

·Review·

## Sperm maturation in the epididymis: a new look at an old problem

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### Abstract

The osmotic challenges facing maturing spermatozoa and their responses to them are discussed in relation to the concept of sperm maturation, defined as the increased ability of more distally recovered epididymal spermatozoa to fertilize eggs when inseminated into the female tract. One explanation could be that the more distal cells are better able to regulate their volume, and reach the oviducts, as a consequence of uptake of epididymal osmolytes. Increased motility, zona binding and oolemma fusion capacities are also acquired within the epididymis and are necessary for those cells that finally arrive at the site of fertilization. (*Asian J Androl* 2007 July; 9: 533–539)

**Keywords:** epididymal function; fertilization; *in vivo*; osmotic challenge; sperm maturation

### 1 Sperm maturation

Sperm maturation is defined as the development of the ability of spermatozoa to fertilize eggs as they progress through the epididymis. The definition was first applied to *in vivo* fertilization where spermatozoa were removed from the caput, corpus or cauda epididymidis and inseminated into the vagina, uterus or oviduct of different species. The ability of spermatozoa to fertilize eggs, as judged by the percentage of pregnant women, litter size, percentage of fertilized eggs flushed from the oviduct or percentage of men with patent ducts after epididymo-vasostomy who subsequently fathered children, is always higher when spermatozoa are obtained from the more distal parts of the tract (Figure 1). In some early and oft-cited work [1], the number of spermatozoa inseminated was not always controlled for, so the greater number of spermatozoa obtained from the cauda might have biased results in the direction of greater success in that epididymal region. Later studies, with both sperm numbers and overall motility controlled for [2, 3], confirmed

this maturational process; however, the nature of sperm motility changes with maturation remained a possible confounding factor, as caput spermatozoa that display circular motion are less able to penetrate the uterotubal junctions [4].

When *in vitro* fertilization techniques became established, similar findings on the increased competence of more distally obtained cells were observed with spermatozoa from the caput epididymidis fertilizing fewer eggs *in vitro* than caudal spermatozoa, whether the eggs were invested in cumulus or the zona was present or not. The conditions of capacitation used were those designed to optimize fertilization by caudal spermatozoa, so again there is an in-built bias towards these cells. Nevertheless, under these *in vitro* conditions when migration through and survival within the female tract was not necessary, cauda spermatozoa always had an advantage over those from the caput in binding to and penetrating the zona and in binding to and fusing with the oolemma when the zona was removed. This functional competence was paralleled by development of motility (acquisition of flagellar beating and development of coordinated axonemal sliding to provide forward propulsion) and morphology (compactness of nuclear and flagellar structures). Therefore, every sperm function required for fertilization seemed to be developed in the epididymis: motility, zona binding and membrane fusion.

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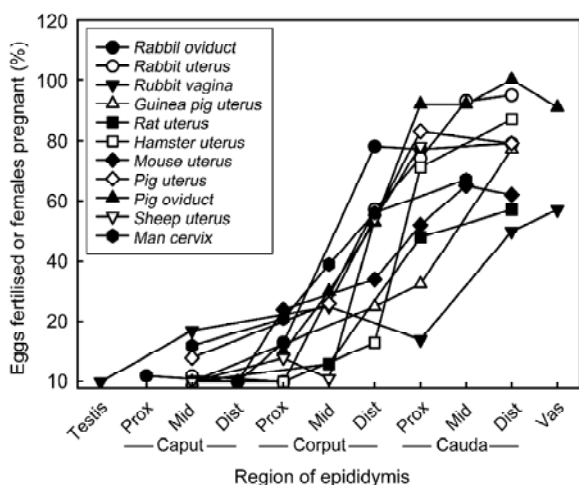


Figure 1. The fertilizing capacity of spermatozoa (ordinate) taken from different epididymal regions (abscissa) after insemination into the vagina, uterus or oviduct from various species.

## 2 Possible importance of low molecular weight secretions

New concepts of sperm function have evolved from several transgenic mouse models in which male infertility stems from a specific sperm morphology: flagellar angulation. This is rare in normal mice [5], but was noticed first in many transgenic mice displaying male infertility together with normal testicular sperm production. The first to be reported was the *c-ros* (a proto-oncogene) knockout mouse [6, 7], but was followed by a natural murine mutant of SHP1 (a phosphatase that dephosphorylates phosphorylated *c-ros*) [8], the transgenic GPX5Tag2 mouse [9] (where the T-antigen is targeted to the caput epididymidis by the caput-specific GPX5 gene promoter), the Apolipoprotein E receptor 2 (ApoER2) knockout mouse [10] (which lacks testicular selenium uptake [11]), the Foxi1 (forkhead transcription factor) knockout mouse [12] and the LRG4 (GPCR) hypo-mutant mouse [13].

