

## On the role of epididymal factors in sperm fertility

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Notwithstanding our improved descriptive knowledge of events occurring to mammalian sperm during epididymal maturation, the nature of the mechanisms involved in the development of such primary functions as forward motility (Bedford, 1975) and zona pellucida recognition (Saling, 1982) remain obscure. This review attempts to summarize the latest information produced by our laboratory in relation to the interaction of epididymal products with spermatozoa and the bearing that this interaction might have on sperm function.

Our interest has been primarily focused on androgen-induced secretory epididymal proteins that associate to sperm during epididymal transit and that our group has described in rats (Cameo and Blaquier, 1976), hamsters (Gonzalez Echeverría, Cuasnicú and Blaquier, 1982) and humans (Tezón *et al.*, 1985b).

Immature hamster sperm, obtained from the proximal segments of the epididymis, were used as a model to induce *in vitro* modifications of their functions (Cuasnicú *et al.*, 1984a). Exposure of these spermatozoa to a preparation enriched with androgen-induced secretory proteins EP2-EP3 (Gonzalez Echeverría, Cuasnicú and Blaquier, 1982) was able to induce a significant increase in homologous zona pellucida binding (Cuasnicú *et al.*, 1984b). Since zona binding seemed to be one of the functions developed by sperm during epididymal transit (Cuasnicú *et al.*, 1984a; Saling, 1982), our results were interpreted as reflecting the *in vitro* advancement of the maturational stage of these spermatozoa which was caused by the added epididymal preparation. Similarly, we were also able to show that immature sperm gained in fertilizing ability when pre-treated *in vitro* with the epididymal extract prior to *in vivo* or *in vitro* insemination (Gonzalez Echeverría *et al.*, 1984).

Our latest results confirm these preliminary findings and support the role of epididymal proteins in sperm maturation. Briefly, our studies show that the preparation of the epididymal proteins mentioned above, in which EP2-EP3 represented 16 % of the total protein, had a biological activity such that, on the average, treated sperm fertilized twice as many oocytes as their untreated controls

(Gonzalez Echeverría *et al.*, 1984). Further purification of the extract produced a fraction of epididymal proteins in which EP2-EP3 represented 60 % of all the protein. When tested in our bioassay, this preparation was able to increase the fertilizing capacity of immature spermatozoa 7-fold at only half the dose of the former preparation (Blaquier *et al.*, 1986). We can calculate that a 10-fold purification of proteins EP2-EP3 from the 6 % present in epididymal cytosol used as starting material, induced a 245-fold increase in biological activity. These data are interpreted as showing not only the removal of an inhibitory factor present in the crude initial preparation, but also as pointing strongly to a role for EP2-EP3 in the development of fertilizing ability.

The active role of hamster epididymal proteins in the promotion of maturation is supported by recent results from Moore and Hartman (1986), showing that immature spermatozoa co-cultured with androgen-stimulated epididymal epithelium gain the ability to fertilize oocytes. In rats, Orgebin-Crist and Fournier-Delpech (1982) were able to demonstrate that incubation of immature sperm with a purified preparation of epididymal secretory proteins increased their zona pellucida binding ability.

From these and other data, a working hypothesis was formulated suggesting that epididymal glycoproteins added to sperm were responsible for at least part of the phenomena observed during maturation and led to the acquisition of fertilizing ability.

Since selective binding to the homologous zona pellucida seems to be the principal mechanism responsible for the species specificity of fertilization observed in mammals (Gwatkin, 1977) and the ability of sperm to recognize and bind to the homologous zona pellucida is developed during epididymal maturation (Cuasnicú *et al.*, 1984a; Saling, 1982), it follows that epididymal proteins which associate to sperm are likely to be part of the structure, being assembled on the sperm surface, which would enable interaction with the complementary site on the homologous zona or, perhaps, act as activators of its assembly or function.

This hypothesis requires that the added epididymal proteins be located on the external surface of the sperm plasma membrane and not be lost during the capacitation process when the sperm sheds many of its coating antigens (Johnson and Hunter, 1972).

Rat spermatozoa were used as a model for these studies mainly because rat androgen-induced epididymal secretory protein DE (Cameo and Blaquier, 1976) is available in a highly purified form (Garberi, Fontana and Blaquier, 1982), and a specific antibody has been developed for precise immunocytochemical studies.

Previous results had indicated that about 70 % of the total protein DE associated with cauda epididymal spermatozoa was lost from the cells when these were capacitated for 4 h *in utero* (Kohane *et al.*, 1980). However, the remaining 30 % of protein DE seemed to have a relevant role in the fertilization process because the exposure of spermatozoa to the antibody, prior to *in vivo* insemination, markedly reduced the fertilization of oocytes (42 % in controls and only 7 % in the experimental group) (Cuasnicú *et al.*, 1984c).

Our most recent results concern the ultrastructural localization of protein DE in rat sperm and its fate during capacitation and after the induction of the acrosome reaction.

