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EXPERIENCE AND HISTORY

Male Reproductive Health

Epididymal research: more warp than weft?

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From a review of some aspects of epididymal structure, function and research done largely in my research area over the last 50 years, I conclude that more is known than is understood of sperm maturation and storage in the epididymis. Highly qualified technicians have not always applied sophisticated modern techniques in well-considered experiments to physiologically relevant and properly-prepared samples, so that our understanding of the biological problem of the nature of the epididymal epithelial influence on maturing epididymal spermatozoa has not kept pace with the outpouring of data generated, much of which is difficult to interpret. We stand at a crossroads of where to aim our limited resources and personnel: should we continue new technology-led studies in many directions, backtrack to test hypotheses and fill in gaps in our knowledge, or consider more biological directions to our research?

SOME OBSERVATIONS

Setting the scene

In 2005, a German newspaper considered the epididymis to be "the third-best male organ"[1] but the first recorded use of "epididymis" in the nonscientific literature was by the neo-classicist (Westminster School-educated), establishment figure (Westminster Abbey-buried) and playwright Ben Jonson (1572–1637). In The Alchemist,[2] Act III, Scene iii: FACE says "…Where is she? She must prepare perfumes, delicate linen, bath in chief, a banquet, and her wit. For she must milk his Epididymis. Where is the Doxy?" Though this word was most likely used as an unfamiliar and peculiar one used for comic effect, at least this sometime author, brawler, drunkard, poet laureate and raconteur understood that prostitutes in King James I's England knew how to expel epididymal contents! Whether London's lay audience of the time left the play knowing more about epididymal function than our current laymen, who balk at "andrology," is debatable.

In my 1996 edition of the play, the spelling has been "modernized" and what Jonson wrote appears as "epididimis!" Even in peer-reviewed scientific publications the noun and its genitive variations are commonly misspelled (with some Andrology Journals changing the proper spelling). Whether from ignorance or laziness, this inattention to detail reflects badly on scientists in the field, who should be as strict in the writing of their manuscripts as they are in performing the experiments they describe in them, by using the authoritative terminology.[3,4]

Epididymal tubular anatomy and physiology

The epididymis is an organ whose structure has been revealed by many simple anatomical techniques. William Hunter was one of several 18th-century anatomists who employed quicksilver to fill the epididymal lumen and even the rete testis, over a period of two weeks from a reservoir attached to the vas deferens. The single column of mercury in the wider coiled tubule of the cauda than that in the corpus coils, as well as the blind-ending spermatocoeles and the multiple efferent ducts in the caput, was demonstrated in stunning beauty by immersing the preparations in turpentine, in which the surrounding tissue is rendered invisible, and presenting them against dark blue or red backgrounds.[5]

The elaborate peritubular venous complexes in the murine initial segment, but not adjoining region, has been demonstrated in impressive corrosion casts, produced from injected compounds that harden and remain after digestion of the tissue.[6] Similar arresting images of a fine interstitial lattice of extravasated India ink[7] have demonstrated that fenestrated capillaries are present only in this, and not the adjoining, region.

A "sperm's eye view" of the surface of the luminal epithelium revealed by scanning electron microscopy[8] and the more recent, heavy metal ion-free technique of helium ion microscopy,[9] has revealed the long kinocilia of the efferent ducts, the long stereocilia of the initial segment, the microvilli of the epididymal principal cells, and the flaps, folds or microplicae of the narrow, apical and clear cells. The surface of the latter changes its form upon luminal perfusion of cell stimulants. Luminal perfusion *in situ* with fluids of different composition containing labeled-inulin has revealed the Na^+-dependence of fluid resorption from the lumen by principal cells (displaying expanded intercellular spaces between them with high Na^+ perfusion) and by clear cell vacuoles (enlarged when Na^+-free), and the greater uptake of horseradish peroxidase by clear cells than by principal cells.[10,11]

Epididymal segmentation

Work on many putative epididymal-specific proteins at the mRNA (hybridization *in situ*) and protein (immuno-cytochemistry) level has revealed that the proteins expressed are often confined to particular segments of the organ, confirming the regional differences in vascularity. From his images Ivell[12] considered the epididymis to be multiple organs, with spermatozoa sequentially experiencing the products of one gene (e.g. CE1) before that of another (e.g. CE5). Segmentation of the organ is well-known to anyone dissecting the organ or seeing tissue sections visualized for endogenous proteins (e.g. β-galactosidase activity), but Turner *et al.*[13] went further to show that the major connective tissue septa that separate segments through which the

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tubule passes, with the epithelial cells in each segment displaying different enzyme activity, were capable of retaining coloured macromolecules only within the interstitium of the injected region.

