

The Use of Zona-Free Animal Ova as a Test-System for the Assessment of the Fertilizing Capacity of Human Spermatozoa

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ABSTRACT

Human spermatozoa are initially incapable of penetrating zona pellucida-free animal (hamster) ova but gain the ability to do so when incubated several hours *in vitro*. Experimental evidence suggests that this ability is associated with the completion of sperm capacitation and the acrosome reaction. Thus it appears that zona-free animal ova can be substituted for human ova in the preliminary assessment of the fertilizing capacity of human spermatozoa when human ova are not readily available.

INTRODUCTION

The fertilizing capacity of human spermatozoa could be assessed by depositing the spermatozoa in the female genital tract at the time of ovulation and later examining the ova for evidence of fertilization. Alternatively, ova can be inseminated *in vitro* (Hayashi, 1963; Edwards et al., 1969; Bavister et al., 1969; Edwards et al., 1970; Seitz et al., 1971; Soupart and Morgenstern, 1973; Soupart and Strong, 1974; Overstreet and Hembree, 1975). The latter approach eliminates many complicating factors inherent with *in vivo* techniques and enables us to conduct precise, analytical studies of the physiology of spermatozoa and of the fertilization processes. The only serious drawback to this approach is that human ova are not readily available in most hospitals and laboratories. While studying ovum-sperm interactions among various animals, we found that human spermatozoa, which were initially incapable of penetrating zona pellucida-free animal (hamster) ova, acquired the ability to do so when incubated several hours *in vitro*. Here, we present supportive evidence that zona-free hamster ova may be used as a substitute for human ova in the preliminary assessment of the fertilizing capacity of human spermatozoa.

MATERIALS AND METHODS

The reasons for using the ova of the golden hamster (*Mesocricetus auratus*) in this study were simply

that these ova could be readily obtained in our laboratories and the background information (Yanagimachi and Noda, 1970a,b; Yanagimachi, 1972a) needed in the interpretation of the results was available. The medium used throughout the experiments for handling the ova and spermatozoa was a modified Krebs-Ringer's solution originally developed by Biggers, Whitten and Whittingham (1971) for the culture of mouse preimplantation embryos. This medium (abbr. BWB medium) was used supplemented with 0.3 percent (w/v) human serum albumin (Fraction V; Sigma Chem. and Miles Lab.). Mature unfertilized ova were collected from the oviducts of superovulated hamsters 15 to 17 h after an intraperitoneal injection of human chorionic gonadotropin (Ayerst Lab.) (Yanagimachi, 1969). The ova were freed from the surrounding cumulus cells by treating them for 15 min at 25°C with 0.1 percent (w/v) bovine testicular hyaluronidase (300 USP units/mg; ICN Pharmaceut.) in BWB medium, then treated for 2 or 3 min (25°C) with 0.1 percent (w/v) bovine pancreatic trypsin (2X cryst., 10⁴ BAEE units/mg; Sigma Chem.) in BWB medium to remove the zona pellucida. Some ova were not treated with trypsin, thereby leaving the zona intact. Both zona-intact and zona-free ova were thoroughly rinsed with BWB medium and placed in 0.1 ml of fresh BWB medium under mineral oil (Squibb) in a plastic Petri dish (3.5 × 1.0 cm; Falcon Plastics). Human semen was obtained from healthy, young adults. Each fresh ejaculate was placed in an air-tight vial (5 × 4 cm) and kept at 25°-37°C for about 30 min to allow liquification then diluted with 10 ml of BWB medium. The diluted semen was filtered through two layers of tissue paper (Kimwipes 900-S, Kimberly-Clark) and centrifuged at 600 g for 4 min (25°C). The supernate was discarded and the sediment (containing the spermatozoa) was resuspended in 10 ml of BWB medium and centrifuged again (600 g for 4 min). After this was repeated once more, the spermatozoa were suspended in 2 ml of fresh BWB medium and gently centrifuged (14g) for 1 min to sediment some of the debris. The supernatant sperm suspension was diluted with BWB medium so that the sperm concen-

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TABLE 1. Sperm penetration into zona-free ova inseminated with fresh, non-preincubated spermatozoa*.

