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Review

Aquaporins and (in)fertility: More than just water transport

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ABSTRACT

Aquaporins (AQPs) are a family of channel proteins that facilitate the transport of water and small solutes across biological membranes. They are widely distributed throughout the organism, having a number of key functions, some of them unexpected, both in health and disease. Among the various diseases in which AQPs are involved, infertility has been overlooked. According to the World Health Organization (WHO) infertility is a global public health problem with one third of the couples suffering from subfertility or even infertility due to male or female factors alone or combined. Thus, there is an urgent need to unveil the molecular mechanisms that control gametes production, maturation and fertilization-related events, to more specifically determine infertility causes. In addition, as more couples seek for fertility treatment through assisted reproductive technologies (ART), it is pivotal to understand how these techniques can be improved. AQPs are heterogeneously expressed throughout the male and female reproductive tracts, highlighting a possible regulatory role for these proteins in conception. In fact, their function, far beyond water transport, highlights potential intervention points to enhance ART. In this review we discuss AQPs distribution and structural organization, functions, and modulation throughout the male and female reproductive tracts and their relevance to the reproductive success. We also highlight the most recent advances and research trends regarding how the different AQPs are involved and regulated in specific mechanisms underlying (in)fertility. Finally, we discuss the involvement of AQPs in ART-related processes and how their handling can lead to improvement of infertility treatment.

1. Introduction

Cellular membranes are permeable to water, small nutrients, and toxins. However, the exchange through the membrane bilayer is rather slow due to its lipidic composition. To maintain cellular health and proper functioning, cells have to transport several substances (e.g. nutrients and water) faster than through simple diffusion [1]. In 1992, the group of Peter Agre identified a transmembrane channel protein responsible for facilitating water diffusion, later called aquaporin (AQP) [2].

In mammalian cells, 13 different AQPs have already been described (AQP0-12), which were organized into groups (Fig. 1). The first group englobes the so-called *orthodox AQPs* represented by seven different homologues (AQP0-2, AQP4-6, and AQP8). These AQPs are described to be most selective for water, due to their low permeability to other small molecules. The second group includes AQP3, AQP7, AQP9, and AQP10, which are called *aquaglyceroporins*. They are characterized by a larger pore size [3], enabling them to be permeable to glycerol, urea, and other small neutral solutes, and also water [4]. The third group of AQPs is composed of AQP11 and AQP12 and called *superaquaporins*. This group of AQPs is not yet fully understood, but research suggests that these AQPs are responsible for the movement of water and neutral solutes in intracellular compartments [5]. Still, these classifications are somewhat outdated, as new nomenclatures are being proposed for some AQPs. For example, AQP3, AQP4, AQP6, AQP7, AQP8, and AQP9 are also known as *ammoniaporins* for allowing the transportation of ammonia [6].

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Additionally, AQPs capable of transporting hydrogen peroxide (AQP3, AQP5, AQP8, AQP9, and AQP11) are known as *peroxiporins* [6,7].

Hence, AQPs are central in the regulation of cellular volume and in the movement of nutrients and/or elimination of metabolic residues [1]. They are involved in proper organ/tissue function, with male and female reproductive systems not being an exception [4]. The development of male and female gametes is a sum of multiple complex processes in which water regulation is essential [8,9]. Water homeostasis is pivotal for spermatogenesis [10] and sperm motility [11,12]. AQPs expression is also essential for folliculogenesis [13], and menstrual cycle [14]. In this review, we present the most up to date research concerning the expression and function of the different AQPs on the male and female reproductive tracts, as well as their role in gametes development until fertilization. Finally, we will discuss the available data regarding AQPs as targets for Assisted Reproduction Technologies (ARTs).

2. Aquaporins in the male reproductive system

AQPs are present in all human tissues, with some specificity regarding their distribution. Herein, we present the data available concerning the expression pattern of AQPs in each cell type/tissue (Table 1 and Fig. 2) and their role (if known) throughout the male reproductive tract.

2.1. Aquaporins in the testis

The testes participate in the creation of the microenvironment that allows the occurrence of spermatogenesis, with the consequential development of germ cells [15]. The testicular environment can be divided into two main areas: the interstitial tissue and the seminiferous tubules [16].

2.1.1. Aquaporins in interstitial tissue

Leydig cells are the predominant cells [17] in the testicular interstitial tissue and produce and secrete testosterone [18]. Rat Leydig cells express AQP0 protein [19] which seems to be involved in the water equilibrium between the extracellular and the intracellular medium [19]. AQP1 expression has also been confirmed in Leydig cells of human adolescents with varicocele, that expression was not observed in healthy subjects [20]. AQP1 expression exclusivity in pathological conditions highlights the extra need for fluid reabsorption/excretion characteristic of the varicocele disease [20]. For AQP5, although mRNA microarrays predict its presence in human Leydig cells, protein expression is yet to be confirmed [21]. Another AQP described in mammal Leydig cells is AQP9. In rats, AQP9 has been suggested to mediate intensive transport of water [22,23] and non-charged solutes [19], between cells and blood vessels and/or interstitial space [23], assisting in the maintenance of cell homeodynamics.

Moreover, AQP1 expression was reported in endothelial cells of the microvessels net throughout the testis. It is believed that this AQP is directly involved in the water diffusion of endothelial cell membranes

Table 1
Aquaporin expression in the male reproductive tract and gametes of mammals.

