Structure of the cumulus matrix and zona pellucida in the golden hamster: A new view of sperm interaction with oocyte-associated extracellular matrices

Ashley I. Yudin¹, Gary N. Cherr ², and David F. Katz ¹

¹ Department of Obstetrics and Gynecology, School of Medicine, University of California, Davis, California, USA; ² Bodega Marine Laboratory, University of California, Bodega Bay, California, USA

Summary. Hamster oocyte-cumulus complexes (OCC), with and without sperm, were structurally analyzed by light- and electron microscopy using freeze substitution. This method has yielded a clear picture of the extracellular oocyte investments, the cumulus cell matrix and the zona pellucida. The cumulus matrix has an overall homogeneous fibrillar structure which appears to attach to cumulus cells at their filopodial extensions. The matrix also extends into the outer regions of the zona pellucida. The zona pellucida has a distinct porous configuration throughout its entire structure. During gamete interaction experiments, capacitated hamster sperm with ultrastructurally intact acrosomes were found throughout the matrix. Sperm had dramatic effects on the matrix, resulting in compression and stretching. Sperm found on the zona pellucida had initiated or completed the acrosome reaction. During the initial stages of the acrosome reaction, the matrix was in contact with the sperm. At later stages of the acrosome reaction, there was a complete loss of matrix material in regions near the sperm.

Key words: Cumulus – Zona pellucida – Extracellular matrix – Sperm – Fertilization – Golden hamster

Immediately prior to fertilization, mammalian sperm must traverse two extracellular egg investments, the cumulus matrix and the zona pellucida. The former is a viscoelastic extracellular matrix consisting of glycosaminoglycans, primarily hyaluronic acid (Ball et al. 1982), while the latter is a highly structured investment containing 3–5 glycoproteins (see Dunbar and Wolgemuth 1984, for review). Mechanisms of sperm penetration of the cumulus and zona remain incompletely understood and are, to some extent, controversial (Talbot 1985). The principal active processes are mechanical forces generated by the motile flagellum and digestion of the extracellular egg investments by sperm enzymes. The latter may be intimately linked to the time course of the sperm acrosome reaction.

It is not clear whether hyaluronidase in the sperm acrosome and/or on the sperm surface is involved in sperm passage through the cumulus matrix (Zao et al. 1985; Corselli and Talbot 1987; see Talbot 1985, for review). In addition, cumulus components may stimulate sperm motility (Bradley and Garbers 1983) and induce the acrosome reaction; they may also enhance the fertilizing ability of sperm (Bavister 1982; Tesarik 1985; Meizel and Turner 1986). Recent studies with the hamster (Cherr et al. 1986; Corselli and Talbot 1987) have suggested that capacitated sperm with intact acrosomes or modified acrosomes (Cummins and Yanagimachi 1986) are capable of traversing the entire cumulus matrix. It has been demonstrated that changes in sperm motility occur when non-acrosome reacted hamster sperm enter the matrix (see Katz et al. 1987, for review). Thus, it has been suggested that flagellar motion plays a necessary, if not sufficient role in sperm passage through oocyte investments (Katz et al. 1987). A thorough understanding of the microenvironment that the sperm encounters during gamete interaction requires geometric and physical characterization of the microstructure of the cumulus matrix and zona, in addition to elucidation of their molecular constituents. As in studies of extracellular matrices from other cells, the ultrastructure of the matrix has been observed by transmission electron microscopy (TEM) following staining with cationic dyes (Talbot 1984). While these compounds exhibit a high affinity for acid mucopolysaccharides (Luft 1971) and stabilize the extracellular matrix to some degree, they do not prevent the dramatic shrinkage during preparation (Talbot and DiCarlantonio 1984a). Clearly, preparative techniques that minimize shrinkage of the cumulus and zona would contribute significantly to such work.

In the present study, we have employed freeze substitution to prepare oocyte-cumulus complexes for light- and electron microscopy. This technique has enabled us to view the fine structure of the cumulus matrix and zona for the first time without some of the artifacts that have typically hampered more conventional approaches. More importantly, preparation of samples in this manner has enabled ultrastructural assessment of the impact of the sperm on the cumulus matrix and zona, as well as the effects of these matrices on sperm functions, e.g., the acrosome reaction and movement characteristics.

Materials and methods

Hamsters

Golden hamsters (Mesocricetus auratus; 8 weeks old) were obtained from Charles Rivers Labs (Wilmington, Massachusetts) and Simonson Labs (Gilroy, California). All were
held in a 14:10 light:dark cycle. For some animals, the light cycle was reversed such that the dark cycle began at 7 a.m.

**Chemicals**

All chemicals used in the preparation of media or injections were purchased from Sigma Chemical Company (St. Louis, Missouri). Supplies for electron microscopy were obtained from Ted Pella (Tustin, California).