Incubation atmosphere	No. (%) of ova penetrated: time after insemination					
	2 h	3 h	4 h	5 h	6 h	7 h
Air	0/40 (0)	0/34 (0)	4/33 (12.1)	...	18/34 (52.9)	22/38 (57.9)
5% CO ₂ in air	0/39 (0)	0/42 (0)	0/34 (0)	4/23 (17.4)	14/30 (46.7)	12/22 (54.5)

*Zona-free ova were mixed with fresh spermatozoa (final sperm conc. 3.6×10^5 /ml) and examined for sperm penetration. At least 4 experiments in each group, using the ova from 4 or more different females.

tration became 1.2×10^5 per ml. One tenth to two tenth ml of this suspension was placed in the central well (18×4 mm) of a plastic organ culture dish (Falcon Plastics) and the surface was immediately covered with mineral oil. The preparation was incubated at 37°C for up to 7.5 h in either air or 5 percent CO₂ in air. The pH value of the sperm suspension in the dish was determined with a glass electrode during and at the end of incubation, and found to be 8.1-8.3 under the air atmosphere and 7.3-7.6 under 5 percent CO₂ in air. The ability of the spermatozoa to penetrate the ova was assessed by transferring a drop (about 0.03 ml) of the sperm suspension to 0.1 ml of BWB medium containing the ova and examining the ova at various time intervals for evidence of sperm penetration. The ova were recorded as "penetrated" (fertilized) when swollen sperm heads or sperm pronuclei were discernible within the cytoplasm. Examination of living ova with phase-contrast microscopy was adequate for the assessment of penetration, but some ova were fixed overnight with a mixture of ethanol and acetic acid (3:1) and stained with 0.25 percent acetocarmum to reveal cytological details. Other samples of the spermatozoa and ova were fixed with 3 percent glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 4°C and processed for electron microscopy (Yanagimachi and Noda, 1970a).