Most work on the *c-ros* knockout model showed that the angulated spermatozoa could not pass the uterotubal junction and never reached the eggs in the oviduct [14] and that the angulation indicated an increased cell volume [15, 16]. Differences in the composition of cauda epididymal fluid from these animals included higher fluid  $K^+$  concentrations [17], higher pH [18] and lower phosphate concentrations [19], and spermatozoa from the null males had lower than normal *myo*-inositol and glutamate contents [17]. In the presence of clear cells and normal vacuolar-ATPase expression, the raised pH is explicable by downregulated NHE2 and NHE3 cation exchangers

in the caput and cauda epididymidis [18]. The reduced intracellular glutamate could be explained by the absence of the  $Na^+$ -dependent glutamate transporter EAAC1 in the caput epididymidis [20], as a result of the failure of the initial segment to differentiate at puberty [21], were the secreted glutamate to be taken up by maturing spermatozoa. As these models have not been examined systematically, there are as yet no common characteristics, apart from the angulated sperm flagella upon release from the epididymis. Raised luminal fluid pH was also found in Foxi1 knockout mice, but in this model apical and clear cell V-ATPase proton pump activity was depleted [12] and phospholipid hydroperoxide glutathione peroxidase PHGPx (GPX4) activity of spermatozoa was reduced in ApoER2 knockout mice [10].

It has been postulated that several small water-soluble components of epididymal fluid (*myo*-inositol, *L*-carnitine, taurine, glutamate) are taken up by spermatozoa during post-testicular maturation, to function as a reserve of intracellular osmolytes against the osmotic challenges that spermatozoa later experience at ejaculation [22]. The increased glutamate content of epididymal fluid and spermatozoa as they traverse the epididymis demonstrated in pigs [23] supports this view. That the epididymal epithelium could influence passing spermatozoa in this way is an interesting concept and would explain many diverse observations on epididymal fluid composition and male infertility and ultimately sperm maturation itself.

### 2.1 Epididymal fluid composition

The high concentration of low molecular weight organic compounds in epididymal fluid, especially in rodents, has long been known [24] and discussed in relation to the high osmolality of epididymal fluid, especially in bats. From what little is known in man, inorganic ions, rather than organic compounds, seem to be the major osmolytes [25]. The effects of high osmolality in dehydrating spermatozoa as a means of enforcing sperm quiescence has been proposed [26], although this implies that the solutes remain extracellular. Were they to enter the cell, they would act only initially to dehydrate the cell, but could then act as intracellular osmolytes. The truth might reflect a combination of actions of both permeant and non-permeant osmolytes. From studies on hypotonically-treated spermatozoa in solutions of putative osmolytes, most epididymal secretions (*myo*-inositol, *L*-carnitine, taurine, glutamate, sorbitol) can cause murine sperm swelling [17], suggesting that the efflux of these compounds anticipated under hypotonic conditions was blocked when the concentration difference between outside and inside the cells was abolished. Noticeably,

glycerophosphocholine (GPC) did not cause swelling and is impermeant towards other cells [27]. In the rat GPC is the first epididymal secretion spermatozoa encountered in high concentration (see [26]) and it might indeed act to withdraw cellular water, which would induce uptake of the permeant osmolytes present in high concentration more distally in the tract.

When subjected to hypertonic solutions, somatic cells are capable of performing regulatory volume increase, the taking up of extracellular solutes (and osmotically obliged water) to counter the osmotic efflux of water. If spermatozoa were able to perform this, it could be a mechanism whereby spermatozoa take up osmolytes. Furthermore, as transport through the epididymis takes approximately a week [26], these osmotic encounters are extremely gradual so that isovolumetric regulation (IVR), the slow movement of osmolytes and water that do not impinge on cell volume, might occur [28–30]. In this scenario, as spermatozoa move through the epididymis they would sequentially encounter impermeant GPC (providing a driving force for water efflux and IVR) and then permeant solutes (*myo*-inositol, *L*-carnitine, taurine, glutamate) that would be taken up into the cells as a result of their high concentration. The result would be osmolyte loads for spermatozoa of the order: cauda > corpus > caput. This speculation of osmotically-driven solute uptake needs to be tested experimentally.

