In vitro maturation of mammalian spermatozoa

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During epididymal transit, mammalian spermatozoa undergo maturation and acquire full fertilizing capacity. The contribution of factors from the epididymal epithelium appears to be essential for this process. Although complete in vitro maturation of epididymal spermatozoa has not been achieved, stages of maturation can be induced under various conditions. The most successful results have been obtained by incubating epididymal spermatozoa with primary cultures of epididymal epithelium. These co-incubation methods promote sperm motility and the capacity of spermatozoa to bind to and fertilize oocytes, and extend the viability of spermatozoa in vitro. Specific androgen-dependent secretory proteins from epididymal principal cells that may be involved in this maturation process have been identified using pulse-labelling techniques.

Sperm maturation is the term given to the process by which the mammalian spermatozoon becomes fully competent to fertilize in vivo or in vitro and refers to the stage after spermatiation, when spermatozoa leave the testis and pass along the lumen of the proximal region of the excurrent ducts (vasa efferentia, and proximal epididymidis). This is a somewhat arbitrary definition, since the male germ cell effectively undergoes a continual differentiation and maturation process from after meiosis to the time of fertilization. However, during transit through the proximal epididymidis (which lasts 2–10 days, depending on the species) various changes to spermatozoa culminate in their acquisition of progressive motility, and the ability to recognize, bind and penetrate the zona pellucida of the ovum and subsequently to fuse with the oolemma (Moore, 1990a). Strictly speaking, this is a potential development of fertilizing capacity, as spermatozoa must undergo an obligatory capacitation process in the female tract or during in vitro incubation, but this will not be discussed here.

The numerous alterations to the sperm cell concomitant with the epididymal maturation process include morphological modifications, such as shedding of the cytoplasmic droplet or alterations to the acrosome (particularly pronounced in marsupial species), and many biochemical changes including the structural stabilization of chromatin and other organelles with disulphide bonds, and changes to the composition and biophysical properties of the membranes (Cooper, 1986; Moore, 1990b, 1992, 1995). Classic ligation experiments carried out by Bedford (1967) and Orgebin-Crist (1967) in rabbits demonstrated that spermatozoa retained in the proximal region of the duct never acquire fertilizing capacity. These investigations established that maturation was not just an inherent ageing process of epididymal spermatozoa but relied upon essential factors in the local luminal environment. Subsequently, it was shown that sperm maturation depends on androgens that control the secretory and absorptive activity of the epithelial cells lining the excurrent duct (Orgebin-Crist et al., 1975) and therefore the composition of the luminal fluid. After their maturation, epididymal spermatozoa may be stored for a variable period in the distal epididymidis and vas deferens before ejaculation. Although the sperm storage features of the epididymis are different from those involved in maturation, a crucial aspect of epididymal function is clearly the maintenance of the viability of spermatozoa at high concentrations.

In laboratory species such as rodents or rabbits, the region of the duct where sperm fertilizing capacity is first acquired has been clearly defined by either in vivo or in vitro fertilization experiments (see Moore 1990b, 1995; Yanagimachi, 1994). For men of proven fertility undergoing vasectomy or patients undergoing various treatments, assessments of sperm motility or the ability of spermatozoa to fuse with zona-free hamster oocytes or both have indicated that sperm maturation also normally occurs (Hinrichsen and Blaquier, 1980; Moore et al., 1983; Dacheaux et al., 1987). Moreover, in men who have undergone epididymovasostomy to restore duct patency, the results of the subsequent fertility outcome suggest that passage of spermatozoa through at least the first centimetre of the epididymidal duct is usually necessary for a fertile ejaculate (Schoysman and Bedford, 1986). However, it is apparent that in patients who lack the vas deferens or have blocked epididymal ducts, and even in normal men, a small proportion of spermatozoa in the very proximal regions of the excurrent ducts (sometimes in the rete testis) may display some progressive motility. Micro-aspiration techniques and Percoll washing protocols have been used to retrieve spermatozoa for successful in vitro fertilization and embryo transfer treatments (Silber et al., 1988). These assisted-conception techniques demonstrate that a few human spermatozoa may require little, if any, contribution from the excurrent duct, to undergo sufficient maturation for fertilization in vitro. Nevertheless, in normal men, the appropriate epididymal maturation is still necessary for an ejaculate to be fertile (Cooper, 1990).

