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Review

Aquaporins in spermatozoa and testicular germ cells: identification and potential role

Ching-Hei Yeung

Centre of Reproductive Medicine and Andrology, University Clinic, Muenster D48149, Germany

Abstract

Mammalian spermatozoa have relatively high water permeability and swell readily, as in the hypo-osmotic swelling test used in the andrology clinic. Physiologically, spermatozoa experience changes in the osmolality of the surrounding fluids in both the male and the female tracts on their journey from the testis to the ovum. Sperm volume regulation in response to such osmotic challenges is important to maintain a stable cell size for the normal shape and function of the sperm tail. Alongside ion channels for the fluxes of osmolytes, water channels would be crucial for sperm volume regulation. In contrast to the deep knowledge and numerous studies on somatic cell aquaporins (AQPs), the understanding of sperm AQPs is limited. Among the 13 AQPs, convincing evidence for their presence in spermatozoa has been confined to AQP7, AQP8 and AQP11. Overall, current findings indicate a major role of AQP8 in water influx and efflux for sperm volume regulation, which is required for natural fertilization. The preliminary data suggestive of a role for AQP7 in sperm glycerol metabolism needs further substantiation. The association of AQP11 with the residual cytoplasm of elongated spermatids and the distal tail of spermatozoa supports the hypothesis of more than just a role in conferring water permeability and also in the turnover and recycling of surplus cellular components made redundant during spermiogenesis and spermiation. This would be crucial for the maintenance of a germinal epithelium functioning efficiently in the production of spermatozoa.

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1 Introduction

It has been known for nearly two decades that spermatozoa have high water permeability compared with other mammalian cell types [1, 2]. When subjected to extreme ranges of osmolality (70–1 500 mmol kg^{-1}), they shrink or swell rapidly, behaving almost like perfect osmometers (human [3, 4]; boar [5, 6]; mouse [7, 8]), enabling the hypo-osmotic swelling test to be used as a simple clinical test for sperm membrane integrity, which is manifested in the swollen shapes and thus reflects cell viability [9]. Nonselective water permeability through plasma membranes is very low, as water molecule is polar and diffusion of polar compounds is hindered by lipid bilayers. Aquaporins (AQPs) are water-selective channels which enable a 10–100-fold higher capacity for water transport across plasma membranes (see Agre *et al.* [10]). Among mammalian cells, 13 members of the AQP family have so far been identified, each being predominantly located in different tis-

Correspondence to: Dr Ching-Hei Yeung, Centre of Reproductive Medicine and Andrology, University Clinic, Domagkstrasse 11, Muenster D48149, Germany.
E-mail: yeungchinghei@yahoo.co.uk
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sues, and most individual cell types having more than one AQP family member [11, 12]. The work on sperm AQP in our laboratory was prompted by the normal existence of osmotic challenges to spermatozoa in the male and female reproductive tracts and the physiological importance of sperm volume regulation. This review presents this background, summarises reports on the identification of sperm AQPs and discusses their roles in male reproduction.

2 Physiological osmotic challenges to spermatozoa and cell volume regulation

Mammalian spermatozoa freshly produced in the testis and discharged into the epididymis are bathed in fluids of increasing osmolality. Extracellular osmolality for posttesticular spermatozoa starts at 280–320 mmol kg^{-1} in the rete testis of various mammalian species, and reaches levels as high as 480 mmol kg^{-1} in the corpus region of the hamster epididymis (see Cooper and Yeung *et al.* [13]). In man, there is no report on the osmolalities of epididymal fluid along the length, whereas that in the vas deferens is about 340 mmol kg^{-1} [14]. Subsequently, on ejaculation, sperm are confronted with a drop in extracellular osmolality, close to that of serum, in fresh seminal fluid (averaged 294 mmol kg^{-1} in men [15]) and female tract fluids (287, 284 and 268–280 mmol kg^{-1} in cervical mucus [16], uterine fluid [17] and oviductal fluid [18], respectively). This represents a drop of ~50 mmol kg^{-1} for human and ~100 mmol kg^{-1} for murine spermatozoa. This necessitates the process of regulatory volume decrease in spermatozoa to counteract the tendency of cell swelling [19]. Therefore, sperm volume regulation is of physiological importance and is further highlighted by study of infertile transgenic mice, the spermatozoa of which show angulation in the tail caused by defects in volume regulation [20, 21]. Inhibition of volume regulation in human ejaculated spermatozoa leads to failure in the penetration of and migration through surrogate cervical mucus, because of reduced swimming velocity of the swollen cells [22]. Sperm volumetric responses have also been shown to be correlated to the fertility of cattle [23]. In man, sperm volume regulation capacity is lower in infertile patients than in fertile men [24] or semen donors [25].