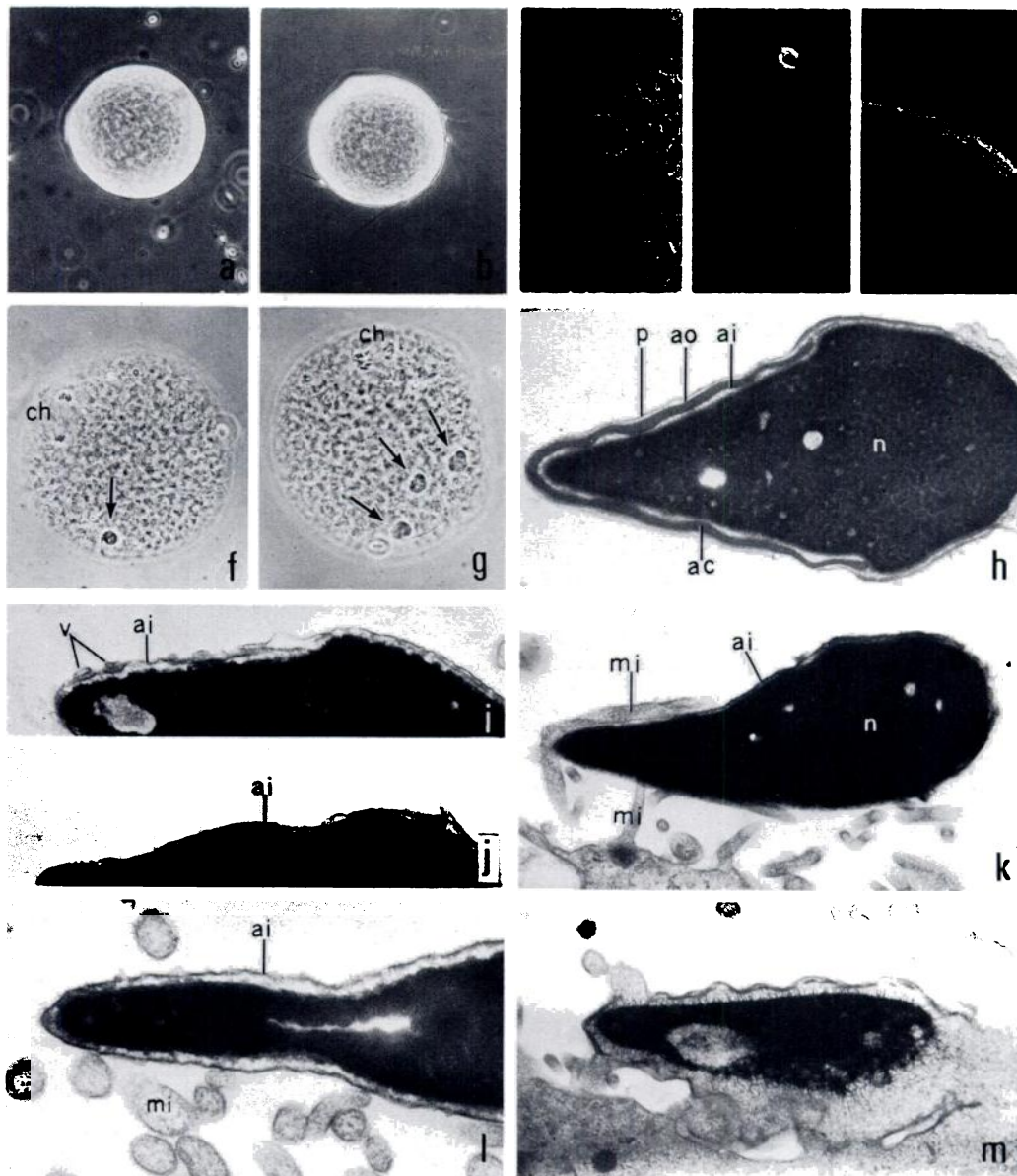
RESULTS AND DISCUSSION

When zona-intact ova were inseminated, none of the ova (0/75) were penetrated by spermatozoa regardless of the duration of preincubation (0-12 h) of the spermatozoa in BWB medium or the time of examination following insemination. The spermatozoa collided with the zona surfaces, but none of them bound to or penetrated the zona; thus a complete failure of sperm penetration into the ova resulted.

When zona-free ova were inseminated with fresh, non-preincubated spermatozoa, sperm penetration (as evidenced by the presence of swollen sperm heads or sperm pronuclei within the ovum cytoplasm) began 4 to 5 h after insemination and the percentage of penetrated ova increased with time. It appeared that sperm penetration occurred a little sooner when the ova were incubated in air alone than when they were incubated under 5 percent CO₂ in air (Table 1). Examination of the ova at various times after insemination showed that the spermatozoa failed to bind to the ovum surface within the first 2 h following insemination (Fig. 1a). The binding began 3 to 4 h after insemination and the number of bound spermatozoa increased up to 10 to 20 per ovum with longer incubation times (Fig. 1b). Many of the bound spermatozoa were motionless and their heads were swollen (Figs. 1c, d) or had transformed into pronuclei (Fig. 1e).

Further experiments were performed to determine whether preincubation of the spermatozoa would affect the time of sperm penetration into the ova. When the spermatozoa were preincubated in BWB medium for 4 h, then mixed with the zona-free ova, penetration began in 1 h, but even faster and more efficient sperm penetration occurred when the spermatozoa were preincubated for 7.0 to 7.5 h (Table 2). When the ova were inseminated with spermatozoa which had been preincubated for 7.0 to 7.5 h, sperm binding to the ovum surfaces was seen at 30 min after insemination when about