Tissue		AQPs	Species	References
Interstitial tissue	Leydig cells	AQP0	R	[19]
		AQP1	H[a]	[20]
		AQP9	R	[22,23]
Seminiferous tubules	Sertoli Cells	AQP0	R	[19]
		AQP3	M, R	[19,27]
		AQP4	H, R	[29,30]
		AQP8	R, M	[34,35], [10]
		AQP9	M, H	[27,36]
		AQP11	R	[39]
	Germ cells	AQP7	R, M, H	[31–33]
		AQP8	R, M, H	[10,32]
		AQP9	H	[37]
		AQP11	R	[39]
Excurrent ducts	*Rete testis*	AQP1	M, R	[41,42]
	Efferent ducts	AQP1	M, R	[35,41]
		AQP9	R, H	[35,43]
		AQP10	R	[19]
	Epididymis	AQP1	R	[42]
		AQP3	R	[19]
		AQP4	H	[29]
		AQP5	R	[46]
		AQP7	R	[47]
		AQP8	R	[34]
		AQP9	R, H	[43,45]
		AQP11	R	[48]
	Vas deferens	AQP1	R	[42]
		AQP2	R	[49]
		AQP9	R, M	[45,51]
Spermatozoa		AQP3	H, P, Ho	[12,71,74]
		AQP7	H, M, P	[11,52,53], [33,71]
		AQP8	H, M	[36,54], [10]
		AQP11	H, M, P, Ho	[54,55], [150], [74]

Legend: H-Human, M-Mice, R-Rat, P-Pig, Ho-Horse.
[a] Expression reported in non-healthy individuals.

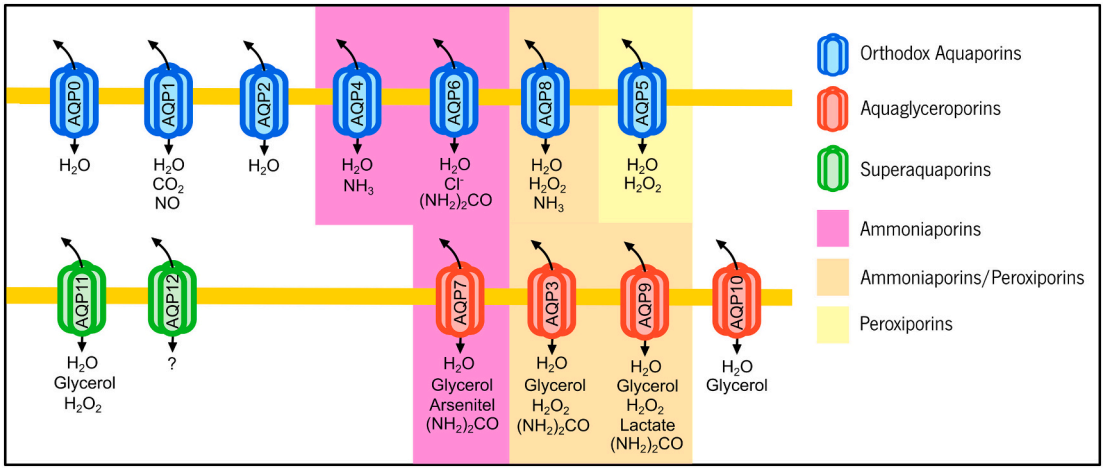

Fig. 1. Aquaporins classification based on their homology and biophysical properties. Up to date, thirteen distinct aquaporins have been identified in mammals (AQP0-12) and they have been classified into three main groups: *orthodox aquaporins*, *aquaglyceroporins* and *superaquaporins*. Although high homology exists among homologues of the same group, each may exhibit a different specificity in its permeability to solutes. For that reason, some AQPs have also been classified according to their permeability to particular solutes (*ammoniaporins* and *peroxiporins*). Legend: AQP – aquaporin.