3 The study of water channels in sperm volume regulation

Water movement in volume regulation is driven by fluxes of ions or organic osmolytes through various ion channels and transporters [26], which have been characterized for spermatozoa, with the chloride channels ClC3 and ICln alongside K^+-channels and K/Cl cotransporter as candidate ion channels [19, 27]. Organic osmolytes found at high concentrations in epididymal fluid, including *myo*-inositol, carnitine, sorbitol, taurine and glutamate, are suggested to be involved in sperm volume regulation [19]. On the other hand, little is known about water channels in spermatozoa, the proper functioning of which would be the pre-requisite for such volume regulation. Although osmotic equilibrium across cell membranes occurs rapidly through water channels, such that water fluxes should not be the rate-limiting step in volume regulation, phosphorylation of some AQPs at different amino acid sites alter water permeability differently and may hinder volume recovery, as suggested for AQP4 in swollen astrocytes (see Pasantes-Morales and Cruz-Rangel [28]). Furthermore, defects in water channels can affect proper volume regulation, as shown in the corneal endothelium of AQP1-deficient mice [29] and in the salivary acinar cells of the AQP5-null mice [30]. Upregulation of AQP5 on osmotic changes has been shown in submandibular acinar cells [31]. There could also be functional interaction between AQPs and ion channels for volume regulation, as shown for swelling-activated Cl^- conductance in AQP4 knockdown astrocytes [32].

4 Water transporters other than AQPs

Besides AQPs, there are other membrane proteins that participate in osmotic water transport, although they are present at lower densities than AQPs and their biological importance relative to AQPs has not been elucidated [33]. Facilitated glucose transporters (GLUT) are among the candidates [34, 35]. Various glucose transporter isoforms including GLUT1, 2, 3, 5 and 8 have been identified in testicular germ cells and spermatozoa in the rat, bull, dog and man [36–42]. In the report that the GLUT inhibitor phloretin inhibits osmotic water fluxes in ovine and human spermatozoa, it was suggested that some of these transporters may be involved in water transport [43]. However, candidacy as water channels in physiological volume regulation has been ruled out, as phloretin cannot inhibit sperm swelling in response to a physiological hypo-osmotic challenge when osmolytes efflux for normal volume

regulation is blocked [44].

Some sodium-solute cotransporters have been suggested to drive water transport in somatic cells [45]. According to Loo *et al*. [46], the sodium–glucose cotransporter SGLT1 can account for half the amount of water reabsorbed by the enterocytes, although its osmotic water permeability is estimated to be only 5% of that of AQP1. The expression of certain SGLT family molecules other than SGLT1 has been indicated in canine spermatozoa [41], allowing speculation of a role in the transport of water alongside that of hexoses. However, there is no evidence for this in swelling experiments using phloretin and cytochalasin B as inhibitors of SGLT [45].

5 Evidence for the identities of sperm AQPs

It is recognized by workers in the field that the qualities of the AQP antibodies are unsatisfactory, especially those against AQP6–12 [47]. Therefore the identities of AQPs in a cell type have to be validated at multiple levels including the encoding mRNA, the specific protein, as well as its cellular localization. To date, AQP7, 8 and 11 are the three AQPs identified in

Table 1. Presence of aquaporins in spermatozoa confirmed at the mRNA level (with or without nucleotide sequence confirmation of open reading frames) and with protein identification and localization.

