Influence of Elevated pH Levels on Structural and Functional Characteristics of the Human Zona Pellucida: Functional Morphological Aspects[1]

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Purpose: *A total of 86 fresh and salt-stored immature human oocytes derived from postmortem ovarian tissue were used for this study.*
Methods: *Oocytes were randomly incubated either in synthetic human tubal fluid medium (untreated zonae) or in a chemically defined medium (treated zonae).*
Results: *Sperm binding experiments using hemizona assay conditions exhibited a 10-fold increased binding of sperm to treated compared to untreated oocytes (272.7 ± 43 versus 24.3 ± 15 sperm bound, respectively; P < 0.0001). pH recordings during incubation showed elevated pH levels of 8.1 compared to pH 7.2 among treated and untreated zonae, respectively. Ultrastructural examination showed a spongy appearance of the surface of treated zonae, whereas untreated zonae appeared compact with smooth surface.*
Conclusions: *The marked increase in sperm binding among treated zonae, together with the ultrastructural findings, suggest that the altered zona surface enhances sperm binding. The physiological maturational process of the zona pellucida might be manipulated in vitro, thus increasing sperm binding to the zona.*

KEY WORDS: spermatozoa; zona pellucida; zona binding; structure of zona; human.

INTRODUCTION

The contributions of sperm-oocyte interaction to infertility diagnostics are well documented. Sperm binding and penetration assays have frequented the literature, reporting on various aspects of sperm-oocyte interaction using either microbisected or whole human oocytes during the assay procedure (1,2). Scientists, therefore, are still urged to probe for methods to improve the understanding of gamete interaction to its full extent.

The zona pellucida serves as a physiological barrier for spermatozoa before fusion with the oolemma and penetration into the ooplasma. It prevents polyspermy (3,4) and supports species specific recognition of spermatozoa (5). Zona pellucida also facilitates sperm binding mediated by carbohydrate side chains (6,7). During or after zona binding, sperm are believed to acrosome react prior their zona pellucida penetration. Key events in the fertilization process can be attributed to the zona pellucida, and therefore functional competence of both gametes is necessary to ensure normal sperm-oocyte interaction and subsequent fertilization. In sperm, fundamental modifications in cell physiology, called capacitation, followed by loss of the acrosomal cap must occur. In oocytes, during natural transition from prophase, metaphase I to metaphase II, the oocyte matures and the zona pellucida acquires its full functional characteristic. This process is accompanied by structural changes of the zona pellucida that renders it more susceptible to sperm penetration (8). The study proceeded in two parts, namely, (i) evaluation of sperm binding capacity of zonae pellucidae that has been exposed to culture

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media containing acidic or basic zona solvents and (ii) description of structural and morphological changes of the zona that occurred during incubation in the different culture media.

MATERIAL AND METHODS

All oocytes used were collected from postmortem material. Great care was taken to ensure that legal, ethical and moral guidelines were adhered to at all times during oocyte collection. Unless otherwise mentioned oocytes were stored in 36.6 mM Hepes buffer, pH 7.4, containing 1.5 M $MgCl_2$ and 0.1% PVP at 4°C. For evaluation of ultrastructural features of the zona, 12 fresh and 18 salt-stored oocytes were used. Prior to testing, salt-stored oocytes were carefully desalted in human tubal fluid medium (HTF) according to Quinn *et al.* (9) supplemented with 10 mg/ml human serum albumin (HSA) using 4 media changes.

Media Used

(i) Medium I: 20 µl 5 mM NaH_2PO_4 (pH adjusted with phosphoric acid), pH 2.5, plus 20 µl of a specially composed double-strengthened HTF-HSA medium mix in a 1:1 (Vol:Vol) ratio. Specially composed double-strengthened HTF medium included the following: NaCl: 203.2 mM; KCl: 9.38 mM; $CaCl_2 \times 2H_2O$: 4.08 mM; $MgSO_4 \times 7H_2O$: 0.4 mM; KH_2PO_4: 0.74 mM; $NaHCO_3$: 450.0 mM; glucose: 5.56 mM; Na-pyruvate: 0.66 mM; Na-lactate: 42.8 mM; penicillin: 120 µg/ml; phenol red: 5 µg/ml.