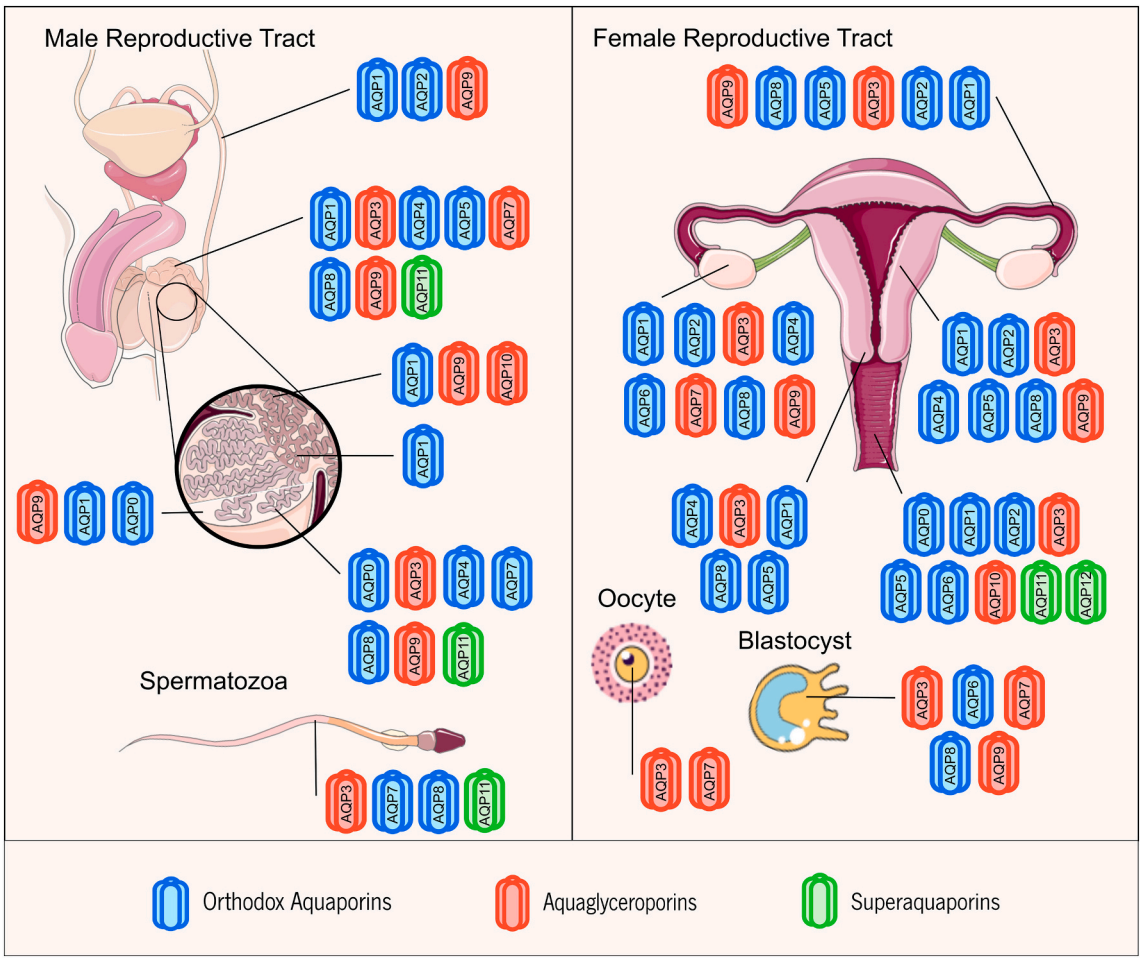

Fig. 2. Aquaporins distribution throughout both the male and the female reproductive tract. Up to date, all the thirteen aquaporins (AQP0 to AQP12) have been described in the female reproductive tract, female gamete and embryo. Only ten AQPs have been identified in the male reproductive tract and spermatozoa (AQP0 to AQP5 and AQP7 to AQP11), among which orthodox aquaporins (in blue), aquaglyceroporins (in red) and superaquaporins (in green). Legend: AQP – aquaporin.

between interstitial tissue and the circulatory system [24].

2.1.2. *Aquaporins in the cells of the seminiferous tubules*

The seminiferous epithelium is mainly composed by Sertoli cells (SCs) and germ cells. SCs are somatic cells responsible for the physical and nutritional support of the developing germ cells [25]. Thus, AQPs present in SCs are suggested to contribute to the creation of a specific environment that allows spermatogenesis and serves as a vehicle for immature sperm motion through the tubules [26]. Similarly to what described for Leydig cells, the orthodox AQP0 is also present in rodent SCs. However, its expression pattern is rather complex and not yet fully understood [19]. Histological cuts of rat seminiferous tubules showed that AQP0 expression levels in SCs changed in different stages of the seminiferous epithelium cycle. Higher expression is seen in the intermediate stages (V-VIII) [19], explaining their detection only in half the circle of the histological cuts. This pattern can be due to the spermatogenic wave and its involvement in specific spermatogenic phases. In fact, AQP0 expression in SCs was associated to the detachment process of elongated spermatids from these somatic cells into the lumen of the seminiferous tubules [19]. As far as AQP3 is concerned, its expression was confirmed in mice [27] and rat SCs [19]. This aquaglyceroporin is suggested to mediate the transport of glycerol [27], which is an essential substrate for germ cells development and thus, spermatogenesis [28]. AQP4 is also present in human seminiferous tubules [29] and rat SCs membrane [30]. In rodents SCs, AQP4 is situated in direct contact with cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel [30]. This interaction indicates a possible role of AQP4 and CFTR in regulating water and ion homeostasis in the seminiferous tubules.

Regarding AQP7, despite its expression not being described in SCs, it was confirmed in germ cells, particularly in later stages of development (secondary spermatocytes and elongated spermatids) [31]. A concurrent study described AQP7 expression also on spermatocytes of rodents [32]. Authors hypothesized that AQP7 may have a role in the drastic decrease in cellular volume in spermatids during spermiogenesis, by modulating water efflux. Interestingly, studies in AQP7 knockout mice showed no alteration in male fertility, particularly in daily sperm production, motility or even on the number of offspring when compared to wild type animals [33]. These results suggest that other AQPs may compensate for the absence of AQP7 expression. For example, AQP8 expression was also confirmed in rat elongated spermatids. This highlights the involvement of both AQPs in spermatids cytoplasmic condensation [32]. Moreover, AQP8 expression was also noted in the cytoplasm of primary spermatocytes. Unlike AQP7, AQP8 was also confirmed in rat SCs. Studies showed that AQP8 immunolocalization delineates the adluminal section of SCs plasmatic membrane [34,35] and is also present in human SCs [36]. Interestingly, AQP8-null mice (like AQP7 knockout mice) are fertile. Once more, it suggests versatility regarding AQPs function and their ability to compensate other AQPs function. However, it was noticed that AQP8-null mice had greater sized testes when compared to wild type [10].

