ANDROLOGY

Use of Failed-Fertilized Oocytes for Diagnostic Zona Binding Purposes After Sperm Binding Improvement with a Modified Medium

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Purpose: Because the availability of prophase oocytes for zona binding testing is limited, we compared sperm binding to the zona of failed-fertilized intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) oocytes after incubation in a standard IVF medium and a specially composed binding improvement medium.

Methods: Semen samples from nine patients and nine fertile donors were separated in parallel by the standard swim-up method in both media. Subsequently, hemizona assays were performed with prophase, failed-fertilized ICSI and IVF oocytes.

Results: Sperm separation resulted in a significantly higher sperm count ($P < 0.01$) and progressive motility ($P = 0.018$) in binding improvement medium. Moreover, spermatozoa cocubated with hemizonae (prophase, failed-fertilized ICSI and IVF oocytes) in binding improvement medium bound significantly more to hemizonae than in the controls ($P < 0.01$). However, the hemizona index did not differ.

Conclusions: Thus, the limited number of human zonae can be increased by the use of oocytes that failed to fertilize during ICSI or IVF. This will lead to a qualitative improvement of the diagnostic spectrum in male-factor infertility.

KEY WORDS: human sperm; zona pellucida; sperm binding; hemizona assay; failed-fertilized ICSI and IVF oocytes.

INTRODUCTION

Severe disorders of fertilization occur in up to 8% of in vitro fertilization (IVF) cycles, and a pathophysiologic diagnosis for specific functional characteristics should assist in identifying defects of sperm-oocyte interactions (1). In this respect, clinical tests of human sperm function have accordingly become a powerful diagnostic tool in assisted reproductive programs (2). One of these assays, which has repeatedly been shown to be an excellent predictor for fertilization in vitro (3,4), is the hemizona assay (5). In this assay, fresh prophase oocytes isolated from postmortem ovaries and usually stored in a high-concentrated salt solution for up to 3 months are used. Since these oocytes are actually immature, they show a smooth surface and compact structure of the matrix scarcely exhibiting holes (6,7). Thus, immature zonae expose only a limited number of sperm binding sites. On the contrary, the fine structure of metaphase-II oocytes is highly porous and spongy, indicating maturity status (6,7). As a consequence of this spongy structure an increased binding surface for sperm is exhibited, which is expressed in an increased binding capability (1,8). However, following fertilization the polyspermy block occurs (9) resulting in a dramatically reduced sperm binding capability of the zona. This is due to the release of the cortical granules with subsequent chemical alteration of the

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zona pellucida. Therefore, oocytes that have extruded their cortical granules, which is the case in failed-fertilized IVF and intracytoplasmic sperm injection (ICSI) oocytes, normally show no or only little sperm binding (10).

Previous studies showed that the sperm binding capability of prophase zonae pellucidae is significantly lower than that of mature metaphase-II oocytes (1) but is still enough to obtain a valid hemizona assay (11). However, the availability of prophase oocytes is very limited, especially in countries like Germany. On the other hand, failed-fertilized oocytes from an IVF or ICSI program, which are available in any IVF unit, do not bind spermatozoa (10) due to the alteration of the zona following cortical granule reaction.

To overcome this gap between the availability of actually nonfunctional zonae in many countries and the need for a better andrological workup including a better understanding of the reasons for male infertility on the molecular level, we treated failed-fertilized human oocytes from an IVF and ICSI program with a culture medium that we developed recently (8). During a short period of incubation, this medium caused a significant structural alteration of prophase zonae from a compact structure to a spongy structure. As a result, sperm binding to the treated zonae increased up to 10-fold.

Therefore, the aim of this study was to investigate whether treatment of failed-fertilized ICSI and IVF oocytes in this medium could also improve sperm binding as in prophase oocytes.

MATERIALS AND METHODS

Spermatozoa from ejaculates of nine patients (original sperm parameters for the patients and sperm donors are shown in Table I) attending the andrological outpatient clinic of the Department of Dermatology and Andrology, Justus Liebig University Giessen, Germany, were parallelly separated by standard swim-up procedure in human tubal fluid (HTF) medium

according to Quinn *et al.* (12) and in the sperm binding improvement medium (BIM) according to Henkel *et al.* (8). In brief, BIM was composed of a mixture (1:1, vol:vol) of 5 mM NaH₂PO₄ (pH adjusted with phosphoric acid), pH 2.5, plus a specially composed double-strengthened HTF medium. The specially composed double-strengthened HTF medium included the following: NaCl, 203.2 mM; KCl, 9.38 mM; CaCl₂·2H₂O, 4.08 mM; MgSO₄·7H₂O, 0.4 mM; KH₂PO₄, 0.74 mM; NaHCO₃, 450.0 mM; glucose, 5.56 mM; Na-pyruvate, 0.66 mM; Na-lactate, 42.8 mM; penicillin, 120 µg/ml; and phenol red, 5 µ/ml. The osmolarity of the mixture was adjusted to 300 mOsmol/kg. Both media contained 10 mg/ml human serum albumin (HSA) HTF-HSA and BIM-HSA, respectively. Ejaculates from nine fertile donors were used as controls in each experiment.