Although extratubular regulatory factors produced by or arriving in one such region might be responsible for synchronizing the expression of certain principal cell proteins at that site, exceptions to any such "blanket" regulation are evident from the varying responsiveness of individual cells within the same segments. Examples of such cell-autonomy are principal cells in the distal rat caput epididymidis showing the glutathione S-transferase P, subunit Yf (GSTsPYf) activity strongly, weakly or not at all,[14] and the glutamate transporter EAAC1 in the mouse epididymis being present in apical, but not principal cells in the distal caput, yet being expressed on the microvilli of principal cells in the cauda, but not in clear cells.[15]

Epithelia cells with circumferential and luminal cytoplasmic extensions

Veri *et al*.[14] showed that some of the GSTsPYf-positive rat corpus basal cells had positively-stained cytoplasm between principal cells extending towards, and occasionally reaching, the lumen; similar luminal extensions of mouse initial segment basal cells were shown when stained for macrophage antibodies F4/80 or Mac-1.[16] Confocal microscope studies of Shum *et al*.[17] clearly showed that the basal cells positive for COX1 and claudin1 could communicate with the lumen, and were thus not wholly basally-situated as hitherto believed.

The antibody studies also showed extensive lateral extensions of basal cells, observed in confocal sections of the rat corpus[14] and grazing sections of the mouse cauda,[16] to form a network of communicating cells around principal and other cells at the base of the epithelium. A similar arrangement of other cells in the mouse epididymis, with cytoplasmic extensions encircling the base of the tubule and reaching the lumen in the mouse initial segment, was shown for enhanced yellow fluorescent protein-labelled dendritic cells.[18] Dual labelling of extra-tubular dendritic cells (each with several extensions to the lumen) and intra-tubular basal cells (each with but one extension per cell) showed them to be distinct cell types.[19]

From their display of macrophage and immune cell features, a role for both of these cell types in detecting luminal sperm antigens (either lost from the sperm cells, or present in the fluid carrying them into the epididymis) and in inhibiting anti-sperm responses by the immune system (not primed before sperm formation to recognize these cells as "self") can be speculated. However, transgenic animals lacking these structures (those lacking the initial segment), show no abnormal immune response to the normal complement of spermatozoa in the lumen.[20]

The "Omes" are here!

As predicted in David Hamilton's Epid IV video presentation, not only are the "Omes" coming, they are here! Advances in techniques have enabled determination of the transcriptomes and proteomes of epididymal tissue, fluid and spermatozoa. Despite vast numbers of epididymal tissue proteins, the epididymal secretome in many species is limited to large concentrations of a rather low number of proteins, which may be involved in lipid transfer to sperm membranes. By whatever means (degradation of the whole protein or just the antigen epitopes) the major proteins may disappear sharply in distinct epididymal regions that differ between species.[21] In man the proteomes of the testis and epididymis,[22,23] epididymosomes[24] and ejaculated spermatozoa[25] have been determined, which show some, but limited, overlap between components.

Despite early views that apical blebbing from epididymal principal cells was an artefact of poor tissue fixation, such apocrine secretion is now considered a true event, with the blebs rupturing to release small vesicles (epididymosomes) into the lumen. These contain proteins that lack the signal sequence necessary for merocrine secretion, and which are transferred to specific domains of spermatozoa in different epididymal regions.[26] Griffiths *et al*.[27] envisaged a scheme in which targeting to specific sperm domains is mediated by docking proteins in the targeted organelle to which lipid carrier proteins bind, before transfer at that site of their bound glycosyl-phosphatidyl-inositol (GPI)-linked protein; followed by uptake of membrane components (e.g., cholesterol) by the lipid carrier and its subsequent removal after docking to a membrane of an epithelial cell. The result is that specific epididymal proteins may be found only on the acrosome, the neck, the equatorial segment, the midpiece or the principal piece of mature (ejaculated) spermatozoa,[22,23] and presumably related to sperm functions expressed in the female tract.

