have a large number of unfertilized aged oocytes that come from fertilization failures after ICSI cycles. However, it has not been investigated if hyaluronidase removal of the cumulus and microinjection affect sperm binding.

We usually perform ZBTs using the intact zona model without egg storage by pooling our aged unfertilized oocytes coming from IVF and ICSI cycles. The purpose of the present study was to investigate and compare, under intact zona assay conditions with unstored eggs, the influence of aging, hyaluronidase digestion of the cumulus and corona layers, and microinjection on the subsequent sperm–zona binding ability of human oocytes.

MATERIALS AND METHODS

Oocytes were recovered from large ovarian follicles by ultrasonic-guided follicular aspiration in cycles

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stimulated with hMG and hCG after pituitary desensitization with a GnRH agonist and used in a therapeutical IVF or ICSI attempt. The details of these techniques and criteria for patient inclusion were described earlier (21–24).

Mature fresh human oocytes were donated by patients for research purposes when a high number of eggs were recovered during therapeutic IVF/ICSI cycles. Mature unfertilized aged oocytes were those that, 44 hr after IVF/ICSI, showed no signs of fertilization (23, 24). Spermatozoa were obtained from samples showing normal values of sperm concentration, motility, and morphology (25). Oocytes and sperm used for experimental purposes were taken after patient consent.

After liquefaction, spermatozoa were washed from seminal plasma with SPM medium (Medicult) by two sequential centrifugation steps of 5 min at 1000 rpm. The final pellet was overlaid with 0.5 ml of IVF medium (Medicult) and incubated for 1 hr at 37°C with 5% CO₂ in air to achieve a swim-up separation of vigorously motile spermatozoa.

Fresh oocytes were freed from their cumulus and corona layers either by mechanical means (dissection with fine needles followed by pipetting with fine glass pipettes) or by a 30-sec period of hyaluronidase (Medicult) treatment. Unfertilized aged ICSI oocytes were already cumulus-free because, before microinjection, the cumulus was removed with hyaluronidase as above. Unfertilized aged IVF oocytes were also already cumulus-free, due in part to having been in culture for 44 hr and also because at 16 hr after insemination the cumulus was partially mechanically removed to search for the presence of pronuclei.

To overcome the problem of the large variation in sperm binding scores, we compared not the number of sperm bound per oocyte but the changes observed in the sperm binding ability of each set of oocytes after priming the zonae with an agent (hyaluronidase) that decreases sperm binding (e.g., ratios of the number of sperm bound to nontreated versus hyaluronidase-treated oocytes). Hyaluronidase (Medicult; 80 U/ml; SPM) was chosen because it did not affect the morphology of the zonae. Some of the cumulus-free fresh and aged IVF and ICSI oocytes were then treated for a 30-sec period with hyaluronidase, after which they were washed four times in IVF medium.

For the ZBT, oocytes were then inseminated in IVF medium with 10⁵ motile spermatozoa/ml. After 4 hr of incubation at 37°C with 5% CO₂ in air, oocytes were vigorously washed by pipetting (10 times) to release loosely bound spermatozoa. Tightly bound

spermatozoa were then counted in an inverted Nikon microscope equipped with a heated stage and Hoffman modulation contrast optics. Only high-quality (with a homogeneous cytoplasmic appearance, without granularities or vacuoles) mature oocytes, with similar zona thicknesses (12–15 µm) and surface appearances, were used, and each ZBT (coupling) was performed only with oocytes from the same woman and sperm from the same donor. Because IVF oocytes contained previously bound spermatozoa in their zonae, these were counted first before performing the ZBT. The different number of eggs used for control and treated groups is due to the fact that, when there was an odd number of eggs present, more eggs were ascribed to the treated group.

The data are presented as mean ± SD. For assessing the influence of oocyte pretreatment within each group, a paired Student's *t* test was performed. The effect of the different pretreatments among groups was first examined by analysis of variance (ANOVA). After that, specific differences between groups were determined by post hoc comparisons. All the performed tests were two-tailed and the differences were considered non significant at *P* > 0.05. Statistical analysis were performed with the computer programs Excel and Statistica (Versions 4.0).