AQP9 expression was detected in human SCs [36], while in germ cells, AQP9 protein expression is present in human primary spermatocytes and adluminal haploid germ cells [37]. Functional studies

performed in mice SCs showed that estrogen treatment caused *Aqp*9 gene down-regulation accompanied by a decrease in glycerol transportation [27]. Glycerol is an beneficial substrate for spermatogenesis and normal testicular morphology [28], evidencing that aquaglyceroporin expression is essential for SC and germ cell physiology and thus, male fertility [27]. A study performed in the testis of adolescents suffering from varicocele, showed that SCs present had decreased AQP9 expression [37]. These authors suggested that AQP9 might have a role in lactate transport from SCs to the developing germ cells, causing a lactate deprivation on developing germ cells of varicocele patients [37]. Lactate is known to be the preferable substrate of developing germ cells for the production of energy [25] and to have anti-apoptotic properties in germ cells [38]. Thus, lactate deficiency due to AQP9 down-regulation may explain the slow spermatogenic rate reported in varicocele patients [37].

Evaluation of AQP11 expression by immunohistochemistry on rat testis showed signal in germ cells, exclusively in the distal tail section of elongated spermatids and in residual bodies inside SCs, probably originated by unviable elongated spermatids phagocytosis and intracellular organelles [39]. The particular location in the tail distal section of elongated spermatids, where the cytoplasm is scarce, and no organelle is known to be located makes it difficult to assign a function to this AQP. However, the authors hypothesized that AQP11 could be located in the sperm membrane to help eliminate the excess of water for the completion of flagellum formation [39].

2.2. Aquaporins in the male excurrent ducts

After spermatozoa are released into the lumen of the seminiferous tubules, the fluid created by SCs assists in the transportation of the immature spermatozoa to the *rete testis* [40]. In this portion of the male reproductive tract, AQP1 expression was confirmed in both mice and rats [41,42]. In a study performed with *Aqp1*-knockout mice, it was reported that these animals presented a dilated *rete testis* when compared to wild type animals. This suggests that AQP1 is crucial for fluid reabsorption in the *rete testis* [41], possibly due to its expression in the vascular endothelium that could be involved in transcellular water transportation. This transcellular transportation is important once 90% of fluid reabsorption in the testis is done by the epithelial cells in the efferent ducts [9]. Here, it is where the concentration of sperm cells is achieved and, for that, water channels are mostly needed. Like in *rete testis*, rat efferent ducts also express AQP1 [35]. Ciliated cells expressed AQP1 on the cilia, while the non-ciliated cells expressed this AQP in the microvilli, apical endosomes, and basolateral cellular membrane [35]. However, unlike *rete testis*, efferent ducts of *Aqp*1-knockout mice do not show any histological alteration, when compared to the efferent ducts of wild type animals [41]. This is suggested to be due to the expression of multiple AQPs in the efferent ducts. In fact, AQP9 expression was reported in the microvilli of the non-ciliated cells of rat efferent ducts [35]. In humans, AQP9 expression was also reported in the apical membrane of non-ciliated cells of the efferent ducts [43]. The importance of different AQPs expression in the same tissue (especially from different groups such as AQP1 and AQP9) can be noted in tissues like the efferent ducts. While the orthodox AQP1 seems to be specialized in transporting water, the expression of the aquaglyceroporin AQP9 may assist in the movement of glycerol and other neutral solutes into the lumen of the tubule [44]. To assist in these events, AQP10 is also present in ciliated and non-ciliated cells of rodent efferent ducts [19].

Epididymis is the subsequent duct located downstream to the efferent ducts. This highly coiled tube can be divided into three different sections: caput, corpus, and cauda [45]. Here, immature sperm cells undergo a series of developmental processes in specific microenvironments created by the multiple epithelial cell types that form this tubule. In fact, it is in the epididymis where sperm cells are stored and mature by gaining more motility and develop ability to fertilize the oocyte [45]. The literature points to specific AQPs expression patterns in the different section of the epididymis. Moreover, many AQP were confirmed to be expressed in the epididymis of animal models but few were studied in human male tissue. Since epididymis support, concentrate and store the developing sperm cells, one would assume that this tissue expresses multiple AQPs. In fact, it was suggested that the multiples cells that constitute the epididymis have cooperative reabsorption of water and solutes between the more luminal cell types (principal and clear cells) and the basal cells increasing the flux of water through the entire width of the epididymis epithelium [19], a process enhanced by the expression of multiple AQPs.

As reported in the testis, AQP1 expression was described in the vascular endothelium adjacent to the epididymis epithelium in rats [42]. There, AQP1 can assist in the final reabsorption of water into the circulation. This process, with the contribution of other AQPs, allows the exchange of water from the lumen of the tubule and the circulatory system and the creation of the microenvironments that permit the maturation, concentration and storage of sperm cells. However, specific mechanisms are yet to be revealed.