(ii) Medium II: 20 µl Na_2CO_3, pH 9.0 plus 20 µl normal double-strengthened HTF-HSA medium composed using: NaCl: 203.2 mM; KCl: 9.38 mM; $CaCl_2 \times 2H_2O$: 4.08 mM; $MgSO_4 \times 7H_2O$: 0.4 mM; KH_2PO_4: 0.74 mM; $NaHCO_3$: 50.0 mM; glucose: 5.56 mM; Na-pyruvate: 0.66 mM; Na-lactate: 42.8 mM; Hepes: 40.0 mM; penicillin: 120 µg/ml; phenol red: 5 µg/ml.

(iii) Medium III: Standard HTF-HSA for control experiments.

Zona Binding

For zona binding experiments, ejaculates from normozoospermic sperm donors ($n = 3$) were prepared using SpermFertil® glass wool columns (Mello Ltd., FRG). Filtrates were washed and resuspended in HTF-HSA. Unless indicated all chemicals used for this study have been purchased from Merck, Darmstadt, Germany, (Na_2CO_3, NaH_2PO_4, H_3PO_4, NaCl, KCl, $CaCl_2 \times 2H_2O$, $MgSO_4 \times 7H_2O$, KH_2PO_4, $NaHCO_3$, glucose (anhydrous), tris, HCl, phenol red) or Sigma, St. Louis, USA, (Na-pyruvate, Na-lactate, human serum albumin (fraction V), Hepes).

Each experiment consisted of 3 incubation droplets containing 10 µl of standard HTF-HSA and 40 µl specially prepared culture medium mixture (medium I or medium II). To each droplet a given number of oocytes (for TEM) or hemizonae (for binding) were transferred. For binding experiments, to each droplet a 10 µl (50×10^6/ml) sperm droplet was added, covered with mineral oil (Sigma #3516), and incubated for 4 hours at 37°C in 5% CO_2. Control zonae were incubated under the same conditions in standard HTF-HSA (medium III). Three binding experiments were conducted using hemizona assay (HZA) conditions. During HZA, 52 matched hemizonae were separately incubated in medium I and II and evaluated for sperm binding. Recordings were made in separate media droplets incubated parallel with the binding experiments, to evaluate pH changes in the droplets during incubation.

Statistical evaluations of binding results were performed with the non-parametric Mann-Whitney two-sample test.

Transmission Electron Microscopy

Because of technical difficulties structural changes were studied using whole oocytes instead of hemizonae. For evaluation of ultrastructure, test oocytes incubated in medium I ($n = 15$; fresh and salt-stored) and control oocytes incubated in standard HTF-HSA (medium III) ($n = 15$; fresh and salt-stored) were fixed over night in 2.5% glutaraldehyde in Sörensen buffer. Standard transmission electron microscope procedure followed. The specimens were embedded in Spurr's epoxy resin. Ultrathin sections (80 nm) were cut on a LKB ultratome (LKB, Bromma, Sweden), stained with uranyl acetate and lead citrate and examined in a Philips 301 transmission electron microscope at 60 kV.

RESULTS

Media composition and pH values during co-incubation, significantly influenced sperm binding

to prophase and metaphase-I human oocytes. Hemizonae coincubated in medium I bound 272.7 ± 43 sperm (mean ± SE) versus those incubated in medium II (24.3 ± 15, $P < 0.0001$). Control hemizonae incubated as controls in standard HTF-HSA medium (medium III) exhibited sperm binding (32.3 ± 10, mean ± SE) comparable to those incubated in medium II (24.3 ± 15, mean ± SE, $P > 0.05$). Results are depicted in Table I.

Results of pH recordings revealed an increase in the pH value after one hour of incubation in medium I, while constant levels were recorded in medium II as well as in controls (HTF-HSA) (Table II). Since the osmolarities of media were adjusted prior to the experiments, the increased pH levels (±0.9) might be responsible for both the spongy appearance of the zonae and the increased zona binding results.