RESULTS

Experimental Group I

To investigate if hyaluronidase digestion of the zona pellucida affects sperm binding, 7 individual ZBTs (couplings) were performed in a total of 26 fresh oocytes which were previously freed of their cumulus layers by mechanical means. Ten of these oocytes were left undisturbed (control oocytes), while the other 16 were further digested with hyaluronidase (treated oocytes). Results showed that this treatment significantly decreased the binding of sperm to the zona (Table I).

Experimental Group II

To investigate if aging affects sperm binding, 10 individual ZBTs (couplings) were performed in a total of 67 fresh oocytes which were left in culture for 44 hr before being freed of their cumulus layers by mechanical means. Twenty-nine of these experimentally aged oocytes were left undisturbed (control oocytes), while the other 38 were further digested with

Table I. Effect of Oocyte Pretreatment on the Mean Total Number of Spermatozoa Linked to the Zona Pellucida^a

	Group I ^c	Group II ^d	Group III ^e	Group IV ^f	Group V ^g
Number of couplings ^b	7	10	9	13	4
Sperm per control oocyte	32.7 ± 20.6	35.0 ± 24.6	49.6 ± 70.8	81.5 ± 92.5	29.0 ± 17.0
Sperm per treated oocyte ^h	9.0 ± 7.4	14.5 ± 11.5	50.6 ± 70.8	22.8 ± 22.5	8.3 ± 4.2
Mean difference of control vs treated	23.7 ± 14.1	21.6 ± 17.1	-1.0 ± 2.5	58.8 ± 70.4	20.8 ± 13.5
Value of the difference ⁱ	<i>P</i> = 0.004	<i>P</i> = 0.003	<i>P</i> > 0.05	<i>P</i> = 0.01	<i>P</i> = 0.05

^a Values are means ± SD.^b In each ZBT (coupling), only oocytes and sperm from the same patient were used.^c Fresh oocytes freed of their cumulus by mechanical means.^d Fresh oocytes left to age for 44 hr and then freed of their cumulus by mechanical means.^e Fresh oocytes freed of their cumulus either mechanically (control) or by hyaluronidase treatments (treated).^f Aged unfertilized ICSI oocytes.^g Aged unfertilized IVF oocytes.^h Except for Group III, treated oocytes were those whose zonae pellucidae were digested with hyaluronidase.ⁱ *P* levels for the significance of the difference between the means of couplings within each group, by paired Student's *t* test.

hyaluronidase (treated oocytes). Results showed that aging did not affect the significant hyaluronidase-induced decrease in sperm binding (Table I).

Experimental Group III

To analyze if hyaluronidase removal of the cumulus layer affects sperm binding, we performed 9 individual ZBTs (couplings) in a total of 44 fresh oocytes. In 20 of these cells the cumulus layer was removed mechanically (control oocytes), while in the other 24 oocytes it was removed by hyaluronidase treatment (treated oocytes). Results showed that this treatment did not influence sperm–zona binding (Table I).

Experimental Group IV

In this group we investigated how hyaluronidase digestion of the zona pellucida affects sperm binding in unfertilized aged ICSI oocytes. Thirteen individual ZBTs (couplings) were performed in a total of 47 unfertilized aged ICSI oocytes. Twenty-one of these cells were left undisturbed (control oocytes), whereas the other 26 were further treated with hyaluronidase (treated oocytes). Results showed that this treatment also significantly reduced sperm–zona binding (Table I).

Experimental Group V

In this group we investigated how hyaluronidase digestion of the zona pellucida affects sperm binding in unfertilized aged IVF oocytes. Four individual ZBTs (couplings) were performed in a total of 23 unfertilized

aged oocytes. Nine of these cells were left undisturbed (control oocytes), whereas the other 14 were further treated with hyaluronidase (treated oocytes). Results showed that this treatment also significantly reduced sperm–zona binding (Table I).

