Addition of an androgen-free epididymal protein extract increases the ability of immature hamster spermatozoa to fertilize *in vivo* and *in vitro*

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Summary. The fertility of spermatozoa from the different epididymal segments of hamsters was tested by in-vivo insemination. Caput and proximal corpus spermatozoa were non-fertile; spermatozoa from the distal corpus epididymidis fertilized 13% (38/290) oocytes and those from the proximal and distal cauda epididymidis 71 and 87%, respectively. When tested by in-vitro insemination, distal corpus spermatozoa penetrated 44% of oocytes while those from the distal cauda fertilized 87% of oocytes. Spermatozoa from the distal corpus recovered in Medium BMOC fertilized 13% (28/219) of oocytes *in vivo*, while those mixed with an epididymal protein preparation (0.8 mg protein/ml) fertilized 24% (49/204; $P < 0.01$) of oocytes.

When distal corpus spermatozoa were inseminated *in vivo* with 0.8 mg epididymal protein preparation 34% (31/90) oocytes were fertilized and only 22% (23/103; $P < 0.05$) oocytes were fertilized when the proteins were obtained from epididymides of animals castrated for 30 days. When distal corpus spermatozoa were preincubated for 5 h in medium without (control) or with protein preparation (0.8 or 1.6 mg protein/ml), a significant increase in in-vitro oocyte penetration was found (25 compared with 45%; $P < 0.05$) when the protein was present at 1.6 mg/ml.

These results confirm and extend previous observations suggesting a role for androgen-dependent glycoproteins secreted by the epididymis in the acquisition of fertilizing ability that occurs during sperm maturation.

Introduction

Using hamster epididymal tubules maintained in organ culture as a model system, we have previously shown (Cuasnicú, González Echeverría, Piazza & Blaquier, 1984a) that androgen stimulation resulted in the synthesis of glycoproteins EP1–EP6 (González Echeverría, Cuasnicú & Blaquier, 1982) and the deposit of some of these on the surface of spermatozoa within the tubules. The resulting sperm population had an increased ability to bind to zonae pellucidae. The effect of androgens was suppressed if protein synthesis was inhibited and was mimicked by an androgen-free epididymal preparation, enriched in the androgen-dependent glycoproteins EP2–EP6 (Cuasnicú, González Echeverría, Piazza, Piñeiro & Blaquier, 1984b) added to the medium. When interpreted in the light of reports stressing the synchronous development of zona-binding and fertilizing ability (Saling, 1982; Fournier-Delpech, Courtens, Pisselet, Delaleu & Courot, 1982), our data were construed to denote an advancement in the stage of maturation of the sperm population caused by the androgenic stimulation of the tissue and involving the glycoproteins EP1–EP6.

However, since sperm maturation is defined in terms of acquisition of fertilizing ability, it was deemed necessary to test the effect of such proteins upon the ability of hamster spermatozoa to penetrate oocytes.

Materials and Methods

Materials. Adult male (10–15 weeks old) and immature female (5–7 weeks old) golden hamsters (*Mesocricetus auratus*) were kept in 14 h light/24 h with food and water *ad libitum*. Females were induced to superovulate by injection of 30 i.u. PMSG followed 48–72 h later by 50 i.u. hCG.

A fraction enriched in androgen-dependent hamster epididymal glycoproteins was obtained as described by Cuasnicú *et al.* (1984b).

In-vivo insemination. Males were killed with ether, the epididymides excised and separated into different segments following the description of Horan & Bedford (1972). Spermatozoa were allowed to seep out of the cut tubules and a drop was diluted in Medium BMOC (Brinster, 1965) to a final concentration of 10^7 cells/ml. When required, an epididymal protein preparation from intact or castrated animals was added to the suspension to obtain the required concentration.

Insemination was performed 8 h after the injection of hCG. An aliquant (0.1 ml) of the experimental sperm suspension, containing 0.8 mg epididymal protein preparation/ml, was injected into a uterine horn and a ligature placed distal to the site of injection. The contralateral uterine horn was injected with an aliquant of the sperm suspension prepared in Medium BMOC alone or with addition of an epididymal protein preparation obtained from castrated hamsters. Oocytes were recovered from the oviducts 17 h after insemination and scored as fertilized when two pronuclei and a sperm tail in the vitellus were observed.