Experimental approaches to investigate sperm maturation are limited. Micropuncture investigations (see Setchell et al., 1994) give valuable information about the epididymal luminal milieu, but sampling the narrow epididymal tubule in the proximal region of the duct is difficult. Ethical considerations
also restrict this type of study in men. In an attempt to mimic the epididymal microenvironment, a number of research groups, including our own laboratory, have used in vitro culture techniques. These methods provide a valuable insight into sperm maturation events, and may eventually have a practical application in clinical medicine, for developing new methods of contraception, and for assessing the effects of toxicants on fertility. Here, we review the progress made with sperm maturation in vitro and describe some recent experiments from our own laboratory. Comprehensive reviews of epididymal sperm maturation are provided elsewhere (Cooper, 1986; Bedford and Hoskins, 1990; Moore, 1990b, 1995).

Incubation or treatment of epididymal spermatozoa with epididymal secretory preparations and other substances in vitro

It is clear that there is a complex series of interactions between epididymal secretions and spermatozoa as they migrate along the epididymis (see for example, Vreeburg et al., 1992; Tulsiani et al., 1993), so it is perhaps surprising that even the simple incubation of immature epididymal spermatozoa in vitro with epididymal protein extracts or other substances has met with some (albeit limited) success. Inherent ageing of immature epididymal spermatozoa in vitro alone will not promote fertilizing capacity. The degree of sperm maturation that has been achieved by simple incubations or in more complex co-culture methods (see below) depends to a large extent on the initial maturity of the spermatozoa being treated. For example, in the first clear demonstration of sperm maturation in vitro, Orgebin-Crist and Jahad (1979) added crude cytoplasmic extracts of sperm-free rabbit epididymis from the distal corpus region to rabbit spermatozoa recovered from the proximal corpus region that had been previously incubated in vitro for 24 h. This procedure significantly increased the fertilizing capacity of the epididymal spermatozoa. Since, in rabbits, the fertilizing capacity of spermatozoa increases substantially as they move from the proximal to the distal corpus region, factors in the epididymal extract were presumably able to induce this final maturation process. Similar experiments in hamsters have shown that epididymal extracts incubated with immature spermatozoa can enhance the fertilizing capacity in vivo and in vitro (Gonzalez-Echeverria et al., 1984). In this case, the extracts were charcoal filtered to remove androgens or other steroids that might have had a direct effect on the spermatozoa.

With spermatozoa retrieved from the more proximal regions of the epididymis of laboratory animals, the development of full fertilizing capacity with simple incubations in vitro has not been reported. However, alterations in the motility patterns of immature spermatozoa have been observed by adding various epididymal preparations or specific substances known to be present within the epididymis. For instance, the L-carnitine concentration in epididymal fluid rises substantially in the corpus region and, when added in vitro to rat spermatozoa recovered from the caput epididymis, will increase their progressive motility (Hinton et al., 1981). Two epididymal secretory proteins, forward motility protein (FMP; Acott et al., 1983) and acidic epididymal glycoprotein (AEG; Pholpramool et al., 1983) also alter the motility pattern of immature spermatozoa in vitro. Whether these substances specifically influence the development of sperm motility in vitro or are merely beneficial to spermatozoa has not been satisfactorily resolved. In hamsters, stiffening of the flagellum beat of immature spermatozoa, which leads to progressive motility, can be induced by sulphydryl oxidation agents in vitro (Cornwall et al., 1986). This observation is consistent with the general increase in disulphide bonds in spermatozoa as they mature (Bedford and Hoskins, 1990); hence incubation conditions that permit or promote sulphydryl oxidation may act by enhancing sperm maturation non-specifically.