Using the sensitive peroxidase-antiperoxidase (PAP) immunocytochemical technique, we were able to show the localization of DE on the external surface of the sperm plasma membrane covering the head of the cell and its absence on the surface of the flagellum (Cameo *et al.*, 1986). Capacitation was obtained by overnight incubation in  $\text{Ca}^{++}$ -free medium supplemented with bovine serum albumin, and synchronous acrosome reactions were induced by the addition of  $\text{CaCl}_2$ . After this treatment, 58 % of the cells had swollen acrosomes and 33 % showed vesiculation of the plasma and outer acrosomal membranes, indicating the occurrence of the acrosome reaction. Immunocytochemical evidence of the presence of protein DE was seen in the vesicles covering the acrosomal region and also in the membrane of the postacrosomal region of the cell (Cameo *et al.*, 1986).

These data are compatible with the postulated role of epididymal proteins in the sperm-zona pellucida recognition process since most authors agree that sperm bind to the homologous zona by the plasma membrane overlying the acrosome (Franklin, Barros and Russell, 1970; Moore and Bedford, 1983). However, the finding that some protein DE remains in the postacrosomal region after capacitation and the acrosome reaction allows some speculation about the different roles of this protein.

It is accepted that the postacrosomal region of the sperm plasma membrane is a specialized structure through which fusion with the oocyte membrane is started (Bedford and Cooper, 1978; Salgi and Phillips, 1980). In addition, it is also well established that a considerable degree of species specificity is retained by this step of membrane fusion in many mammals (Hanada and Chang, 1972). Therefore, one may speculate on the existence of a second species-specific site on the sperm membrane that would recognize its complementary structure on the homologous oolemma after the zona pellucida has been penetrated. Both the localization of protein DE on the postacrosomal region and the blocking effect of the specific antibody on fertilization (Cuasnicú *et al.*, 1984c) support a possible role of the protein in this step of gamete interaction.

### **Sperm maturation in man.**

The significant advances in our understanding of the mechanism of sperm maturation in laboratory animals achieved in the last few years contrasts with the scarcity of similar progress with respect to man. This may be attributed to the difficulty in obtaining suitable samples in adequate numbers and also to ethical limitations in the performance of experiments.

It was only after the description of the zona-free hamster oocyte penetration test by Yanagimachi *et al.* (1976) that the investigation of the existence of a functional sperm maturation process in the human epididymis became possible. In the first study in this field, Hinrichsen and Blaquier (1980) showed that sperm

recovered from the cauda epididymis segment was able to penetrate 27 % of the offered eggs, while sperm from the caput and corpus were unable to undergo the fusion process. These results were later confirmed by Moore, Hartman and Pryor (1983) who demonstrated that, while caput sperm were entirely unable to fuse with hamster oocytes, those cells recovered from the corpus and cauda segments penetrated 15 and 43 % of the oocytes, respectively.

Since sperm maturation has been shown to be strictly androgen-dependent in all mammals (Orgebin-Crist, Danzo and Davies, 1976), a most useful primary approach to the identification of the factors involved in this process has been the analysis of androgen-induced secretory products of the organ that interact with the maturing spermatozoa.

To be able to study the influence of androgens on human epididymal function we adapted an organ culture system to human tissues that had originally been developed for rat and rabbit epididymides. Samples were obtained from patients undergoing castration as a treatment for prostatic carcinoma, and the tissues were cultured for periods up to 8 days (Tezón and Blaquier, 1981).

It was observed that once the cultured tissues were depleted of their endogenous androgen, the preparation, stimulated with 0.1  $\mu\text{M}$  of androgen (a concentration within the physiological range for this organ), responded by an increased synthesis of DNA and RNA (Tezón and Blaquier, 1981). This model was further characterized showing the presence of active  $5\alpha$ -reduction of testosterone and the binding of androgen to specific intracellular receptors (Tezón *et al.*, 1982).

We then attempted to determine whether some of the regulatory mechanisms of epididymal function such as the determination of the number of specific androgen binding sites within the epithelial cells (Tezón and Blaquier, 1983) and the activity of  $5\alpha$ -reductase (de Larminat *et al.*, 1978), known to be controlled by androgens *in vivo* in experimental animals, were also operating in cultured human tissues.

Our findings showed that the number of receptor sites seems to be controlled by androgens in cultured human epididymis since after 6 days in culture androgen-exposed tissues contained twice as many receptors sites as their controls (Vazquez, de Larminat and Blaquier, 1986).

Conversely, we could not demonstrate a clear-cut effect of androgen on the regulation of  $5\alpha$ -reductase activity, notwithstanding the biochemical similarities between the epididymal enzyme in humans (de Larminat *et al.*, 1980) and in experimental animals (de Larminat and Blaquier, 1978; Monsalve and Blaquier, 1977). Furthermore, our experiments explored (and eventually discarded) the existence of a possible masking effect of changes in NADPH concentration due to culture conditions and also established that most of the enzyme activity was associated with the epithelial cells during the culture period.