AN INTERLUDE

Reflections on what is known and what is understood

We now know a lot about epididymal structure, cells and their secretions. We know much about regulation of and inter-relationships between epididymal epithelial cells and the function of mature spermatozoa. We know something about sperm storage: the nature of the luminal environment in limiting the exposure of developing sperm cells to activating conditions *in situ*, and the presence of "decapacitation factors" temporarily limiting the response to environmental challenges encountered in the female tract or *in vitro*. We know little about sperm maturation: the mechanisms whereby spermatozoa within the epididymal lumen develop their ability to respond to environmental challenges encountered later in the female tract or insemination medium. We need to know more about sperm functioning within the sequentially changing epididymal milieu.

For example, although much is known about the regional localization of sperm membrane ion channels and transporters, and about how changes in ion transport into and out of mature sperm cells can initiate phosphorylation of intracellular proteins responsible for motility when they removed from the epididymis,[28] next to nothing is known about the mechanism of the changes in the ability of these proteins to be phosphorylated as spermatozoa migrate through the tract. Are there time-dependent changes in flagellar proteins that occur irrespective of the extracellular medium? Do the luminal ions, osmolytes or proteins affect intracellular changes in spermatozoa?

Inter-regulation of epithelial cells

From electro-physiological studies Shum *et al*.[29] summarized the interrelationships between epithelial cells as basal cell stimulation of clear cells (promoting luminal acidification) and of principal cells (promoting Cl^- and HCO_3^- secretion); principal cell secretions stimulating clear cells (promoting acidification); and luminal and circulating angiotensin II stimulating apical and basal aspects of basal cells. Such interrelationships along the length of the duct could explain the long-known regional differences in luminal fluid composition in the rat epididymis,[30] which ensure that distally-migrating spermatozoa experience sequentially a sudden increase in glycerophosphocholine (GPC) thereafter constant, decreasing mono-valent cations (but an increasing K^+/Na^+ ratio), and then increasing *L*-carnitine followed even more distally by increasing *myo*-inositol.[31] From the small osmotic deficit observed in all regions (the difference between measured osmolality and calculated osmolarity) it is possible to calculate the ionic strength (IS), which decreases distally.[32]

Do separate protein types function in sperm storage and maturation?

There appear to be two major types of secreted epididymal protein that bind to maturing spermatozoa; could each play a different role in male fertility? Merocrine secretion liberates proteins with signal peptides, which may be N/O glycosylated and peripheral proteins, held on the surface by electrostatic forces in the low IS intra-luminal environment. Such temporary proteins could act as decapacitation factors, keeping the sperm quiescent in the epididymis, and are probably lost upon ejaculation when the IS rises. Apocrine secretions, on the other hand, include proteins lacking a signal sequence, which are GPI-linked or otherwise lipidated, and which are integral membrane proteins that are retained upon ejaculation, although they may migrate to other sperm domains exposed during fertilization. Such permanent proteins may well participate in fertilization events of sperm zona- or vitellus-recognition or fusion.

Volumetric regulation of spermatozoa

Epididymal luminal osmolality increases distally in all species, in parallel with increasing concentrations of organic osmolytes. It has been postulated that osmolyte uptake in maturing spermatozoa, by the gradual process of iso-volumetric regulation, would not only prevent osmotic dehydration,[33] but provide sperm cells with a reserve of osmolytes that they could discharge passively along with water during the rapid regulatory volume response to the influx of water as the cells experience a drastic decrease in osmolality at ejaculation or transfer to insemination medium.[34] Murine cauda epididymidal spermatozoa are isotonic with the surrounding epididymal fluid[35] and are expelled into hypotonic media at ejaculation.[36] This presumably holds for the epididymal spermatozoa of other species that also enter hypotonic medium at ejaculation in men[37] and rats.[38] This is a nice story, but are osmolytes really accumulated by spermatozoa during epididymal transit (demonstrated so far only for *L*-carnitine) in normal animals? Is there a lower concentration of osmolytes in transgenic spermatozoa with angulated flagella? Are osmolytes lost when spermatozoa are removed to hypotonic medium? Are maturing epididymal spermatozoa in all regions isotonic to their surrounding epididymal fluid?