The maturation process may also involve the removal or unmasking of determinants from the surface of the spermatozoon. An example of this is the induction of physiological acrosome reactions (by solubilized zona) in mouse spermatozoa when they are washed after incubation under capacitating conditions (Biegler et al., 1994).

Epididymal epithelial cell cultures

Various methods of culturing epididymal cells have been devised to mimic, more closely, the microenvironment of the epididymal lumen in vitro. These methods have proved invaluable for investigating the specific interactions between epididymal spermatozoa and epithelial cells, as well as for identifying and characterizing epithelial cell secretion. Indeed, it may be essential to establish viable human epididymal epithelial cultures because the paucity of tissue and ethical restriction severely limit research at present.

Initial attempts to culture epididymal epithelium from laboratory animals or humans involved organ culture techniques that used isolated fragments of tubules with and without spermatozoa in the lumen (Orgebin-Crist and Jahad, 1979; Tezon and Blaquier, 1981). For short incubations, these preparations in which the morphological integrity of the epithelium, basal lamina and peritubular cells remain largely intact proved successful in terms of secretory activity and sperm maturation but they could not be maintained for more than a few days and there was limited access to the tubule lumen. These early experiments established the need for androgens to be present in the medium (Vazquez et al., 1986) and now usually both testosterone and dihydrotestosterone are added to epididymal cultures. A much more sophisticated epididymal organ culture was established by Klinefelter and Hamilton (1984, 1985), which involved perfusion of rat caput epididymal tubule segments. This method maintained the structural and functional integrity of the interacting epithelial, luminal and sperm compartments. During the first three days of culture, spermatozoa became progressively motile on dilution, implying that certain aspects of maturation had occurred.

For a more defined cell culture system, procedures that reduce the epididymal epithelium to a purified single-cell suspension of principal cells were developed (Kierszenbaum et al., 1981; Joshi, 1985). Unfortunately, the disruption of the epithelial architecture and the lack of basal lamina and other peritubular elements compromised the cells, which rapidly de-differentiated and lost function in culture. Most of the current methods for the primary culture of epididymal epithelium use washed fragments or plaques of epithelium prepared by repetitive collagenase digestion (Moore et al., 1990). In our laboratory, this protocol has enabled us to generate plaques of epididymal
epithelium from rodent (Moore et al., 1986) or human tissue (Moore et al., 1992) that evert overnight in culture and form contiguous spheres of epithelium with the apical surface facing outwards (Fig. 1). These tissue balls eventually attach and plate out on the bottom of the Petri dish. The principal cells remain polarized and continue to secrete proteins for many days and sometimes weeks in the presence of androgens (Akhondi and Moore, 1994) and there is seldom fibroblast overgrowth. Various techniques have been adopted to maintain normal epithelial cell function, including the use of semi-permeable support and extracellular matrix to produce polarized monolayers (Byers et al., 1986; Cooper et al., 1990; Klinefelter, 1992). In the case of human tissue, the major obstacle to a successful epithelial culture is the large amount of connective tissue around the epididymal tubule which, if not removed, prevents adequate fragmentation. The origin of the tissue is also important. Samples from vasectomy volunteers or from patients with testicular cancer where no anti-androgen treatment has been given produce much better cultures than do samples from men given anti-androgen or oestrogen (sex change patients).

Fig. 1. Preparations of human epididymal epithelium in culture. (a) Typical epithelial fragments from the corpus epididymidis region after collagenase treatment and fragmentation. Scale bar represents 500 µm. (b) After 5 days in culture, epithelium plates out onto the bottom of the Petri dish. Scale bar represents 500 µm. (c) Electron micrograph showing that epididymal epithelium retains architecture in culture. Scale bar represents 2 µm.
Co-culture of spermatozoa with epididymal epithelial cell cultures and other cell cultures