FIG. 1. Human spermatozoa before, during and after penetration into zona-free hamster ova: a) Freshly ejaculated spermatozoa shortly after being mixed with ova; no binding to ovum surface, living specimen; b) Five hours after spermatozoa and ova were mixed; note that several spermatozoa have bound to the ovum surface, living specimen; c-d) Swollen sperm heads in ovum cytoplasm, 3-6 h after freshly ejaculated spermatozoa were mixed with ova; compare the size of the swollen sperm head with that of the head of a spermatozoon outside



the ovum (the top of d), fixed and stained specimen. e) A sperm pronucleus, 6 h after freshly ejaculated spermatozoa were mixed with the ovum, living specimen; arrow indicates the posterior region of sperm tail which is still outside the ovum in this particular case. f-g) Monospermic (f) and polyspermic (g) ova inseminated with spermatozoa preincubated to 7-7.5 h; 1 h after insemination; arrows indicate swollen sperm heads, fixed and stained specimens. h) A sagittal section through the head of a freshly ejaculated spermatozoon showing intact acrosome. i-j) Sagittal halves of heads of spermatozoa preincubated for 7-7.5 h, showing that the acrosome was vesiculated (i) or absent except for the inner acrosomal membrane (j). k) A sagittal section of the head of a spermatozoon which is about to fuse with an ovum, 25 min after the ovum was mixed with spermatozoa preincubated for 7-7.5 h; note that the acrosome is absent except for the inner acrosomal membrane. l-m) Sections through the head of a spermatozoon fusing with ovum, 45 min after the ovum was mixed with spermatozoa preincubated for 7-7.5 h; the anterior region of the sperm head (l) is still outside the ovum, while the posterior region of the head (m) has fused with the ovum.

Abbreviations: ac, acrosome; ai, inner acrosomal membrane; ao, outer acrosomal membrane; ch, ovum chromosomes at early telophase of the second meiotic division; mi, microvilli of ovum; n, nucleus; p, plasma membrane; v, vesicles formed by multiple fusions between plasma membrane and outer acrosomal membrane.

Magnifications: a-b, $\times 250$; c-e, $\times 460$; f-g, $\times 250$; h-j, $\times 18,500$; k, $\times 15,000$; l, $\times 37,500$; m, $\times 13,500$.

TABLE 2. Effect of preincubation of spermatozoa on the timing of sperm penetration into zona-free ova*.

Preincubation time (h)	No. (%) of ova penetrated: time after insemination				
	30 min	1 h	1.5 h	2 h	3 h
4	0/32 (0)	8/40 (20.0)	12/36 (33.3)	12/32 (37.5)	18/26 (69.2)
7.0-7.5	6/31 (19.4)	45/47 (95.7)

*Zona-free ova were inseminated with preincubated spermatozoa (final sperm conc. $3-6 \times 10^5$ /ml) and examined for sperm penetration. Gas phase during sperm preincubation, 5% CO₂ in air. At least 4 experiments in each group, using the ova from 4 or more different females.

one fifth of the ova had swollen sperm heads within the cytoplasm. The disappearance of all or the large majority of the cortical granules from these ova as well as many of the ova with spermatozoa only bound at their surfaces indicated that sperm penetration (sperm-ovum fusion) and activation of the ova had occurred shortly after insemination. By 1 h after insemination virtually all the ova contained swollen heads. The number of swollen sperm heads or pronuclei in one ovum varied from one to seven with one to three being most common (Figs. 1f, g).

The results described above clearly indicate that ejaculated human spermatozoa are initially incapable of binding to and penetrating zona-free hamster ova, but gain the ability to do so when they are incubated in the medium for 4 h or more. Obviously the physiological properties of the spermatozoa which have been incubated in the medium are different from those of freshly ejaculated spermatozoa. The difference can not be attributed to a difference in the motility of the spermatozoa since both fresh and incubated spermatozoa are equally motile. No distinctive morphological differences in these spermatozoa were detected by light microscopy. Electron microscopy, however, revealed a profound difference in the structure of the acrosomes of these spermatozoa. All of the fresh spermatozoa (with the exception of apparently "dead" spermatozoa) had intact acrosomes (Fig. 1h), while many (30-60 percent) of the spermatozoa preincubated for 7.0 to 7.5 h had either "vesiculated" acrosomes (Fig. 1i) or no acrosomes (Fig. 1j). All the spermatozoa which were about to fuse (Fig. 1k) or were fusing (Figs. 1l, m) with the ova lacked intact acrosomes. It appears, therefore, that freshly ejaculated spermatozoa with intact ac-