A hole in the fabric of our epididymal understanding

If the matrix of epididymal research results is considered as a piece of cloth, then the warp (the "vertical" threads extending the length of the material), which represents the contribution of individual scientists to their research area (each pursuing a few projects, proteins, ion channels or new RNA species; each burrowing down under a meaningful biological process; each increasing the size of the epididymal database), has by no means been matched by the weft (the "horizontal" thread interwoven with the warp, extending over the entire width of the material), which should act to hold it together as cloth of substantial strength, and provide a solid understanding of the biological processes under study. Consequently, we currently have some flimsy epididymal material with many holes and a lot of loose ends.

While there has been a dramatic increase in data over the last 20 years, particularly following the application of new techniques to the epididymis, there has been a less than convincing increase in understanding of the biological problem set out in the initial papers on sperm maturation:[39,40] is sperm maturation time- or secretion-dependent? If the latter, how do the secretions act? Are any or all the changes in the epididymal fluid (osmolality, IS, K^+/Na^+ ratio, osmolytes, integral and peripheral proteins) related to or necessary for sperm maturation? How do they affect the intra-cellular machinery of spermatozoa? Can macromolecules enter the spermatozoon *via* the fusion of epididymosomes? Is the sequential interaction of these luminal components with spermatozoa necessary for sperm maturation or just fortuitous?

Are we currently pursuing ideas purely to obtain grant funds, to satisfy some grant-awarding body's stipulations, to provide a student with a degree, to lengthen our CVs? Have we lost the sight of where our research should be heading, that of understanding a complex biological problem of reproductive importance and of evolutionary significance? Should we be mending the broken fabric rather than weaving a longer narrative?

ONE IDEA FOR THE FUTURE

Pieces of the epididymal jigsaw puzzle

Discoveries of important epididymal proteins are often followed by a knockout or knockdown of their genes in animal models, in attempts to determine their function. The phenotype of such males is not predictable, with often the same phenotype (e.g. male infertility related to angulated sperm flagella) appearing in different gene knockouts;[41] presumably as a result of other proteins being up- or down-regulated during development as a consequence of, and over- or under-compensating for, the missing gene. More pertinent information on gene function can now be provided by their age- and region-specific epididymal gene knockouts. An alternative to this "negative" approach of removing a piece of the jigsaw puzzle, is to knock in (or over-express) a gene against a backdrop of normal gene components. Another "positive" approach, now that we know so much of the composition of epididymal fluid and spermatozoa in different regions, would be to start putting pieces of the jigsaw together *in vitro*.

Towards an artificial epididymis

Such a model would allow immature spermatozoa to be sequentially provided with the ions, osmolytes and proteins they normally encounter, in the order in which they experience them during their epididymal sojourn, under sterile conditions and in medium of physiologically relevance and varying pO_2, pCO_2, HCO_3^-, pH, temperature, etc. To prevent any dilution of the immature testicular spermatozoa (TS) they could be placed in dialysis sacs. These would permit access of nutrients and oxygen, and escape of waste products, from an artificial rete testis fluid (aRTF: low K^+, low osmolality, high Na^+, high IS), bathing the spermatozoa.