Figure 1 shows photomicrographs of fresh human oocytes incubated in standard HTF-HSA (A) and in medium I (B). Figure 2 shows photomicrographs of salt-stored human oocytes incubated in HTF-HSA (A) and in medium I. Ultrastructural evaluation of the zonae pellucidae of both fresh and salt-stored oocytes showed fine granular matrixes of light to medium electron density. There was no difference between both these groups. However, zonae pellucidae of oocytes incubated in standard HTF-HSA showed a compact, granular and medium electron dense appearance. The surfaces were smooth and homogenous (Fig. 1A, 2A). The treated zonae, on the contrary, showed numerous openings in the matrixes of the surface, thus a marked spongy appearance (Fig. 1B, 2B).

DISCUSSION

Using medium that contains acidic (NaH_2PO_4) zona pellucida solvents (medium I) for sperm-oocyte co-incubation we were able to improve sperm binding to immature human zonae pellucidae significantly ($P < 0.0001$), compared to control samples in medium supplemented with basic (Na_2CO_3) zona pellucida solvents (medium II). Control samples exhibited sperm binding in range previously reported for prophase and metaphase-I oocytes (10). The number of zona bound sperm recorded in medium I showed a 10-fold increase compared to zonae treated with medium II. The ultrastructural findings indicate that the marked spongy appearance of the surface of treated zonae is the result of the effect of medium I on fine structure of the zona matrix, causing a manifold increased surface. These structural changes are possibly due to the slightly increased pH levels in the medium, namely pH 8.1, during treatment (medium I) compared to pH 7.2 (medium II). Structural organization of zona surface of treated immature (prophase/metaphase I) oocytes compares well with the zonae of mature (metaphase II) oocytes described by Tesarik *et al.* (8) and Familiari *et al.* (11) using TEM and SEM, respectively. Similarly, a spongy structural organisation of zona components was described in mice and hamsters (12).

The dramatic increase in sperm binding to treated zonae together with the ultrastructural alterations suggest that the changed surface observed enhanced sperm binding. Provided that there are no genetically or post-translationally caused alterations of zona proteins itself, which may also result in reduced sperm binding, exposure of more sperm binding sites on the zona pellucida leads to improved sperm binding. Consequently, our results confirm the speculation of Familiari *et al.* (11) that sperm binding is closely correlated to binding surface of the zona pellucida. Therefore, sufficient sperm binding, which seems to be dependent on zona efficiency and binding ability of the sperm itself, is a prominent prerequisite for fertilization. Increased zona binding is known to be directly correlated with fertilization *in vitro* (10). Physiological disaggregation in texture of zona proteins takes place during natural transition of metaphase I to metaphase II oocytes. The process, called "zona maturation," is not only influenced by the oocyte, but also by the surrounding cumulus cells. In fact, Tesarik and Kopecný (13) showed by means of autoradiography and histochemistry an active secretion of proteoglycans from both, the human metaphase II oocyte and cumulus cells into the zona pellucida.

Table I. Mean (±SE) Number of Bound Sperm Under Hemizona Assay Conditions (three experiments)

Number of sperm bound to hemizonae		
Medium I	Medium II	Medium III (Control)
272.7 ± 43.6*a* *n* = 24	24.3 ± 15.2*b* *n* = 20	32.3 ± 10.0*c* *n* = 8

a vs. *b*: $P < 0.0001$.
a vs. *c*: $P = 0.0008$.
b vs. *c*. $P > 0.05$.

Table II. Results of pH Measurements in the Different Media and Control After 3 h of Incubation in 5% CO_2 and 20°C and 37°C, respectively

	Before incubation	After 3 h incubation at 20°C	After incubation at 37°C		
			1 h	2 h	3 h
Incubation medium					
Medium I	7.68	7.68	8.10	8.20	8.21
Medium II	7.10	7.27	7.33	7.31	7.28
Medium III (control)	7.02	7.27	7.33	7.30	7.27

Many *in vitro* fertilization failures can be attributed to premature addition of spermatozoa to meiotically immature oocytes (8). Although, human oocytes can be successfully matured *in vitro* (14–16), maturation rate as well as fertilization rate, is relatively low (17,18). An asynchrony in nuclear and zonal maturation resulting in immaturity of the zona pellucida, might be a cause for failed fertilization in *in vitro* matured human oocytes (10). Therefore, adequate timing of both nuclear and zonal maturation seems to be of major importance. During *in vivo* "zona maturation," secretions of the cumulus cells might alter the microenvironment that exists between cumulus cells and the oocyte. These changes might include pH changes, resulting in disaggregation and fenestration of the zona pellucida similar to the observations of the present report.