	AQP7	AQP8	AQP11
Testicular mRNA[a]			
Mouse	ppds 26–29[b] [52], round spd [53]	High at ppds 26–29 [52]; somatic ORF [62]	Absent before ppd 22 [52]; high in round spd [53]; Northern [68]; somatic ORF + shorter variants [69]
Rat	Round spd [54]	High in stages IV–VII[c] [54]; ORF not sequenced [78]	High at stages XIII–III [54]; Northern [67]; somatic ORF [69]
Human	Somatic ORF + shorter variant [58]	Somatic ORF + shorter variants [58]	Somatic ORF [69]
Sperm mRNA[d]			
Mouse/rat	M: somatic ORF [62]	M: somatic ORF [62]	M, R: somatic ORF; M: shorter variants [69]
Human	Somatic ORF [58]	Absent[d] [58]	Somatic ORF [69]
Sperm protein in Western blot			
Mouse/rat	M: 24, 33 kDa [62]	M: 27, 32 kDa [62]	M: 34 kDa + minor bands; R: 33 kDa [69]
Human	27–30 kDa [58]	26, 37 kDa [58]	
Protein localization in germ cells			
Mouse/rat	M: spd [72]; R: spd [87] + residual body [70, 78]	M: all germ cells [79], only elongated spd [62]; body [69] R: all germ cells [79, 88], elongated spd + residual body [62, 71] + spc [71]	M, R: elongated spd + residual
Human	Round and elongated spd [58]	Spg, spc, round + elongated spd [58]	
Protein localization in spermatozoa			
Mouse/rat	M, R: CD + anterior tail [59, 70, 72, 78, 87]	R: CD [78], tail [62]	R: end piece of tail [69]
Human	CD + tail (not end piece) [58, 75]	Punctate on CD + tail [58]	

Abbreviations: AQP, aquaporin; CD, cytoplasmic droplet; M, mouse; ORF, open reading frame; ppd, post-partum day; R, rat; spc, spermatocytes; spd, spermatids; spg, spermatogonia.
[a]For verification of sperm AQP, its mRNA must be found in the testis in which the AQP is synthesized (unless it is of epididymal origin and incorporated during maturation, for which there is no evidence); [b]Mouse post-partum days 26–29 coincides with the appearance of elongating spermatids; [c]Rat spermatogenic stages IV–VII coincides with the elongation of spermatids; [d]The absence of sperm mRNA cannot rule out past synthesis of the protein in the testis, whereas its presence is evidence for it.

rodent and human spermatozoa by these criteria (Table 1).

Because of chromatin packaging during spermiogenesis, proteins used for the formation of spermatozoa in the testis have to be synthesized by the latest in elongating spermatids [48], by delayed translation (uncoupled from transcription), before the mRNA is degraded as spermiogenesis progresses [49]. As mature spermatozoa do not undergo conventional gene transcription and translation, possess a condensed nucleus and sparse cytoplasm, any presence of mRNA of existing proteins only represents remnants from the spermatid [50]. Therefore, the absence of mRNA from spermatozoa gives no indication for the presence or absence of the sperm protein, whereas the presence of mRNA provides evidence for its past synthesis in the testis during sperm formation. On the other hand, round or early elongating spermatids must contain the mRNA of all sperm proteins of testicular origin, including any AQP members found on mature spermatozoa.

5.1 AQP0–6

The AQP0 is expressed specifically in the eye [47] and not in germ cells [51]. According to the mRNA expression profiles of rodent spermatogenic cells provided by global cDNA studies, either by deduction from testicular developmental profiles (mouse [52]) or by using germ-cell type-enriched fractions (mouse [53], rat [54]), *Aqp0–6* mRNAs are absent from rodent spermatogenic cells, although some are expressed in somatic cells.