## 2.2 Natural male infertility

There are several animal models of male infertility in which acutely angulated spermatozoa are a chief characteristic [31]. These “sterile studs”, although completely healthy animals and capable of mating, never sire offspring. In bulls, evidence that the problem was of epididymal origin came from multiple ejaculation studies in which the semen profile improved as epididymal reserves were depleted with continual voiding. Such studies suggest that spermatozoa entering the cauda epididymidis (emptied at ejaculation) were not damaged but that residence in a hostile cauda environment promoted the condition. Some animals did not display such an improvement and still retained approximately 50% abnormal cells after multiple ejaculation, suggesting that even spermatozoa coming from the proximal cauda epididymidis were affected in these individuals.

Studies in which luminal contents from the epididymides of such infertile bulls were fixed revealed that the regions in the epididymis at which angulation was first found varied between individuals. In some males, flagellar angulation occurred only as the spermatozoa entered the cauda epididymidis, as predicted from the multiple ejaculation studies, others when spermatozoa

entered the corpus and yet others when spermatozoa entered the caput. Once angulated, they remained in this shape, attesting to the irreversible nature of the phenotype and that the cause could occur anywhere along the length of the epididymal duct.

Only few studies have been undertaken to ascertain the cause of the morphological normality: in a review by Cooper [32], osmotic differences in cauda epididymal fluid from boars and bulls were contradictory and changes in Na<sup>+</sup>, K<sup>+</sup> and GPC (a potential osmolyte) were small and inconsistent. It is likely that the infertility, as for the infertile transgenic mice, is related to unopposed volume changes in the affected spermatozoa.

## 2.3 Transgenic male infertility

Some of the transgenic mouse models were similar to the naturally infertile bulls in displaying different extents of flagellar angulation within the epididymis: low angulation within the epididymal canal is typical of the *c-ros* knockouts but a more extensive and more proximal appearance of angulation occurs within the epididymis of ApoER2 knockout mice; an even greater extent occurs in GPX5Tag2 mice [33]. The extensive angulation of *c-ros* knockout mouse spermatozoa occurring upon release from the epididymis into routine, sperm preparation media can be abolished by treatment with detergent, signifying that the flagellar bending is enforced on the spermatozoon by its membrane and does not reflect an axonemal defect. Some spermatozoa from ApoER2 knockout mice [10] also respond in this way (others display midpiece anomalies), but those from the GPX5Tag2 line [9] remain angulated even when the membrane has been removed. It is likely that the sustained presence of angulated spermatozoa within the epididymis, during which oxidation of flagellar components occurs, “fixed” the tail in the hairpin bend formation that remains even when the membrane restraint is removed; this mirrors the irreversibility of angulation in some bovine ejaculates.

The angulation *in situ* might reflect an osmotic imbalance, because the osmolality of distal caudal fluid from the GPX5Tag2 mouse is lower than the wild type controls. However, this was not the case in the ApoER2 and *c-ros* knockouts [31]. This does not seem to reflect epididymal structure, as the *c-ros*, SHP1 mutant and ApoER2 KO mice all lack the initial segment, whereas the GPX5Tag2 mice have an initial segment, although it is probably hypertrophied and possibly malfunctioning. In the one Dag defect animal examined, the affected boar did have an initial segment [33].

## 2.4 Osmotic considerations

The recent measurements of rodent epididymal fluid

have confirmed that it is hyper-osmolal to blood serum in many species; that female tract fluids are generally isotonic to blood means that upon ejaculation spermatozoa are confronted with a large osmotic challenge. This has been confirmed in both man and mouse. The tonicity of seminal vesicle fluid is more than 100 mmol/kg lower than that of epididymal contents, as is post-coital uterine fluid, so from the moment of ejaculation spermatozoa are exposed to hypo-osmolal forces that are maintained in the female tract [31]. Studies with ejaculated spermatozoa are necessarily complicated by the fact that they can only be studied after they have come into contact with accessory gland fluids that comprise most of the ejaculate. These are now known to be hypo-osmolal to vas deferens contents and the osmolality of human semen measured within 5 min of production approximates that in the female tract [34]. It then rises during liquefaction [34, 35] and asymptotically thereafter [36–39] to give the high values that are often quoted as being representative of semen [40]. This has repercussions for sperm physiology because spermatozoa have been exposed to, and have possibly responded to, both natural hypo-osmotic forces (during ejaculation) and artefactual hyper-osmotic challenges (during liquefaction in the collection vessel) before they can be examined experimentally. Clinically, they are then subjected to further hyper-osmotic and hypo-osmotic challenges when transferred to IVF medium.