In conclusion, functional ability of the zona pellucida to bind sperm is closely connected to mor-

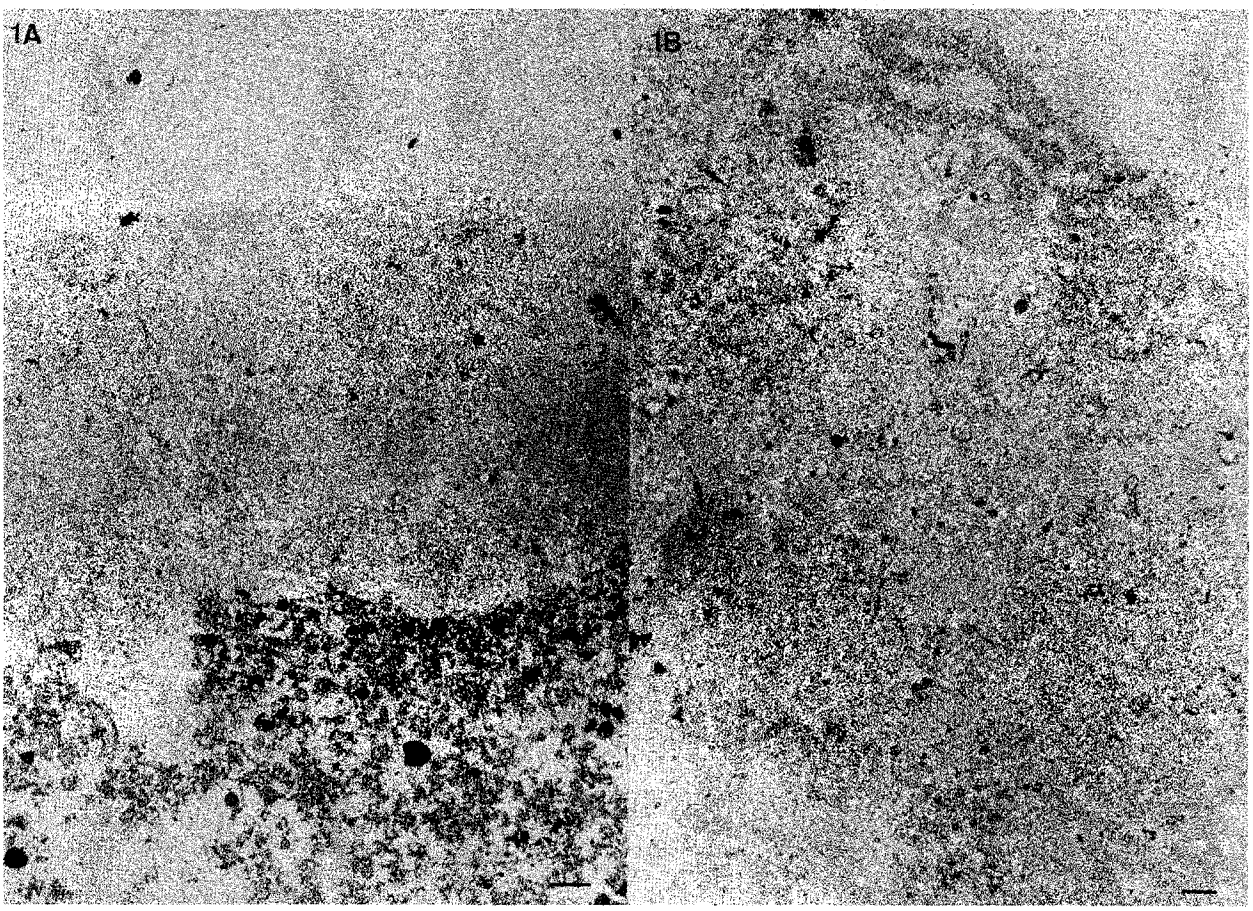

Fig. 1. Electron micrographs from fresh human oocytes. Untreated zona (A) shows a compact, granular appearance with smooth surface, while zona treated with medium I (B) exhibits numerous openings and a spongy appearance. (Bar: 1 μm)