AQP3 expression was confirmed in basal cells across all sections of rodent epididymis [19] whereas AQP4 expression was only confirmed in human epididymis [29], with no further information available on the cell-specific expression of this AQP on the epididymis. Contrastingly, AQP5 expression has not yet been described in human tissue, but is present in principal cells in the corpus and cauda sections of rat epididymis [46]. AQP7 expression is also present in rat epididymis [47]. The presence of this protein was confirmed in the basolateral membrane of adjacent principal cells in the caput section of the epididymis. In corpus and cauda epididymis section, AQP7 expression was found in the plasma membrane of principal cells and delineating some basal cells [47]. Moreover, a study performed in rat models confirmed the expression of AQP8 in basal cells of the epididymis [34].

AQP9 seems to be one of the most abundant AQPs in the epididymis, and its expression was already described in humans with similar expression patterns to what is reported in rodents [43]. AQP9 is expressed through all sections of epididymal epithelium, especially in the apical microvilli of principal cells [45], likely to maintain metabolic substrate supply for sperm cells [44]. Similarly, AQP11 expression was also confirmed in the microvilli of principal cells of rat epididymis, although in the more distal region of the caudal section [48].

After their maturation in the epididymis, spermatozoa are then guided to the *vas deferens*. This section of the male reproductive tract can be divided into three different sections (proximal, middle, distal), and like the epididymis, separate regions have different AQP expression patterns. AQP1, for example, is only expressed in the luminal and basolateral cell membrane of principal cells of rat *vas deferens* distal section [42]. Similarly to what described for the efferent ducts, this AQP is responsible for the movement of large amounts of water possibly for the purpose of concentrating sperm cells in the *vas deferens* [45]. In adult rats, AQP2 is also differentially expressed throughout the sections of the *vas deferens*. It has a more intense expression in the principal cells of the middle and distal sections, when compared to that of the principal cells of the proximal section, where no expression is apparent [49]. Interestingly, AQP2 expression levels change through postnatal development in rats, that at four weeks of age exhibit the pattern of expression detected in adult animals. This finding suggests that AQP2 expression is coordinated via translational or post-transcriptional mechanisms [46]. Rodent principal cells also exhibit AQP9 expression [45], probably to ensure fast uptake of glycerol by sperm cells [44] as AQP9 does in the liver [50]. A study conducted using an *Aqp9*-knockout mouse model showed an increased concentration of glycerol in serum although these animals were fertile, highlighting the ability of AQP9 in transporting this substrate [51]. A role for AQP9 in the excurrent ducts related to its peroxyporin activity is not ruled out.

2.3. Aquaporins in spermatozoa

Throughout their journey from the seminiferous tubules until reaching the oocyte, spermatozoa encounter multiple microenvironments created by the epithelial cells of both male and female reproductive tracts, in order to ensure maximum fertilization capacity. However, all the effort of the epithelial cells would be in vain if the sperm cells were not able to withstand the changes in the composition of the various milieu, and accommodate the passage of substances from the extracellular to the intracellular medium (and vice-versa).

To assist in those events, multiple AQPs have been described in the membranes of mammalian spermatozoa. In humans, AQP3 expression was described on the tail of sperm cells [12], whereas AQP7, AQP8, and AQP11 expression, which were already described in developing germ cells [31,32,39], have been non-consensually noted in mature spermatozoa. While AQP7 expression is most commonly situated in the middle piece, some authors also reported less intense expression through all sections of the spermatozoa, suggesting different pattern expressions in different individuals [11,52,53]. In the case of AQP8, its expression is mostly described in the middle piece of human spermatozoa [36,54]. Finally, AQP11 seems to be expressed in the intracellular organelles of the head and middle piece, although its expression has also been referred throughout the membrane of the tail [54].

The functional role of AQPs in spermatozoa has been related to: a) changes in cell volume occurring during spermiation; and b) changes in the osmolality of the surrounding environment that spermatozoa experience during their journey throughout the male and female reproductive tracts. Throughout their passage, sperm are exposed to the mild hypertonic environment of the cauda epididymis (~415 mOsm/kg in mouse) and to the isotonic one in the uterine body (~310 mOsm/kg in mouse), which is crucial for motility activation. In the latter case, this osmoadaptation/osmoregulation is related to a proper osmotic balance allowing sperm to have the adequate shape and function. Not only does this adaptation rely on ion channels but also on water channels [36]. Thus, various AQPs have different roles and functions in the processes that spermatozoa undergo during their journey to fertilization, that ultimately allow fertilization.

As aforementioned, osmoadaptation is crucial during the transition of sperm from the male to female reproductive tracts, due to the hypertonic environment of the epididymal cauda and the isotonic environment of the uterine body that activates sperm motility [12]. This was described for mice, which are known to ejaculate into the uterus and not into the vagina like humans. In such an osmoadaptation, there is an increase in cell volume, so that AQPs are needed to protect spermatozoa from detrimental swelling. In this osmoadaptation, AQP3 would play a pivotal role [12]. This was studied in much detail using knockout mice. Mouse sperm with deficient AQP3 were seen to activate their motility adequately when exposed to a hypotonic environment, but they showed an impaired ability to regulate cell volume upon entering the uterus and the oviduct, which resulted in an increased tail bending [12]. Specifically, the absence of AQP3 was found to lead the tail to deform, forming a hairpin-like structure due to mechanical membrane stretch. Therefore, AQP3 was suggested to serve as an osmosensor specialized in the efflux of water that is involved in the osmoadaptation of sperm cells during their transit throughout male and female reproductive tracts, and a deficiency in this protein results in reduced fertilizing ability due to impaired motility in the oviduct [12].