When epididymal spermatozoa are incubated with epididymal epithelial cell cultures, they can undergo maturation which in some instances leads to the development of sperm fertilizing capacity. However, it has not proved feasible as yet to bring about all the changes to spermatozoa that are required for acquisition of full fertility by co-culture. This is not surprising; spermatozoa in situ undergo sequential modifications as they migrate along the epididymis and this is difficult to mimic in vitro. Perhaps a good example of this is the in vitro development of the fertilizing ability of hamster epididymal spermatozoa after co-culture with epithelium from the proximal cauda epididymidis (Moore and Hartman, 1986). If spermatozoa are retrieved from the distal corpus region, they still exhibit weak progressive motility, have poor zona binding and thus low fertilizing ability in vitro. However, when co-incubated for 6 h with epithelial cell cultures from the cauda epididymidis, binding of spermatozoa to eggs markedly improves and under capacitating conditions, spermatozoa exhibit characteristic agglutination, suggesting that their cell surface has been modified. But fertilizing capacity after such co-culture remains low because sperm motility is not enhanced. Only when spermatozoa are artificially held for three days in the distal corpus region of the epididymis by ligation of the duct can fertilizing capacity be induced by co-culture. In this case, the spermatozoa exhibit good motility owing to an ageing effect but have low fertility until they are co-incubated with epithelial cultures (Fig. 2). When even less mature spermatozoa from the caput epididymidis are incubated with epithelial cultures from the corpus region, motility is enhanced for 24 h but other aspects of maturation, such as zona-binding, cannot be improved (Smith et al., 1986). Attempts to undertake sequential co-cultures of rodent spermatozoa have been unsuccessful, as it has not been possible to maintain cell viability for more than 24 h. In this respect, promising results have been obtained recently in our laboratory with a co-culture system involving human tissue. Primary cell cultures of caput or corpus epithelial cell preparations have been maintained for up to 12 weeks without overgrowth of fibroblasts (determined with monoclonal antibodies to cytokeratin). When human cauda epididymal or washed ejaculated spermatozoa are co-incubated with these cultures, they maintain good viability for 8 days (50% with progressive motility < 20 μm s⁻¹), provided some medium is replenished every other day. A small proportion of these spermatozoa was still viable after 17 days of co-culture and retained intact acrosomal membranes (Akhondi and Moore, 1994). This extended cell viability is associated with the close attachment of spermatozoa to the apical surface of epididymal principal cells in culture (Fig. 3). In contrast, the co-incubation of human caput spermatozoa with epididymal epithelial cultures enhances sperm motility for only about 24 h, although over this period the ability of spermatozoa to bind to salt-stored human zona increases significantly (Moore et al., 1992). These changes are promoted by androgen-dependent factors from epididymal principal cells, since cultures maintained in the absence of testosterone or dihydrotestosterone fail to induce maturation. Toxicants such as ethane dimethanesulphonate (EDS) can interfere with in vitro sperm maturation by acting on epididymal epithelium in culture and preventing the normal secretion of androgen-dependent proteins (Klinefelter et al., 1992). The action of EDS is probably due to the alkylation of cytoplasmic proteins, which renders them incapable of being secreted.
In our laboratory, co-incubation with cultures other than epididymal epithelial cells has been unsuccessful in promoting sperm maturation, although in some cases they may enhance sperm motility and viability (Akhondi and Moore, unpublished observation). Human oviductal cells in culture have a beneficial effect on washed ejaculated human spermatozoa in terms of viability, synergistic induction of capacitation and induction of hyperactivated motility (Chian and Sirard, 1995; Kervancioglu et al., 1995; Pacey et al., 1995) and these effects seem to be more specific than with other epithelial cell lines (i.e. Vero (monkey kidney) cells; Kervancioglu et al., 1995).