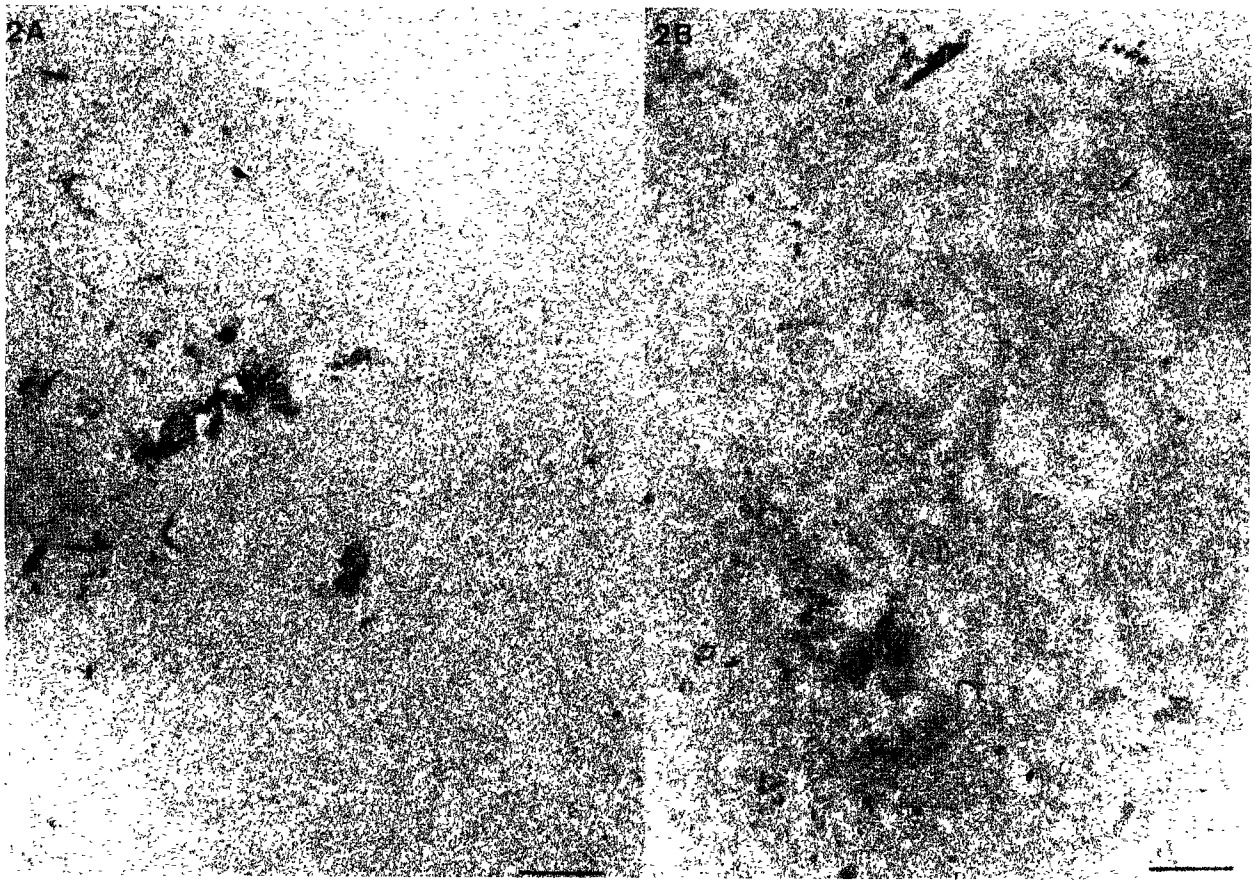

Fig. 2. Electron micrographs from salt-stored human oocytes. Untreated zona (A) shows a compact, granular appearance with smooth surface, while zona treated with medium I (B) exhibits numerous openings and a spongy appearance. (Bar: 1 μm)

phological appearance. Thus, it is important to underline the functional morphological aspects during evaluation of specific functions of the gametes.

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