Most of the functional studies about AQPs in mammalian sperm have been conducted with AQP7. This includes knockout studies in mouse, and the relationship of AQP7 with sperm quality and fertilizing ability in humans. With regard to knockout studies, Sohara and colleagues surprisingly reported no differences between the *Aqp7* knockout model and the wild type control specimen regarding testis and epididymis morphology, sperm quality (daily sperm production and motility) and fertilizing ability (in vitro) and offspring [33]. This suggests that AQP7 knockout mice were neither sterile nor had abnormal sperm function and morphology. On the other hand, blocking potassium channels with quinine results in sperm volume increase, which is prevented when AQPs are inhibited with $HgCl_2$ [33]. In another study using an *Aqp7* knock-out murine model, spermatozoa did not differ between knockout and wild type animals. However, both water influx and efflux occurred faster in the knockout than in the wild type, which was due to an upregulation of the expression of AQP9 in the latter [55]. This could be a way of compensating the absence of AQP7 in knockout animals, suggesting that in spermatozoa aquaglyceroporin AQP7 functions primarily as a solute channel rather than a water channel. With regard to the relationship between the relative content of AQP7 and fertilizing ability in humans, Saito and colleagues compared fertile controls and infertile patients and associated the absence of AQP7 in ejaculated spermatozoa with impaired motility and infertility [11]. In another trial, Yeung and colleagues compared fertile controls and infertile patients and also found that the relative content of AQP7 in sperm evaluated with flow cytometry was correlated with sperm motility and was higher in fertile donors than in infertile patients [36]. As far as the relationship between AQP7 and sperm quality is concerned, Moretti and colleagues found that human sperm selected through swim up and separated into motile and immotile fractions have different AQP7-localization. Indeed, a higher percentage of sperm cells from the motile population showed staining of AQP7 labeled in the pericentriolar area, midpiece, equatorial segment and in the tail while the abnormal and immotile fraction had AQP7 labeling in the cytoplasmatic residues, coiled tails, and entire head and acrosome. In addition, spermatozoa with normal morphology and progressive motility showed clear dotted AQP7 staining, whereas weak, diffuse AQP7 immunoreactivity was observed in sperm cells with cytoplasmic residues (translucent vacuoles and swollen and badly assembled mitochondria), cytoplasmic droplets (immature) and coiled tails [53]. Therefore, this link with sperm morphology indicates that defective spermatogenesis and epididymal maturation lead to an abnormal localization and expression of AQP7. In light of all the aforementioned, current evidence supports the idea that AQP7 is crucial during spermatogenesis, differentiation into spermatozoa (spermiation) and epididymal maturation, glycerol metabolism in sperm and volume reduction of spermatids [36,56].

Regarding AQP8, earlier studies suggested that since phloretin, an inhibitor of GLUT transporters, affected the permeability of human and sheep sperm to water, these transporters could be involved in water transport across the sperm plasmalemma [57]. This hypothesis was later dismissed by Callies and colleagues (2008), who did not find evidence that sodium-dependent solute and glucose transporters were involved in the transport of water in mouse spermatozoa [55]. In fact, phloretin does not inhibit orthodox AQPs but rather aquaglyceroporins which, as will be later discussed, are related to sperm cryotolerance [58]. Since AQP8 is an orthodox AQP, it was reasonable that when Yeung and colleagues used two inhibitors ($HgCl_2$ and phloretin) to determine the role of AQPs in the regulation of sperm volume, they observed that $HgCl_2$ but not phloretin was effective in blocking quinine-induced swelling [36]. This suggested that AQP8 rather than aquaglyceroporins (targeted by phloretin) is involved in the regulation of sperm volume. These results were also in agreement with Callies and colleagues who found that Hg^{2+}- and Ag^{+}-sensitive channels are involved in the transport of water, thus supporting a role for AQP8 [55]. Taking these evidences into consideration, it is conceivable to assume that AQP8 is the major permeation route of water in the middle piece of spermatozoa [55]. It is also possible that this peroxiporin is responsible for the elimination of ROS (especially H_2O_2) from sperm mitochondria, as demonstrated in a study made in seabreams [59]. In fact, the inactivation of an ortholog of AQP8 (AQP8b) and consequent ROS accumulation in sperm mitochondria, resulted in mitochondrial membrane depolarization, which reduced production of ATP and diminished progressive sperm motility [59]. Moreover, decreased ATP production suggested the inability of sperm to go through capacitation (high ATP levels are a marker for sperm hyperactivation), which is a crucial process for sperm to achieve