The system was applied to the study of androgen-induced secretory proteins of the human epididymis. After examination of the behaviour of 20 different samples, a pattern emerged showing the existence of 5 protein bands, separated by PAGE, whose synthesis was consistently stimulated by the addition of androgen to the culture media (Tezón *et al.*, 1985b). The bands corresponded to

molecular weights of 13 900, 21 000, 29 000, 38 000 and 69 000 daltons and were produced by the caput and corpus regions of the organ. Initial evidence suggested that proteins with the same electrophoretic behaviour could be obtained after mild salt extraction of spermatozoa recovered from the cauda epididymis, while no equivalent band was obtained from sperm recovered from the caput region. These results suggested that the secretory proteins were loosely associated with spermatozoa during epididymal transit.

To explore this phenomenon further, a polyclonal antibody was raised against the fraction of ejaculated sperm antigens thought to be of epididymal origin (Tezón *et al.*, 1985a). After purification by adsorption with semen from vasectomized donors, this antiserum reacted with cells of human epididymal epithelium while it failed to react with human prostate and testis. Furthermore, the antiserum localized the antigens to the anterior portion of the head of epididymal spermatozoa (acrosomal cap) and a gradient of increasing intensity was observed in sperm cells from the successive segments (Tezón *et al.*, 1985a). In contrast, the antibody failed to recognize spermatozoa recovered from the testis. In addition, the ability of the antiserum to precipitate androgen-induced radioactive proteins produced by the cultures supports our assumption of the identity between these proteins and those extracted from ejaculated sperm.

This antiserum became a useful tool to explore the possible biological significance of epididymal antigens in sperm function. In a recent report (Blaquier *et al.*, 1987), we examined the content and distribution of antigens in ejaculated spermatozoa from fertile donors and infertile patients. The most relevant finding was that while 88 % of all spermatozoa from the 18 fertile donors had the antigens localized on the acrosomal cap, 40 % of our infertile patients showed a localization of antigens different from that in the controls. This was accompanied by a significant reduction in the amount of antigens present in their spermatozoa. These differences could not be correlated with parameters in the routine semen analysis.

These data led us to speculate that, if the human epididymal proteins present on the sperm surface played a role similar to that described in animals, their abnormal localization on spermatozoa from infertile patients might be related to their infertility because of the specificity of the region of the human sperm membrane which initiates fusion with the oocyte (Sathananthan and Chen, 1986).

Following a different experimental approach, we have also employed antiserum to investigate the fate of epididymal antigens during capacitation and attempted to correlate these findings with the percentage of fertilization of human oocytes in our IVF-ET program.

Aliquots of spermatozoa were obtained from each sample at different stages of capacitation: crude semen, after washing and swim-up, after incubation for 18 h at 37 °C in Ham's F-10 supplemented with 10 % of maternal serum and, finally, from left-over media after incubation with the human oocyte in cumulus to obtain fertilization. In each sample we determined the proportion of cells in the population that were positively stained after indirect immunofluorescent labelling with our antiserum, as well as the localization of the antigens.

Although preliminary, the results are suggestive of a pattern linking higher fertilizing ability with a faster loss of antigens from the sperm surface during capacitation. 37 % of the spermatozoa of a subgroup of 6 men, who were able to fertilize more than 85 % of their wives' oocytes, were intensely fluorescent after swim-up; only 9 % of these spermatozoa had a stained acrosomal cap after overnight incubation with oocytes (76 % reduction in sperm with epididymal antigens). In contrast, a group of 12 men, who fertilized less than 50 % of the oocytes, presented 66 % of fluorescent sperm after swim-up, 49 % of which remained positive after attempted fertilization (26 % reduction in sperm with epididymal antigens).

This group of results suggests that epididymal antigens may behave like the acrosome stabilizing factors found in other species (Langlais and Roberts, 1985); they could be useful markers of capacitation in human sperm. Clearly, more research is needed to understand the role of epididymal factors in the development of sperm fertilizing ability.

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### **Résumé.** *Antigènes épидидymaires et fertilité chez l'homme.*

Chez le rat et le hamster on a pu démontrer que quelques-unes des protéines produites par les cellules épидидymaires, sous l'effet des androgènes sont liées aux spermatozoïdes; ce revêtement des spermatozoïdes augmente leur capacité de reconnaître la zone pellucide et de féconder l'ovocyte. On peut proposer que ces protéines forment, sur la membrane cellulaire des spermatozoïdes, une région spécialisée pour la reconnaissance et l'adhésion à la zone pellucide.

Avec un système de culture organotypique des tubules épидидymaires humains on a identifié après stimulation par des androgènes une sécrétion de protéines qui se lient aux spermatozoïdes. Des anticorps spécifiques pour ces protéines ont été utilisés pour étudier leur localisation et leur détermination quantitative dans les spermatozoïdes de donneurs fertiles et de patients stériles. Environ 30 % des hommes stériles ont montré une altération de ces paramètres; il pourrait exister une relation entre cette altération et leur stérilité.

Nous avons aussi étudié lors de la fécondation *in vitro*, le devenir de ces protéines pendant la capacitation du sperme. On a observé une corrélation entre la rapidité du détachement des protéines épидидymaires de la surface des spermatozoïdes et leur fécondité.

On considère que ces résultats ont une importance expérimentale, mais aussi qu'ils peuvent être utilisés pour expliquer et peut-être améliorer quelques cas de stérilité.

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