Despite early reports of the absence of AQP1 from ovine and human spermatozoa by western blotting [44, 55], a positive finding was claimed in canine spermatozoa, although there was no report on the localization of the protein [56]. In addition to localization in vascular endothelial cells, the presence of AQP1 on the germ cell membrane and in the cytoplasm of elongated spermatids was observed in the testis of high-grade varicocele patients, but not on their ejaculated spermatozoa [57]. *AQP1* mRNA with an encoding sequence identical to that of somatic cells has been identified in human testes with complete spermatogenesis, but not in ejaculated spermatozoa [58]. Nevertheless, the latter study confirmed the absence of the protein from spermatozoa by western blotting.

5.2 AQP7

As it was cloned and identified first in the rat testis [59], reports on AQP7 on germ cells and spermatozoa have been most consistent among AQPs (Table 1). The diffuse staining of the cytoplasm, in addition to the plasma membrane (also for AQP8), may represent the degraded protein in view of the dynamic formation and differentiation of spermatids (Figure 1A and B). It is unclear whether the shorter-than-expected mRNA species of AQP7 and AQP8 displaying incomplete ORFs (open reading frames), as revealed in the human testis [58], are alternatively spliced variants or degraded RNA products. AQP7 is located all along the sperm tail except the end piece, as shown clearly in human spermatozoa (Figure 1E).

5.3 AQP8

Similar to AQP7, AQP8 was first identified through its cloning from testicular cDNA [60]. In contrast to AQP7, however, cellular localization of this AQP in the testis is most controversial. In rats and mice, this ranges from restriction to certain spermatogenic cell types to all germ cells, but not to somatic cells (see Table 1), or even absence from germ cells with expression exclusively in Sertoli cells [61]. Notwithstanding differences in antibody qualities causing confusion in the cellular localization, it is quite likely that differences among species exist, as our own work confines AQP8 in mouse to round and elongated spermatids only [62], but shows staining in all germ cells in the human testis [58]. Despite the abundance of mRNA in the rat testis, Northern blotting failed to detect the full-length species in the human testis [63]. However, recent attempts using reverse transcriptase PCR identified the entire encoding sequence, as well as shorter variants not found in control somatic tissues [58]. On the other hand, evidence from mRNA analysis, western blotting and protein localization (see Table 1), as well as functional studies in the mouse [62] and humans [58], support the presence of this AQP on spermatozoa in a punctated pattern on the cytoplasmic droplet and along the tail (Figure 1F).

5.4 AQP9

Testicular *Aqp9* mRNA has been reported in spermatogenic cells in both rats and mice. *In situ* hybridization has revealed mRNA in immature germ cells [64] and cDNA microarrays have shown high expression in pachytene spermatocytes [53, 54] with upregulation at the onset of spermatid elongation [52]. In the human testis, the entire ORF of the mRNA has been detected and the protein was occasionally observed in a