Hemizona assay was performed with 83 human prophase oocytes collected from postmortem material (from women aged 18 to 41 years), 48 failed-fertilized oocytes after ICSI (mature oocytes), and with 25 failed-fertilized oocytes after IVF (mature oocytes). Great care was taken to ensure that all legal and ethical guidelines were adhered to at all times during oocyte collection. Oocytes had been stored in 36.6 mM HEPES (Sigma, Deisenhofen, Germany) buffer, pH 7.4, containing 1.5 M MgCl₂ (Merck, Darmstadt, Germany) and 0.1% PVP (MM 40,000) (Sigma). All other chemicals used were purchased by Merck. Prior to use, oocytes were carefully desalted in HTF-HSA overnight. The medium was changed four times. Afterwards oocytes were microbisected by means of a micromanipulator (Luigs-Neumann, Ratingen, Germany), and hemizona assays according to Burkman *et al.* (5) were performed (Fig. 1). Each experiment

Fig. 1. Scheme of the standard hemizona assay according to Burkman *et al.* (5). Human oocytes are microbisected by means of a micromanipulator. Subsequently, remnants of the ooplasm are flushed out and matching hemizonae are coincubated with patient or donor sperm.

Table I. Original Sperm Parameters (Mean $\pm$ Standard Error of the Mean) of Patients and Donors Examined in this Study^a

	Sperm count (10 ⁶ /ml)	Motility (%)	Progressive motility (%)
Patients	44.0 $\pm$ 17.7	52.0 $\pm$ 2.7	28.1 $\pm$ 3.4
Donors	55.8 $\pm$ 8.1	53.1 $\pm$ 1.8	29.3 $\pm$ 2.5

^a No difference between patients and donors can be observed.

Fig. 2. Comparison of the sperm count after sperm separation in HTF-HSA medium or BIM-HSA. Using BIM-HSA yielded significantly ($P < 0.01$) more sperm after swim-up.

consisted of a number of incubation droplets (HTF-HSA and BIM-HSA) that each contained prepared spermatozoa ($50 \mu\text{l}$ of 0.5×10^6 motile cells/ml). To each droplet one hemizona was transferred, covered with light mineral oil (Sigma), and incubated for 4 hr at 37°C in 5% CO_2 . Apart from the zona binding results, recordings were made on motility (global motility and progressive motility) and sperm count after sperm separation in the two respective media.

Statistical evaluations were done with the nonparametric Wilcoxon two-sample test using MedCalc 2.20 (MedCalc Software, Mariakerke, Belgium).

RESULTS

Using the BIM-HSA for sperm separation resulted in a significantly higher sperm count after sperm separation than the HTF-HSA ($P < 0.01$) (Fig. 2). Although

Fig. 3. Comparison of motility and progressive motility after swim-up in HTF-HSA or BIM-HSA. Spermatozoa separated in BIM-HSA show a slightly higher motility ($P = 0.0771$) and a significantly higher progressive motility ($P < 0.018$) than those separated in HTF-HSA.

Fig. 4. Comparison of the number of sperm bound to human zona pellucidae derived from different sources (prophase, failed-fertilized ICSI, and failed-fertilized IVF). Zonae treated with BIM-HSA generally exhibit a significantly higher ($P < 0.01$) number of spermatozoa bound to their surface than those treated with HTF-HSA.

global motility was not affected by BIM-HSA ($P = 0.0771$), progressive motility was significantly increased after swim-up in BIM-HSA compared with HTF-HSA ($P = 0.018$) (Fig. 3). With regard to zona binding, spermatozoa separated and coincubated with hemizonae in BIM-HSA showed significantly higher zona binding than samples separated and coincubated in HTF-HSA ($P < 0.01$) (Fig. 4). This was the case for any type of zonae used in this study (prophase oocyte, failed-fertilized ICSI oocyte, and failed-fertilized IVF oocyte). However, the hemizona index, which is the actual diagnostic parameter for sperm binding testing in the hemizona assay, was not affected by treatment of sperm and zonae in BIM-HSA ($P > 0.2$) (Fig. 5). Concerning sperm binding, hemizonae (from prophase, failed-fertilized ICSI and failed-fertilized IVF oocytes) incubated in HTF-HSA did not differ, although sperm binding to prophase zonae was higher in tendency.

Fig. 5. Comparison of the hemizona index of 83 prophase oocytes coincubated with spermatozoa in HTF-HSA and BIM-HSA, respectively. No difference can be observed between groups.