Three experimental paradigms can be envisaged: (1) a gradual change in the composition of fluid bathing the dialysed cells from aRTF to an artificial cauda epididymidal fluid (aCEF: high K^+, high osmolality, low Na^+, low IS), mimicking that of the caput, corpus or cauda, by means of a simple gradient maker. This would indicate any effects of fluid changes *per se* (e.g. rising K^+) on the spermatozoa. (2) Effects of low molecular weight organic epididymal secretions (GPC, *L*-carnitine, *myo*-inositol) on sperm function could be tested by their addition at various concentrations to aCEF either alone, in combination, or in the more physiological sequence of rising GPC, then *L*-carnitine, then *myo*-inositol. Controls would be an addition of these components in alternative sequences. (3) A different approach would be required to monitor the effects on stored spermatozoa of epididymal proteins, which are too large to penetrate dialysis sac pores. Here open-ended dialysis sacs could be used, with drying out and dilution of spermatozoa prevented by overlying mineral oil, through which epididymal macromolecules (e.g. RNAse 10, clusterin, HE1) could be injected either alone, in combination, or in the more physiological sequence of RNAse 10, then clusterin, then HE1 (proteins and sequence depending on species); with a scrambled order of addition as control (**Figure 1**).

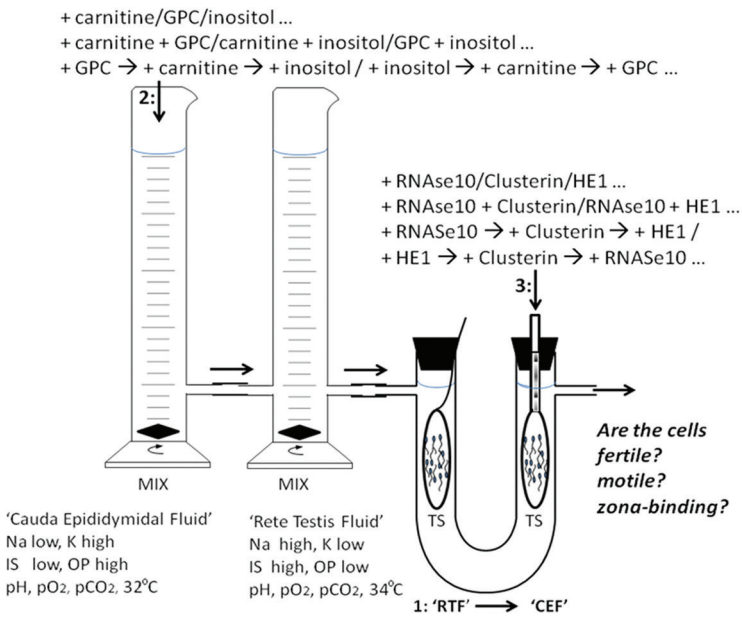

Figure 1: Design of an artificial epididymis for maturing spermatozoa *in vitro*. TS are retained in a dialysis sac placed initially in artificial RTF from a reservoir (right-hand measuring cylinder). Fluid flow continues after connection (1) of the left-hand cylinder containing artificial CEF to facilitate a gradual change of bathing fluid from the former to the latter. To the latter (2) can be added low molecular weight organic components of *L*-carnitine, GPC, *myo*-inositol either alone, in combination or in the physiological sequence (GPC → carnitine → inositol). Relevant epididymal luminal proteins (e.g., RNAse10, clusterin, HE1) would have to be added to spermatozoa in a dialysis sac through a column of oil (3), again alone, in combination or in the physiological sequence (RNASE10 → clusterin → HE1). During and after the perfusion the spermatozoa are checked for motility, zona-binding and fertility. CEF: cauda epididymidal fluid; GPC: glycerophosphocholine; HE1: human epididymal protein 1; IS: ionic strength; OP: osmolality; RTF: rete testis fluid; TS: testicular spermatozoa.

In each case the ability of the spermatozoa to become motile, zona-binding, oocyte-binding or fertile *in vivo* or *in vitro* could be examined at various times of incubation. The rather obvious 1970s-era setup shown in **Figure 1** indicates that it arose from papers read and ideas jotted down in that decade, but provides the concept; downsizing to current small fluidic chambers may provide better control of small numbers of cells, with single-cell analysis determining sperm membrane potential, fluidity, enzyme or transport activity; the level of protein phosphorylation; or even, if maturation occurs, the fertilizing ability *in vitro* of rare human TS!

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COMPETING FINANCIAL INTERESTS

I declare no competing financial interests that would prejudice the conclusions of this work.

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