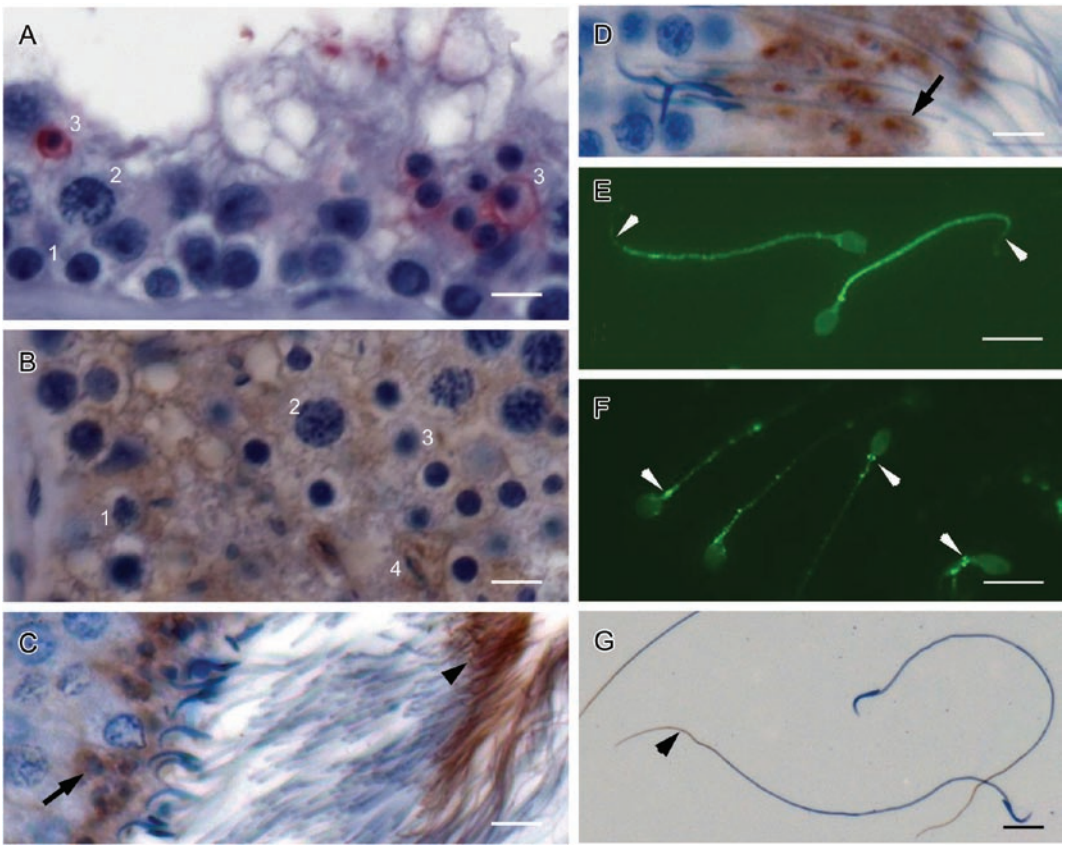

Figure 1. Localization of AQP7 (A, E), AQP8 (B, F) and AQP11 (C, D, G) in germ cells and spermatozoa. (A): Human testis with AQP7 absent from spermatogonia (1) and spermatocytes (2), but present in round spermatids (3). (B): Human testis with AQP8 localized on the plasma membrane of all germ cells (1–3) and elongated spermatids (4), and possibly cytoplasm of Sertoli cells. (C), (D): Rat testis with AQP11 localized in caudal cytoplasm of elongated spermatids (arrow, D) later in the proximal residual cytoplasm (arrow, C) and distal tail of testicular spermatozoa (arrow head, C). (E): AQP7 along the whole tail of human ejaculated spermatozoa except the end piece (arrow heads). (F): AQP8 in cytoplasmic droplets (arrow heads) and tail of human ejaculated spermatozoa. (G): AQP11 in end piece (arrow head) of rat epididymal spermatozoa. Bars = 10 μm; A–D have the same magnification. Corresponding negative controls can be seen in previous reports [58, 62, 69].

few spermatocytes and Sertoli cells [58]. In both mouse and human spermatozoa, western blotting revealed a signal band of the expected size, but it persisted after specific adsorption of the antibodies, casting doubt on the true identity of the protein band [58, 62]. Whereas the expression of AQP9 in the efferent ducts and epididymis is well established, and its role in fluid absorption from the tubule lumen is well accepted, there is no evidence for its presence in spermatozoa on the basis of immunohistochemical staining of luminal spermatozoa [61, 65]. Therefore, evidence so far supports the absence of AQP9 from spermatozoa.

5.5 AQP10

This is an aquaglyceroporin expressed mainly in the duodenum and jejunum (see Ishibashi [47]. It is a pseudogene in the mouse [66], and the protein is not expressed in the rat testis [51].