In-vitro fertilization. This was performed as described by Gwatkin (1977). Sperm suspensions (10^7 cells/ml) were prepared in Medium BMOC as described above and, when desired, epididymal protein preparation was added to a final concentration of 0.8 or 1.6 mg protein/ml. Freshly recovered oocytes (10 in 20 μ l BMOC), with their follicular cells, were added to 20 μ l sperm suspension and this mixture was incubated under a layer of mineral oil for 5 h at 37°C in 5% CO₂:95% air. Spermatozoa were then examined for the presence of activated motility. The oocytes were removed and replaced by 8–10 fresh oocytes previously treated with hyaluronidase (450 U/ml in PBS containing 1% polyvinylpyrrolidone, 5 min at 37°C) to remove the follicular cells. Incubation was continued for 3 h and the oocytes were observed for penetration.

Pronounced inter-assay and individual variations required that all comparisons (*in vivo* and *in vitro*) were made between aliquants of the same sperm samples and within the same experimental protocol. Results were analysed with Wilcoxon's signed rank test for statistical significance.

Results

Choice of spermatozoa for treatment with epididymal proteins

The sperm population to be tested had to be sufficiently immature to be susceptible to substantial modifications by treatment but with enough fertile cells to serve as internal control of the working order of the technique.

Each female was inseminated in one uterine horn with mature spermatozoa from the cauda epididymidis as control, and with spermatozoa from different epididymal segments in the other (Table 1). While spermatozoa recovered from the caput and proximal corpus epididymidis (segments *a* and *b* of Horan & Bedford, 1972) were totally unable to fertilize any oocytes, spermatozoa from the distal corpus (segment *c*) consistently fertilized 13%, while spermatozoa from the proximal cauda (segment *d*) showed higher fertility (71%), not different from that obtained with fully mature spermatozoa from the distal cauda (segment *e*, 87%). As seen in Table 1, sperm

Table 1. Fertility of spermatozoa recovered from different segments of the hamster epididymis when inseminated *in vivo*

Segment	No. of females inseminated	Epididymides*		Mean $\pm$ s.e.m. fertility (%)
		Left	Right	
Caput	6	0/60 (0)	0/44 (0)	0
Proximal corpus	10	0/57 (0)	0/64 (0)	0
Distal corpus	22	20/152 (13)	18/138 (13)	13 $\pm$ 3.29
Proximal cauda	20	88/122 (72)	119/168 (71)	71 $\pm$ 5.08
Distal cauda	58	209/235 (89)	222/262 (85)	87 $\pm$ 2.67

* Values are no. of oocytes fertilized/total no. of oocytes tested with the % in parentheses.

samples obtained from the right and left epididymis of each animal were inseminated separately and the similarity of the results indicates the synchronous development of maturation on both sides and also provides a measure of confidence in the reproducibility of the technique employed.

It was therefore decided that spermatozoa from the distal corpus epididymidis were the most suitable population for our work, and were used routinely for all subsequent experiments.

Spermatozoa recovered from different epididymal segments were also tested for their fertilizing ability *in vitro*. In 9 different experiments, spermatozoa recovered from the distal corpus penetrated 32/73 (44%) oocytes, while cauda epididymal spermatozoa penetrated 64/74 (86%) oocytes. As a control of this technique, aliquants of the different cauda epididymal sperm samples were also inseminated *in vivo* and an almost identical (87%) fertilization rate was obtained.

Effect of epididymal protein on the fertilizing ability of immature spermatozoa inseminated in vivo

Spermatozoa from the distal corpus epididymidis were recovered in Medium BMOC with or without added epididymal protein fraction (0.8 mg total protein/ml) obtained from intact or castrated hamsters. After incubation for ~ 5 min, the samples were inseminated into superovulated females. Each animal received the control sperm suspension (in Medium BMOC alone or Medium BMOC containing epididymal protein fraction from castrated animals) in one uterine horn and the test suspension in the other. In the first group of 16 experiments, the fertility of spermatozoa exposed to epididymal proteins (49 eggs fertilized/204; 24% mean fertility, median 17%) was compared to that of controls in Medium BMOC (28/219 eggs; 13% mean fertility, median 6%) and was significantly elevated ($P < 0.01$).

In a second series of 7 experiments, the effects of an epididymal protein preparation from intact and castrated hamsters were compared. While 34% (31/90, median 46%) oocytes were fertilized by spermatozoa suspended in Medium BMOC + epididymal proteins from intact males, only 22% (23/103, median 20%) oocytes were fertilized by spermatozoa exposed to an epididymal protein fraction from castrated animals ($P < 0.05$).