**Media**

Dulbecco's phosphate-buffered saline (PBS) was used to wash all gametes. The concentrations of salts were 144 mM NaCl, 3.15 mM KCl, 8.24 mM Na2HPO4, 1.48 mM KH2PO4, 0.93 mM CaCl2·2 H2O and 0.40 mM MgCl2·6 H2O. PBS was stored at 4°C. All gamete incubations were carried out in a modified Tyrode's buffer in which the salt concentrations were 123 mM NaCl, 3.1 mM KCl, 0.3 mM Na2HPO4·H2O, 24.9 mM NaHCO3, 2 mM CaCl2·2 H2O, 0.4 mM MgCl2·6 H2O. Media for sperm capacitation and fertilization (FM) also contained 0.5 mM taurine, 0.5 mM glucose, 0.25 mM pyruvate, 12.5 mM Na-lactate and 12.5 mg bovine serum albumin (BSA). The pH of this solution was adjusted to 7.3 with CO2.

**Oocyte collection**

Mature female hamsters were stimulated to superovulate by an intraperitoneal injection of 25 IU of pregnant mare serum gonadotropin (PMSG), followed by injection of 25 IU of human chorionic gonadotropin (HCG) at least 60 h later. They were sacrificed 12.5 h after the HCG injection. In some experiments, female hamsters were tested for their stage of the estrous cycle so that unstimulated ovaries could be used. The products of naturally stimulated ovaries tended to exhibit less variability.

Female hamsters were monitored for receptivity to males; if receptive, they were removed and sacrificed 4-5 h after the onset of the dark cycle. Excess ovaries were rinsed in PBS (pH 7.2) and dissected to remove peripheral tissue.

Mature follicles were then ruptured, the external granulosa wall removed and the oocyte-cumulus complex was gently teased from the follicle (Talbot 1983). Isolated oocyte-cumulus complexes were thrice rinsed and placed in FM. OCC's were kept at 37°C and after isolation were maintained in an atmosphere of 5% CO2, 95% air. After 30 min, OCC's were either fixed or incubated with sperm.

**Sperm collection**

Caudal epididymal sperm were obtained and capacitated according to Meizel and Turner (1983), as modified by Cherr et al. (1986). Only sperm suspensions with >70% motility, of which 70% of the cells exhibited hyperactivated motility after 3.5-4.0 h of incubation, were used in the gamete interaction studies.

**Sperm-egg interaction**

OCC's (10) pipetted into 500 μl fresh FM were overlaid with paraffin oil (37°C) that had been previously equilibrated in CO2 and buffer. Sperm suspensions were generally diluted prior to combining with OCC's, such that a final ratio of 20-100 sperm/OCC was achieved. Under these conditions, 6-10 sperm were found within an OCC after 20 min. Following introduction of sperm, samples were coincubated at 37°C under 5% CO2 and 95% air for either 10 or 20 min prior to fixation.

**Processing for electron microscopy**

OCC's were prepared for electron microscopy by modifying a previously reported technique for freeze substitution (Murata et al. 1985; see Gilkey and Staehelin 1986, for review). OCC's with and without sperm were prefixed at the appropriate times by adding 100 μl of 2.5% glutaraldehyde-buffered in 0.1 M cacodylate (pH 7.3). After 5-10 min, the OCC's were transferred to fresh 0.1 M cacodylate-buffered 2.5% glutaraldehyde to which 0.25% acrolein had been added. OCC's were fixed for 1 h, and then thoroughly washed in 0.1 M cacodylate overnight. Samples were post-fixed in 0.1% tannic acid for 2 h. Thereafter samples were washed for at least 4 h in 0.1 M cacodylate. Washed OCC's were placed in a 10% DMSO — 0.1 M cacodylate solution for at least 30 min.

OCC's were then placed in 3-mm gold freeze-fracture caps; excess liquid was removed with filter paper. Gaseous freon-22 was injected into a small well and bathed in liquid nitrogen (LN2) until it liquified and eventually solidified. A hexagonal wrench was then inserted into the frozen freon to liquefy it. The cap containing the OCC was then immersed for 30 sec and immediately placed in LN2. This process was continued until all OCC were in LN2. The caps containing OCC were then transferred to 100% methanol to which 0.1% OsO4 had been added. The caps containing OCC were then transferred to 100% methanol. After 4-8 h, they were placed in a Revco freezer at -80°C. After 72 h the 100% methanol was replaced with 0.1% OsO4 in 100% methanol, and placed in a freezer at -20°C for 4-8 h. After removal of the H2O, the sample usually floated free from the gold caps. Samples were brought up to room temperature (1 h), washed twice in 100% methanol, and then placed overnight in a 50:50 mixture of Spurrs' epoxy resin and 100% methanol. The samples were infiltrated with resin for 4 h and then embedded overnight.

Samples were sectioned with glass and diamond knives. The 0.5-μm thick sections were stained according to del Cerro et al. (1980), a procedure that enhanced the appearance of the extracellular matrix. Samples for TEM, sections of the OCC were cut on a diamond knife and stained with uranyl acetate and lead citrate. Observations and photography were performed on a Philips 410 TEM.

Samples used for scanning electron microscopy (SEM) were prepared as previously described and then criticalpoint dried before sputter coating with gold. Micrographs were taken on a Philips 501 SEM.

**Results**

**Cumulus and zona material**

After puncturing a mature follicle, the OCC was immediately expelled. The mature OCC usually continued to expand until it had increased in diameter by approximately 10%. Whether this expansion was due to continued hydration, dilution of follicular fluid or rebounding from elastic compression is not yet clear. However, it should be noted that