5.6 AQP11–12

These two newest AQPs belong to the so-called superaquaporins. They differ from the others not only in their unusual Asn-Pro-Ala (NPA) motifs (the first of the two highly-conserved hydrophobic NPA motifs forming the water pore in the prototypical AQPs contains a cysteine instead of an alanine) but also in their intracellular localization [47, 67]. Whereas AQP12 is specifically expressed in pancreatic acinar cells, AQP11 has been found in many organs, but most abundantly in the testis at the mRNA level (mouse

[68], rat [67]). Most recently, localization of the protein has been restricted to the late stages of spermiogenesis, first appearing in step 16 of spermatid elongation in the caudal cytoplasm and gradually becoming concentrated in vesicular organelles [69]. The final destiny of the protein is the end piece of the sperm flagellum just before spermiation, as well as dense bodies of the residual cytoplasm phagocytosed by Sertoli cells (Figures 1C, D and G). In humans, although the encoding mRNA has been localized in both the testis and spermatozoa (Table 1), the localization of the protein is unknown owing to the lack of an appropriate antibody.

6 Putative role of AQPs in spermatozoa and testicular germ cells

This section only includes AQP7, 8 and 11 as there is no convincing evidence for the existence of the other AQPs in germ cells and spermatozoa.

6.1 AQP7

When AQP7 was first identified and localized in round and elongated spermatids, it was natural for the authors to propose a role in the reduction of cell volume in the development of round spermatids into testicular spermatozoa [70, 71]. However, this assigned significance in spermiogenesis cannot be substantiated by the *Aqp7*$^{-/-}$ mice, which show only a mild phenotype with normal testicular and epididymal morphology [72], normal testicular weight, normal daily sperm production, normal sperm morphological and kinematic characteristics, as well as *in vitro* and *in vivo* fertility [73]. *Aqp7*$^{-/-}$ epididymal spermatozoa also have normal cell volume and are capable of water influx and efflux [62]. However, such negative findings cannot completely rule out a role of AQP7 in water transport in spermatids and spermatozoa, as AQP8 is also expressed by the same cell types. Indeed a moderate overexpression of *Aqp8* mRNA has been detected in the *Aqp7*$^{-/-}$ testis [62]. Nevertheless, compared with AQP8 (see below), such a role would be limited in normal physiology, if it exists at all.

Whereas AQP7 may be redundant in spermatogenesis, sperm AQP7 may have a role in the utilization of glycerol, which can be transported through this aquaglyceroporin channel as an energy substrate, as shown in cardiomyocytes [74]. The presence of glycerol in the rat epididymis and its metabolism by spermatozoa has been reported [75]. In a study of 22 infertile men, sperm AQP7 was nondetectable in five of them who showed slightly lower sperm motility than the other patients [76]. In a cohort of 50 men, AQP7 expression in ejaculated sperm was correlated with their progressive motility, with low values for both parameters in infertile patients compared with donors [58]. In addition to the different extents of expression, there are also individual differences in the molecular weight of human sperm QP7 ranging from 27 to 30 kDa. The relationship between sperm AQP7 and glycerol utilization in energy metabolism remains to be established. In this respect, reanalysis of sperm motility in *Aqp7*$^{-/-}$ mice using various energy substrates is warranted. Nevertheless, in line with fertile *Aqp7*$^{-/-}$ mice, an *Aqp7*-null man has been reported as fertile [77].

6.2 AQP8

In view of the expression of AQP8 in developing testis coinciding with Sertoli cell barrier formation, it was speculated that this AQP may have a role in the formation of testicular luminal fluid in addition to the role of volume reduction of spermatids during elongation [78]. Intriguingly, despite a marked increase in testis weight of the *Aqp8*$^{-/-}$ mice, seminiferous tubular diameter, sperm count, sperm gross morphology and fertility remain normal [79]. Water retention *per se* is ruled out in this case, as the water content of the knockout testis is unchanged. Probably there is functional compensation for AQP8 by AQP7 in these transgenic animals. However, AQP7, which is insensitive to Hg^{2+} in contrast to AQP8, does not normally have a role in germ cell water transport, as the water permeability of wild-type testicular plasma membranes can be abolished by Hg^{2+} [79].