Sperm–epithelial interactions during in vitro maturation

The myriad of changes occurring to spermatozoa during maturation have confounded attempts to identify particular epididymal epithelial factors specifically involved in the development of fertilizing capacity. Since epididymal epithelial secretory proteins may undergo additional processing at the sperm surface, following the fate of a particular determinant by immunolocalization investigations in situ has often produced perplexing observations which may be difficult to interpret (Jones et al., 1985; Moore et al., 1994). Perhaps one of the most important aspects of co-culture experiments is that intimate interactions between spermatozoa and epithelial cells can be examined in detail. While these systems are still far removed from the actual conditions prevailing in the lumen of the epididymis, the fact that spermatozoa may undergo maturation changes which require androgen-dependent epithelial cells indicates that conditions are at least mimicked to some extent in vitro. Therefore, mainly immunolocalization or pulse labelling techniques with \[^{35}S\]methionine or \[^{3}H\]thymidine have been used to correlate the acquisition of determinants on spermatozoa during co-incubation with epididymal cultures with their maturation. A number of proteins in both rodent and human tissue have now been implicated. For example, an early study using the hamster co-culture system identified a 34 kDa determinant with a monoclonal antibody (C5) that was secreted by principal cells of the proximal corpus epididymidis in vivo or in vitro (Smith et al., 1986). During co-culture conditions, this epitope was transferred onto the post-acrosomal and annulus region of spermatozoa and was associated with development of their progressive motility. We have shown that this glycoprotein is also expressed by rat epididymal cells in culture and binds to spermatozoa. Klinefelter et al. (1992) also reported on a 34–36 kDa group of proteins, expressed by rat epithelial cells in culture, that bind to spermatozoa. Treatment with EDS inhibited sperm maturation and diminished secretion of the 34–36 kDa (and 36–38 kDa) proteins to almost undetectable amounts. In our laboratory, a 44 kDa secretory protein that binds to rat spermatozoa during co-culture has been implicated in the maintenance of sperm viability and fertilizing capacity (Akhondi and Moore, 1993). This protein is also expressed by human epididymal epithelial cells, specifically binding to washed human ejaculated spermatozoa, which then maintain viability for a prolonged period of co-incubation (Akhondi and Moore, 1994). Our preliminary findings indicate that this protein is a protease inhibitor (Akhondi and Moore, unpublished observation). In this connection, a major human epididymis-specific cDNA encodes a protein with sequence similarity to extracellular protease inhibitors (Kirchhoff et al., 1991). Such inhibitors may regulate the processing of sperm surface components during maturation, may serve to prevent the degradation of viable spermatozoa by proteases released from dead cells, or could be decapacitation factors that modify or mask membrane epitopes involved in zona binding or the acrosome reaction (Biegler et al., 1994). Previous co-culture investigations using immature human epididymal spermatozoa have also identified proteins with molecular masses of 20–22 kDa, 38–40 kDa and 66–69 kDa as being important for maturation (Tezon et al., 1985; Moore et al., 1992).

Conclusion

In the past, in vitro maturation of human spermatozoa was seen as a possible clinical procedure for enhancing the fertilizing-capacity of samples retrieved from the excurrent ducts of infertile men for various assisted-conception techniques. The advent of intracytoplasmic sperm injection (ICSI) has now largely circumvented the need for human spermatozoa to meet even the minimal maturation requirements in vitro (Palermo et al., 1992). Nevertheless, in vitro sperm maturation techniques still remain important in both laboratory species and humans for investigating sperm–epithelial interactions leading to the development of fertilizing capacity. The practical and ethical problems associated with experiments in human medicine mean that in vitro studies using primary cell cultures and, in future, epididymal epithelial cell lines are probably the only approaches that can be undertaken. There is also a promising role for in vitro maturation techniques in reproductive toxicology investigations (Klinefelter et al., 1992).

A number of epididymal secretory proteins that participate in sperm maturation in vitro have now been identified, but their complete characterization remains to be determined. An important development has been the greater use of specific cDNA libraries to examine gene expression in the epididymis...
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(e.g. Kirchhoff et al., 1991, 1993). When used in conjunction with in vitro sperm maturation and epididymal epithelial cultures, it is now possible to identify rapidly genes encoding specific factors involved in sperm maturation.

References

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