fertilization. Still, it is worth noticing that there is not a total homology between the AQP8b and mammalian AQP8 [59] and its expression pattern. For instance, in rats, while found at the inner mitochondrial membrane of hepatocytes and Sertoli cells, AQP8 is absent in the mitochondria of spermatozoa [60]. However, in a recent study, its expression was reported in the mitochondrial membrane of human spermatozoa [54], highlighting AQP8 involvement in ROS management in spermatozoa, as was reported in other cell types [61]. In fact, low H_2O_2 concentration is important for sperm capacitation in order to initiate the cascade of events that allow oocyte fertilization. However, if too high, H_2O_2 inhibits this process [62]. This opens up the possibility of peroxiporins, like AQP8, to regulate the concentrations of H_2O_2 to initiate the process of sperm capacitation. However, the inhibition of AQPs and its effect on sperm function will be further discussed in another topic. Interestingly *Aqp8*-knockout mice are fertile, with no differences being found in sperm concentration and morphology between AQP8 null and wild type mice [10], although sperm motility and ability to fertilize were not evaluated. Because of the crucial role of AQPs on sperm function, it has also been investigated whether infection with human Papillomavirus (HPV) could affect the expression and function of AQPs in human spermatozoa. Interestingly, while neither AQP3 nor AQP7 were found to interact with HPV, AQP8 was found to colocalize (laser scanning confocal microscopy) and co-immunoprecipitate with HPV-L1 protein, which could explain why HPV reduces the sperm permeability to water and increases their sensitivity to oxidative stress via interaction/inhibition of AQP8 [63]. The same study also described that AQP8, as well as AQP3 and AQP7, are found expressed in the head of human spermatozoa [63] around the acrosome which could highlight a possible role of these AQPs in acrosomal reaction. Despite not being yet fully understood, sperm capacitation need tightly regulated H_2O_2 concentrations to allow hypermotility and acrosome reaction, being both these processes critical for natural fertilization [62]. In fact, acrosome swelling is required for the proper completion of acrosomal reaction [64]. Taking into consideration that 2 of the 3 AQPs found in human spermatozoa head are capable of monitoring H_2O_2 concentrations and acrosomal swelling (AQP3 and AQP8) one could assume that these water pores are involved in acrosome reaction. However, we could not find any studies that were able to confirm this possibility.

In spite of all the aforementioned, Yeung and colleagues compared fertile controls and infertile patients and found that while sperm cells with higher levels of AQP8 showed less sperm tail coiling in response to the induction of swelling, there were no significant differences regarding the relative content of this protein. Therefore, AQP8 is now suggested to play a vital role for water transport [36], but more research is needed to address its relationship with sperm fertilizing ability in humans and animal models.

The last AQP encountered in the mammalian spermatozoa is AQP11. Unfortunately, knockout animal studies of this particular AQP are, to this day, impossible to perform due to the fact of kidney failure and consequent premature death [65]. Nevertheless, its expression pattern indicates some important functions. AQP11 presence on the membrane of intracellular organelles [54] could be important for the maintenance of microenvironments essential for sperm function. As an example, AQP11 expression in the endoplasmic reticulum is essential for H_2O_2 transportation and thus, in the assistance of redox homeostasis and pathways signaling in kidney cells [7]. A possible role of AQP11 in the elimination of H_2O_2 and other metabolic waste from mitochondria and other organelles, similar to what discussed for AQP8, would be interesting to explore. For now, a study performed with pig sperm cells, that have a similar expression pattern to the one seen in human sperm, noted that AQP11 expression was correlated with higher sperm quality [54]. The spermatozoa with higher AQP11 expression had also higher membrane integrity and motility, further evidencing the relevance of AQP11 on sperm function. However, more studies are needed to access the function of AQP11 and other AQP homologues in sperm physiology. This information is of extreme relevance per se and valuable as it could help in the development of technologies capable of enhancing ARTs.

3. General aquaporin inhibitors: effects on sperm motility and oxidative stress

Since inhibitors are not specific enough to target one AQP, but they usually tackle one of the three AQP families, this section summarizes the results reported from a global perspective.

The function of AQPs with regard to ROS (hydrogen peroxide) has been investigated in humans, by comparing normospermic and subfertile patients, after inhibition of all AQPs with $HgCl_2$. Elimination of hydrogen peroxide is impaired when AQPs are blocked, and this effect is much more apparent in normospermic than in subfertile men which, prior to inhibition, already show reduced permeability to hydrogen peroxide, perhaps due to a reduced AQP-amount. Remarkably, sperm motility and elimination of hydrogen peroxide are related to AQP [54]. In another study that used $HgCl_2$ as a blocking agent, Alyasin and colleagues found that samples incubated with $HgCl_2$ for 60 min have reduced sperm motility and mitochondrial membrane potential. However, this effect was reversed when samples were incubated with $HgCl_2$ and 2-mercaptoethanol. All these data corroborate the idea that AQPs are involved in the regulation of sperm motility and mitochondrial function [66]. The function of AQPs in spermatids and spermatozoa is related to the control of their volume during spermiation, the removal of residual cytoplasm droplet at the end of epididymal maturation, the passage from the epididymal tract to the cervical mucus and the migration through the female reproductive tract. In addition, they also play a role in the elimination of hydrogen peroxide and the transport of glycerol and urea. Besides cell volume regulation and cytoplasm removal during sperm maturation, the role of AQPs also extends to reactive oxygen species elimination. Specifically, the function of AQP7 appears to be related to glycerol metabolism, that of AQP8 is vital for the regulation of the sperm volume, and that of AQP11 is very important during the elimination of surplus cell components that are made redundant during spermiogenesis and spermiation [39].