Similarly, despite the high expression of AQP7 in spermatozoa, the role of water transport has been assigned to AQP8, as water influx and efflux for volume regulation under a physiological osmotic challenge can be completely inhibited by Hg^{2+} [62]. In men, the expression of AQP8 by ejaculated spermatozoa is inversely correlated with the extent of coiling of the sperm tail in the native semen [58], which is probably a phenomenon reflecting abnormal cell swelling [80].

6.3 AQP11

As a superaquaporin, the intracellular localization of AQP11 is well recognized as a characteristic

different from that of the other AQPs. Although the water permeability of AQP11 was in doubt when it was initially expressed in plasma membranes [67], a high water conductance has since been shown when it is expressed in liposomes [81]. In *Aqp11* knockout mice [68], cells in the proximal renal tubule show vacuolation, which results in the formation of polycystic kidney. Unfortunately, as the animals die of renal failure before puberty, this transgenic model sheds no light on the role of AQP11 in the testis, in which the protein is first expressed when the first wave of spermatogenesis is near completion [69]. Testicular AQP11 is clearly a marker for the process of spermiation. The strong protein expression coincides with the contraction of the caudal cytoplasm of the elongating spermatid and follows the fate of the residual cytoplasm during the formation and eventual elimination of the residual bodies by phagocytosis and digestion inside the Sertoli cells.

It is known that various AQPs are involved in transport of small molecules besides water, such as urea, chloride, heavy metal salts, ammonia, hydrogen peroxide and others, and thus may have unexpected cellular roles [12, 82]. A role of AQP11 in the efflux of water and nonmetabolizable substances across membranes of organelles has been proposed, although the identities of the transported molecules have proved elusive [83]. In the testis, AQP11 may have a role in the degradation of surplus cytoplasmic components of spermatid origin, which may even be used to produce ATP by Sertoli cells after phagocytosis [84]. Furthermore, failure in such clearance would upset the homeostasis of the germinal epithelium and disrupt normal spermatogenesis [85], or may even result in a breakdown of self-tolerance leading to autoimmune orchitis [86].

On the other hand, the appearance of AQP11 in the distal sperm tail at the final stage of spermiogenesis (Figure 1C) may imply a function in the elimination of the excess components of outer dense fibers and axonemal microtubules at completion of flagellar assembly. It is interesting to note that in the mature spermatozoon, the presence of AQP11 exclusively on the end piece (Figure 1D) complements the restriction of AQP7 and AQP8 to the more anterior part of the tail (Figures 1E–G). As there is no intracellular organelle in the end piece, it is likely that this superaquaporin is located on the plasma membrane. As intracellular localization has been recognized as a characteristic of AQP11 so far, this would represent a unique feature in understanding the role of AQP11.

7 Summary and conclusion

Although AQP7 and AQP8 were first cloned from the testis, and together with AQP11, have been well recognized in the frontier of AQP research to be expressed in abundance in the testis, these testicular AQPs have been largely neglected in the field of male reproduction. Interest and progress have been dampened on one hand by the lack of specific inhibitors, poor antibody quality and ubiquitous presence of such AQPs, and on the other by the lack of reproductive phenotypes in the knockout mouse models. The latter could be a result of compensatory expression of testicular AQP7 and AQP8, and the premature death of AQP11 knockout animals due to kidney failure, thus preventing the manifestation of any adult testicular phenotype. Nevertheless, research has so far established the identification and localization of these AQPs in the testis and mature spermatozoa and hinted at their major potential roles. AQP7 may contribute toward glycerol-related energy metabolism of spermatozoa, AQP8 enables water fluxes required for physiological cell volume regulation in the constantly differentiating germinal epithelium and venerable mature spermatozoa and AQP11 may be crucial for the turnover and recycling of surplus cellular components made redundant during spermiogenesis and spermiation. Clinical data associating these AQPs with fertility or infertility barely exist. Further studies in testicular and sperm AQPs are important in clarifying the maintenance of a germinal epithelium functioning efficiently in the production of spermatozoa and sperm physiology in natural fertilization.

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