Between Group Analysis

According to the paired analysis within groups (Table I), the pretreatment (hyaluronidase digestion of the zona pellucida) was more effective in fresh and in experimentally aged oocytes (Groups I and II) than in unfertilized aged oocytes from ICSI (Group IV) or IVF (Group V) cycles, the levels of significance of the differences being lower for Group V than for Group IV. However, the between-group analysis (Table II) showed that this order of effectiveness is an apparent one when we rely on the mean ratio as a success index (e.g., ratio of the number of sperm bound to nontreated versus treated oocytes). This index showed that hyaluronidase digestion of the zona pellucida decreased the ratio of sperm binding to the zona pellucida by three to four times in Groups I, II, IV, and V, with no statistical significant differences being noticed between these groups (Table II).

DISCUSSION

The present study shows that unstored aged mature unfertilized ICSI oocytes have the same sperm binding potential as fresh oocytes under intact-zona assay conditions. Similar results were obtained with aged IVF

Table II. Effect of Oocyte Pretreatment on the Mean Ratio Between the Number of Spermatozoa Linked to the Zona Pellucida of a Control versus of a Treated Oocyte^a

	Group I	Group II	Group IV	Group V
Number of couplings ^b	7	10	13	4
Ratio control/treated ^b	4.21 ± 1.85	3.21 ± 3.08	3.30 ± 0.66	3.43 ± 0.85

^a Values are means ± SD.^b No significant differences were detected among groups.

oocytes under the same conditions, which were used as a further internal control.

To compare the sperm binding ability of differently prepared oocytes and overcome the problem of the large variation in sperm binding scores, we compared the changes observed in the sperm binding ability of each set of oocytes after priming the zonae with an agent that decreases sperm binding. For this, hyaluronidase was preferred because it did not affect the morphology of the zonae. In this way, each set of oocytes coming from the same patient was divided into a control group (zonae not exposed to hyaluronidase) and a treated group (zonae exposed to hyaluronidase). By this method the sperm binding ability of the different sets of oocytes could be compared by using the ratio of the number of sperm bound to nontreated versus treated oocytes.

It is worth noting that hyaluronidase digestion of the cumulus did not affect sperm binding, contrary to what was found when the enzyme was applied to the zona pellucida. This is explained by the fact that a short exposure (30 sec) of the cumulus to the enzyme allows digestion of only the hyaluronic acid that exists between cumulus cells. In practice, after this digestion, corona cells must then be detached from the zona pellucida by mechanical means, further showing that this short enzyme exposure does not reach as far as the zona.

To decrease the number of variables being analyzed simultaneously, we first studied the influence of aging and of the enzymatical removal of the cumulus by using only fresh oocytes. To study the effect of aging, we determined the sperm binding ability in control and hyaluronidase-treated fresh oocytes after having left them to age for 44 hr in culture. To determine if hyaluronidase removal of the cumulus layer could affect the results, we compared the sperm binding scores of fresh mature oocytes in which the cumulus was either mechanically or enzymatically removed. In both studies, results showed that aging (Group II) or enzymatical removal of the cumulus layer (Group III) did not affect the sperm binding ability of the oocytes.

By using these two further controls, we could then compare the sperm binding ability of fresh (Group I) and aged (Group IV) unfertilized ICSI and IVF (Group V) oocytes. When between-group analysis was performed by using the mean ratio of nontreated versus treated oocytes, the sperm–zona binding was found to be decreased by three to four times in all four groups (I, II, IV, and V), which demonstrates that the sperm binding ability of fresh and unfertilized aged ICSI and IVF oocytes is similar.

In conclusion, our results, using unstored mature unfertilized aged oocytes coming from IVF cycles and used under intact-zona assay conditions, confirm the results of others, which have shown that pooling, storage, aging, and previous sperm exposure do not affect sperm binding (1–6, 15–20). Our results expand the present knowledge by showing that hyaluronidase removal of the cumulus layer and microinjection also do not affect sperm binding, thus showing that aged mature unfertilized ICSI oocytes can also be used safely for tests of sperm binding.

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