### 3 Explaining sperm maturation

All these observations have a bearing on sperm maturation, as defined at the beginning of this article, because it involves the transfer of epididymal spermatozoa to the female tract for assessing their fertilizing competence. Kann and Raynaud [41] studied sperm maturation in the hamster by inseminating caput and cauda spermatozoa into the uterus of superovulated female hamsters and examining eggs recovered 14 h later. Menezo medium was used for collection and insemination and caput spermatozoa failed to fertilize eggs, whereas cauda spermatozoa achieved 88% success. When motility was initiated in caput spermatozoa by addition of caffeine and bovine forward motility protein (FMP), 22% of eggs were fertilized by caput spermatozoa.

In a highly interesting follow-up study, Serres and Kann [42] found that releasing caput spermatozoa into the Menezo insemination medium (290 mmol/kg, termed “isotonic”) induced a flagellar angulation, in both motile and immotile cells. The site of this angulation depended on the position of the cytoplasmic droplet so that when it was near the head there was a neck angulation, when it

was in the middle of the midpiece the flagellum was bent there, but most of the spermatozoa were angulated at Jensen’s ring, at the end of the midpiece. They noticed that when released into “hyperosmolar” fluid of 400 mmol/kg such spermatozoa had straight flagella. Cauda spermatozoa were motile and displayed no such angulation at either osmolality. Caffeine, which induced irregular non-progressive motility in caput spermatozoa had no effect on angulation, whereas FMP induced progressive movement in caffeine-stimulated cells and abolished the angulation. Changes in membrane permeability were surmised to explain the phenomenon of FMP-induced prevention of angulation prevention in the absence of large changes in osmolality of the medium.

To describe routine sperm preparation media as “isotonic” (meaning leading to no change in cell volume) is common usage, but 290 mmol/kg is clearly hypo-osmolal to epididymal fluid in the hamster [43]. Unlike most species, where osmolality rises more-or-less in a linear fashion along the length of the duct, in the hamster, maximum osmolality (approximately 400 mmol/kg) occurs in the mid-corpus region, with distal cauda and vas fluids being lower (Figure 2). This means that when transferred to so-called “isotonic” medium of 290 mmol/kg, spermatozoa from the caput suffer an osmotic insult of approximately 110 mmol/kg, which could explain the observed angulation, because water would enter the cell osmotically. Conversely, when released into “hypertonic” medium of 400 mmol/kg, they suffer no osmotic insult at all, so there is no reason for the cell to swell, hence the observed straight flagellum.

Therefore, the poor fertilizing capacity of non-stimu-

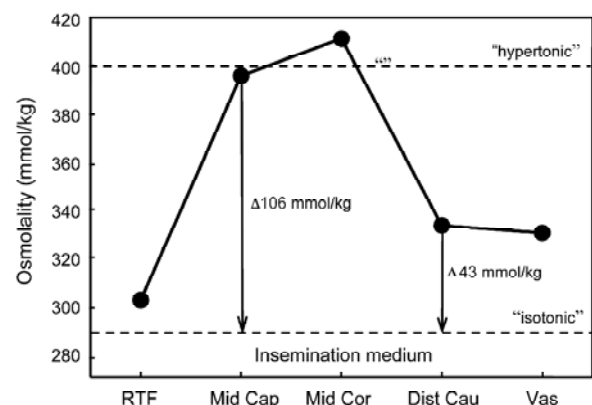


Figure 2. The osmolality of epididymal fluid (ordinate) from various epididymal regions of the hamster (abscissa). The osmolality of the insemination medium and that of fluids considered by many to be isotonic and hypertonic are indicated, as well as the osmotic challenge facing spermatozoa from the mid caput and distal cauda epididymidis.

lated caput spermatozoa could be explained by their inability to reach the oviducts, as found in the *c-ros* knockout mice, and, for the same reason, an inability to regulate volume when presented with a large osmotic challenge and angulated flagella failing to negotiate the uterotubal junction. When stimulated with caffeine, the few cells that had sufficient osmolytes might have had enough vigor to pass through the uterotubal junction. These cells must also have had sufficient epididymal secretions (e.g. P26h) to facilitate binding to the zona pellucida [44]. Apart from the expected osmolyte-loaded status of the cauda spermatozoa, another reason for their being successful in achieving fertilization under the same conditions is that the conditions were not really “the same”: the lower osmolality of epididymal fluid in the cauda epididymidis of the hamster compared with that in the caput means that the more mature spermatozoa suffered less of an osmotic insult during insemination, and being mature, were more likely to be better equipped with osmolytes to withstand the challenge.