Effect of epididymal proteins on the fertility of spermatozoa inseminated in vitro

Epididymal protein fraction from intact hamsters was added to the sperm suspension at 0.8 or 1.6 mg protein/ml, and the mixture was incubated for 5 h to capacitate the spermatozoa before addition of the oocytes to be fertilized. No significant difference was observed between the number of oocytes penetrated by control spermatozoa and those incubated with the lower concentration of epididymal protein ($n = 10$), but a significant increase in fertility from a mean of 25% (21/83, median 20%) to 45% (41/92, median 43%) ($P < 0.05$) was obtained when spermatozoa were incubated with 1.6 mg protein/ml.

Discussion

The secretion and attachment of epididymal proteins to spermatozoa during the maturation process seem to be common to several species (rat: Fournier-Delpech, Bayard & Boulard, 1973; Olson & Hamilton, 1978; Lea, Petrusz & French, 1978; Kohane, González Echeverría, Piñeiro & Blaquier, 1980a; rabbit and hamster: Moore, 1980, 1981; González Echeverría *et al.*, 1982; ram: Voglmayr, Fairbanks, Jackowitz & Colella, 1980; bull: Vierula & Rajaniemi, 1980) and has been characterized in molecular terms as a high-affinity type of interaction (Wong & Tsang, 1982) with a receptor present on the sperm surface. The suggestion for a physiological role for these moieties originated in the work of Orgebin-Crist & Jahad (1979) and was substantiated by the results of Moore (1981) showing that antisera against rabbit and hamster epididymal proteins associated to spermatozoa were able to interfere with the fertilizing ability of mature spermatozoa. Moreover, the selective localization of these acquired proteins on the plasma membrane covering the head of the spermatozoon (Moore, 1981; Kohane, Garberi, Cameo & Blaquier, 1979) is in keeping with their proposed participation in gamete interaction.

The experiments described in this report represent a further attempt to establish a relationship between the interaction of androgen-dependent epididymal proteins with spermatozoa and the acquisition of fertilizing ability. Using the organ culture system we had already demonstrated the androgen-induced synthesis of glycoproteins EP1–EP6, their addition to the surface of spermatozoa and the concomitant increase in binding to the zona pellucida (Cuasnicú *et al.*, 1984a). Subsequently, we showed that cycloheximide prevented the effect of androgen, thus implicating *de-novo* synthesis of protein. An epididymal extract enriched in glycoproteins EP2–EP6 but devoid of androgens was able to mimic the effect of androgen (Cuasnicú *et al.*, 1984b). Although the increase in zona pellucida binding is thought to represent an advancement in the maturation state of the sperm population (Saling, 1982; Fournier-Delpech *et al.*, 1982), we felt it necessary to determine the effect of epididymal glycoproteins on the fertilizing ability of spermatozoa. Our present results provide evidence demonstrating that exposure of immature spermatozoa to an epididymal preparation enriched in glycoproteins EP2–EP6 was able to produce significant increases in fertilizing ability *in vivo* and *in vitro*.

A strong argument can be put forward for the specificity of the effect observed in our experiments since it could not be duplicated by an equal amount of epididymal proteins obtained from castrated animals. The concentration of the androgen-dependent glycoproteins decreases after castration, becoming undetectable 30–45 days after gonadectomy in the hamster (González Echeverría *et al.*, 1982) and decreasing by over 90% after only 14 days of castration in the rat (Kohane, Piñeiro & Blaquier, 1983).

The *in-vitro* conditions for testing fertilizing ability minimize the chances of modification of the epididymal proteins and eliminate the participation of uterine factors which may act after insemination *in vivo*. To the best of our knowledge, the only previous attempt to modify immature sperm fertility by direct exposure to epididymal factors was that reported by Cooper & Orgebin-Crist (1975) who did not succeed in increasing the fertility of rabbit testicular spermatozoa by incubation with fluid from the cauda epididymidis. Apart from the difference in the experimental animals used, the discrepancy in results may arise from the selection of the sperm population tested and the source of epididymal factors used. While glycoproteins are secreted in the proximal segments of the epididymis (Lea *et al.*, 1978; Moore, 1980; Kohane *et al.*, 1980a), their accumulation on spermatozoa (Kohane, Cameo, Piñeiro, Garberi & Blaquier, 1980b) suggests that cauda epididymal fluid may be a depleted source of these factors. Moreover, the failure to attain maturation of spermatozoa retained in the caput epididymidis (Cummins, 1976; Gaddum & Glover, 1965; Bedford, 1967) also suggests the cumulative action of factors produced at different levels of the epididymal duct.

Our present results provide further support for the notion that a structure assembled or activated on the sperm surface with the intervention of epididymal secretory glycoproteins is in some way responsible for the increased zona pellucida recognition and fertility seen during sperm maturation.

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