rosomes can not fuse with zona-free ova, whereas the incubated spermatozoa that have undergone the acrosome reaction can readily do so. Previous studies (Yanagimachi and Noda, 1970b; Yanagimachi, 1972b; Noda and Yanagimachi, 1976) have shown that both hamster and guinea pig spermatozoa fuse with zona-free hamster ova provided the spermatozoa have undergone the acrosome reaction. Acrosome-intact spermatozoa do not fuse with zona-free ova regardless of whether the ova are from the same species (Yanagimachi and Noda, 1970b; Yanagimachi, 1972b; Noda and Yanagimachi, 1976) or a different species (Yanagimachi, 1972a). In other words, the zona-free ova of one species (e.g., the hamster) can be used for the assessment of the acrosome reaction of the spermatozoa of other species as well as the same species.

The acrosome reaction, which is a prerequisite for the successful penetration of spermatozoa through the zona pellucida (Austin and Bishop, 1958; Yanagimachi and Chang, 1964; Yanagimachi and Noda, 1972; Bedford, 1972), is believed to occur only after the spermatozoa have undergone a physiological change called "capacitation" (Austin, 1958, 1967; Bedford, 1970; Yanagimachi and Usui, 1974). The acrosome-reacted spermatozoa, therefore, can be considered to have completed the process of capacitation. The human spermatozoa that underwent the acrosome reaction in the incubation medium must have completed the process of capacitation. These acrosome-reacted (and capacitated) human spermatozoa cannot penetrate the zona pellucida of hamster ova, but must be able to a) penetrate the zona pellucida of human ova and b) fuse with zona-free ova of the human, as well as other species. According to Soupart and Strong (1975), acrosome-

reacted human spermatozoa are capable of fusing with zona-free human ova. They also state that acrosome-intact human spermatozoa can fuse with zona-free human ova. However, the evidence they presented (Fig. 3B of their paper) is not convincing enough to substantiate the latter statement. It is our current belief that unless spermatozoa have been exposed to highly abnormal conditions, only acrosome-reacted spermatozoa are able to fuse with zona-free ova.

From the evidence presented above, it is highly probable that we can use zona-free animal ova as a substitute for human ova to assess capacitation and the acrosome reaction of human spermatozoa. Since only acrosome-reacted (capacitated) spermatozoa can fuse with zona-free ova, one may assess whether the spermatozoa have completed capacitation and the acrosome reaction by examining the ability of the spermatozoa to penetrate (fuse with) the ova immediately after being mixed with the ova. Human spermatozoa, if they are fertile, should be able to develop the ability to fuse with zona-free animal ova in the proper incubation media. An important precaution is that positive results do not necessarily imply that the spermatozoa in question would be fertile under normal *in vivo* conditions. Normal fertilization involves many processes other than sperm capacitation, acrosome reaction and ovum-sperm fusion. The positive results should be interpreted merely as an indication that the spermatozoa are capable of capacitation, acrosome reaction and fusion with the ova. Negative results, on the other hand, clearly indicate that the spermatozoa are unable to accomplish all or some of these processes and would probably be unable to effect fertilization *in vivo*. Beyond doubt, normal human ova are the most ideal subjects for determining the fertilizing capacity of human spermatozoa. We merely suggest here that where and when human ova are not available, zona-free animal ova could be used as a substitute for human ova in the preliminary assessment of the fertilizing capacity of human spermatozoa. In this study we used the ova of the golden hamster simply because of convenience. The ova of other laboratory animals, such as the rat and mouse, might also be useful for this purpose.

ADDENDUM

We have recently performed experiments in which cumulus cell-free human ova were in-

seminated in BWW medium (gas phase, 5 percent CO₂ in air) with freshly ejaculated (washed) human spermatozoa. We observed that zona penetration by spermatozoa began between 4 and 5 h after insemination.

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RECOMMENDED REVIEWS

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