### 3.1 Development of volume regulating ability

Direct volume regulation measurements have not been made in the hamster but before volume measurements were made in the mouse, the ability of maturing spermatozoa to regulate volume could be assessed by the percentage of cells undergoing angulation in medium of female tract osmolality. Caput spermatozoa showed greater angulation than corpus spermatozoa [22] correlating with poorer volume regulating capacity [16].

Around the same time as these discoveries, another group [45] was trying to separate hamster epididymal spermatozoa from epithelial contamination by density gradient centrifugation. They not only achieved this but found that there were always two populations of spermatozoa differing in density, whether taken from the caput or the cauda epididymidis. What changed upon maturation was an increase in the proportion of high density spermatozoa. As these spermatozoa are capable of losing water they must have developed the mechanisms of RVD, in contrast to the low density population that were incapable of doing so. Although not mentioned in the paper, micrographs of the low density population revealed them all to be angulated (R. Sullivan, personal communication), confirming this assumption. In retrospect, this paper can be seen as the first to demonstrate the maturational development of volume regulation of spermatozoa in the epididymis.

### 3.2 Cytoplasmic droplets and volume regulation

The angulation of epididymal spermatozoa from the naturally infertile males and in the transgenic mice oc-

curs at the cytoplasmic droplet at the end of the midpiece. This change in morphology provides the smallest membrane area for the increased volume, limiting damage done to the membrane through stretching. Similar angulation of spermatozoa occurs to bovine spermatozoa incubated in penetrating the cryoprotectant glycerol [46] and murine spermatozoa in which volume regulation has been blocked [47]. The importance of the droplet must stem from its being the largest repository of cytoplasm through which osmolytes and water can pass. Within the epididymal canal it migrates in most species from the neck to the annulus within the caput and corpus epididymidis [22] and, despite statements in the literature to the contrary, the droplet is not lost from the cell in the epididymis, with the exception of some Australian marsupials [48]. Wherever the droplet lies on a spermatozoon, it could act as the site of entry of osmolytes that would then be distributed throughout the rest of the spermatozoon. A role for the droplet in osmolyte efflux during volume regulation is supported by the immuno-cytochemical localization of voltage-gated  $K^+$  and chloride channels responsible for volume regulation in the droplet of murine and human spermatozoa [49, 50].

The association of higher fertility rates of bulls with better volume regulating spermatozoa [51] and higher volume regulating ability of human spermatozoa from fathers compared with patients [52] suggests that volume regulation by spermatozoa is an important property.

However, the loss of the droplet at or around ejaculation seems to be important for fertility because its retention is associated with infertility in several domestic species [48]. In all the infertile transgenic mice mentioned above, flagellar angulation to some extent has occurred in the epididymis, which is further accentuated upon release into medium, and might make loss of the droplet impossible with consequences for post-ejaculatory function. In the boar this might be a result of obstruction of oviductal binding sites [53]. That the improved binding of droplet-free spermatozoa to the oviduct is associated with spermatozoa displaying better volume regulation [54] suggests that the droplet *per se* is not required for volume regulation; a view consistent with its postulated osmolyte loading function within the epididymal canal.

## 4 Summary

As spermatozoa migrate passively through the epididymis they may acquire from epididymal secretions low molecular weight, water-soluble compounds by a process of iso-volumetric regulation. These may be expended, along with obligatory cellular water, when the

cells encounter hypo-osmolal fluids of the male accessory glands and female tract fluids. This process of regulatory volume decrease (RVD) serves to maintain cell volume and prevent flagellar angulation that hinders sperm migration in the female tract. The channels responsible for RVD are located on the sperm cytoplasmic droplet. It is postulated that the inability of immature spermatozoa from the caput epididymidis to fertilize eggs as successfully as mature spermatozoa from the cauda epididymidis reflects their lowered osmolyte content, which is inadequate for complete volume regulation when suspended in hypotonic insemination medium. This in turn leads to angulated flagella that prevent migration through the uterotubal junction and failure to reach the eggs.

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