DISCUSSION

Using the BIM-HSA medium according to Henkel *et al.* (8) for both sperm separation and sperm-oocyte coincubation, we were able significantly to improve sperm binding not only to immature human zonae pellucidae as described previously (8), but also to failed-fertilized oocytes obtained from the ICSI and IVF program. The highest increase was visible in prophase zonae. However, using failed-fertilized oocytes sperm binding to the zona pellucida still increased by a factor of 3 to 5, resulting in a number of tightly bound spermatozoa being above the cut off of 20 bound spermatozoa per hemizona which should be reached for getting a valid result in the hemizona assay (11). The hemizona index on the other hand, which is the actual predicting parameter of the hemizona assay, remained unaffected ($P > 0.2$) by the treatment. The improved zona binding can be explained by structural changes of the zona pellucida due to the slightly increased pH during culture in the BIM-HSA medium (8), resulting in an increased exposure of sperm binding sites. This obviously also happens in zonae derived from failed-fertilized ICSI and IVF oocytes. However, in these oocytes molecular changes that are associated with fertilization and leading to reduced sperm binding took place (13). Although this polyspermy block already happened in failed-fertilized ICSI and IVF oocytes, a recovery of sperm binding ability following incubation in BIM-HSA seems to be possible.

In addition to the zona binding improvement, we observed a significantly higher number of spermatozoa as well as a significantly higher percentage of progressively motile spermatozoa after swim-up in BIM-HSA. This phenomenon seems to be evident from the increased concentration of bicarbonate, which is 4.5 times higher in the BIM-HSA medium than in the regular HTF-HSA. Bicarbonate has repeatedly been shown to improve sperm motility in different species (14–17), including humans. Parkkila *et al.* (18) identified a protein in the equatorial segment of human and rat spermatozoa that is immunologically related to the erythrocyte bicarbonate/Cl exchanger. This observation suggests a specific carrier protein for bicarbonate, allowing it to cross the plasma membrane. Inside the sperm cell, bicarbonate seems to have an effect on respiration (15), and on microtubule sliding velocity (19), resulting in enhanced sperm motility. In the hamster, increased concentrations of bicarbonate also enhance hyperacti-

vated motility, which is responsible for improved zona penetration (16,20).

Boatman and Robbins (16) showed that when hamster sperm were treated with a medium containing a high concentration of bicarbonate at early stages of capacitation, this anion also seems to stimulate acrosome reaction. However, during the short-term culture in BIM-HSA we used no changes in acrosome reaction could be observed (21). Because neither the capability of zona pellucida to induce the acrosome reaction nor the potential of spermatozoa to undergo the acrosome reaction, changed under the incubation conditions in BIM-HSA (21), this is an indication that the enhanced zona binding was not due to changes in the sperm population. The percentage of spermatozoa that were capable of undergoing the acrosome reaction, i.e., those that are already acrosome-reacted plus partly reacted sperm, is similar to that reported by Coddington *et al.* (22) and Franken *et al.* (23) using a monoclonal antibody and transmission electron microscopy, respectively (21).

Because asynchrony in nuclear and zonal maturation may result in immaturity of the zona pellucida, this might be a cause for failed fertilization in in vitro matured human oocytes (1) despite normal sperm functions. According to Tesarik *et al.* (7) there are two possible solutions to this problem: (i) in vitro maturation of oocytes, which has already successfully been performed (24); and (ii) establishment of conditions during insemination with husband's sperm, that restores penetrability of the zona pellucida. On the other hand, Winston *et al.* (25) did not recommend failed-fertilized human oocytes for reinsemination, because of the poor success rate. However, Nagy and co-workers (26) fertilized 1-day-old failed-fertilized human oocytes by ICSI with good success. They reported no difference in fertilization rate of oocytes when male-factor cases and non-male-factor cases were compared. Moreover, they did not find a difference between patients showing good fertilization and those showing poor fertilization in normal IVF. This implies that failure of fertilization of mature metaphase-II human oocytes in such cases might be caused by zona dysfunction and encourages us to investigate the possibility of improvement of fertilization by treatment of failed-fertilized oocytes with BIM-HSA. Since the superior binding recorded for zonae treated with BIM-HSA is due to disaggregation in texture of zona proteins causing an increase in binding surface, and the increased concentration of bicarbonate present in the medium is responsible for improved motility, including hyperactivation, this

attempt seems possible. Increased zona binding is known to be directly correlated with fertilization (1). An essential prerequisite, however, is that reinsemination take place in the window for effective fertilization, which has been estimated as 9 to less than 22 hr after retrieval of oocytes (26,27).

In conclusion, the results indicate the practical utilization of a simple and effective laboratory method to use failed-fertilized oocytes from ICSI and IVF for diagnostic purposes in the hemizona assay. Thus, the limited number of human zonae pellucidae can be increased and will lead to a qualitative improvement of the diagnostic spectrum in male-factor infertility. In addition, the method also seems to be suitable to improve specific gamete dysfunctions closely correlated with fertilization failure.

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