4. Aquaporins and sperm cryopreservation

Although cryopreservation is currently the best method for long-term storage of mammalian sperm, freezing and thawing induce injuries on sperm integrity and function, which may result in reduced fertilizing ability [67–69]. One of the causes for these cryoinjuries is the formation of ice crystals that occurs while temperatures decrease beyond 0 °C. Cryopreservation media contain non-permeable cryoprotectants that increase the osmolality of the surrounding cell environment and induce water to flow out from the intracellular space [70]. Therefore, water is sequestered from inside the cell and this reduces the likelihood of ice crystal formation. In this context, the presence of AQPs in sperm cell membrane and their potential involvement during exposure to cryopreservation media has attracted the interest from many researchers in the last years [1]. In addition, not only is sperm osmoregulation important during freezing but also during thawing, as they are diluted in isotonic media.

4.1. Aquaporins and sperm cryotolerance

The studies conducted thus far on the potential involvement of AQPs in mammalian sperm cryopreservation have: a) tried to establish a relationship between the relative levels of the most relevant AQPs (AQP3, AQP7 and AQP11) and sperm cryotolerance; and b) investigated how sperm cryotolerance is altered when AQPs are inhibited. With regard to the first kind of studies, it has been found that the relative abundances of AQP3 and AQP7, but not those of AQP11, provide pig sperm with greater ability to withstand cryopreservation (evaluated as sperm survival and motility recovery at post-thaw) [71].

In cattle, sperm showing higher relative levels of AQP7 but not of

AQP3 appear to be related to higher cryotolerance. In contrast, Fujii and colleagues observed that not only AQP7 but also AQP3 is linked to sperm cryotolerance in these species [72]. Another study, also in cattle, investigated the role of AQP11 in sperm cryopreservation and found that not only is the relative content of AQP11 related to higher sperm resilience to cryopreservation but also with fertilization outcomes of frozen-thawed sperm evaluated both in vivo and in vitro [73]. With regard to horse sperm, the relative content of AQP3 and AQP11, but not that of AQP7, has been found to be related to their cryotolerance, so that those fresh ejaculates that have higher relative levels of AQP3 and AQP11 are more likely to have higher viability after thawing [74]. Therefore, the relationship between AQPs and sperm cryotolerance varies across species, since AQP3 and AQP7, but not AQP11, are relevant in pigs; AQP3, AQP7 and AQP11 are related to sperm cryotolerance in cattle; and AQP3 and AQP11, but not AQP7, are important in horses.

4.2. Inhibition of aquaporins and cryopreservation

Three different inhibitors (acetazolamide; phloretin; and propanediol) have been used to block AQPs and examined how this affects sperm cryopreservation outcomes in pigs and horses [58,75,76]. Propanediol was used as an inhibitor of orthodox AQPs and aquaglyceroporins; acetazolamide was used as an inhibitor of orthodox AQPs [77]; and phloretin was used an inhibitor of aquaglyceroporins. $HgCl_2$ was not used, since it is a general blocker of all AQPs. The results in both pigs and horses were similar, since acetazolamide, which is an inhibitor of orthodox AQPs barely affected sperm cryopreservation outcomes, which confirmed that either they are absent from ejaculated sperm in these two species or play a marginal role with regard to sperm cryotolerance. In contrast, phloretin which is an inhibitor of aquaglyceroporins had a detrimental impact on post-thaw sperm motility and survival, thus confirming the relevance of this family of AQPs during sperm cryopreservation. Finally, propanediol increased post-thaw sperm viability, lowered membrane lipid disorder and increased mitochondrial membrane potential, suggesting that it functions as a cryoprotectant rather than as a blocker for AQPs and aquaglyceroporins [58,75]. Interestingly, the effects of these inhibitors depended on the intrinsic freezability ejaculate, so that inhibition through phloretin had a more detrimental impact in ejaculates with high freezability, which would match with the individual differences between good and poor freezability ejaculates in terms of the relative abundance of AQP3 and AQP7 [71].

4.3. Relocalization of aquaporins in response to sperm cryopreservation

Finally, cryopreservation has also been found to induce changes in localization of AQP7 in pig sperm, which is mainly located in the connecting piece in fresh sperm but is relocated over the mid-piece in frozen-thawed sperm [71,78], which could result from the damages inflicted on the sperm membrane occurring during cryopreservation, as has also been reported for other proteins [67]. In contrast, such a relocalization does not occur in cattle [72,79], nor has been reported for AQP3 and AQP11.

5. Aquaporins in the female reproductive tract

The female reproductive tract is composed of multiple complex organs and is characterized by the existence of reproductive cycles in tissues such as the ovary and the uterus. In the occurrence of these and other events, fluid secretion and compartmentalization assisted by transmembrane transporters are essential. In fact, the multiple cell types that form the female reproductive tract express multiple AQPs (Table 2 and Fig. 2). In the next section, that information will be presented and the putative roles of AQPs on tissue function will be discussed.

Table 2
Aquaporin expression in the female reproductive tract and gametes of mammals.

Tissue	AQPs	Species	References
Vagina	AQP0	R	[80]
	AQP1	R, H	[81,82]
	AQP2	R, H	[81,82]
	AQP3	R, H	[81,82]
	AQP5	H	[81]
	AQP6	H	[81]
	AQP10	R	[80]
	AQP11	R	[80]
	AQP12	R	[80]
Cervix	AQP1	H[a]	[85]
	AQP3	H[a], R, M	[85,86]
	AQP4	M	[86]
	AQP5	M	[86]
	AQP8	H[a], R, M	[85,86]
Uterus	AQP1	H	[14]
	AQP2	H	[14]
	AQP3	H, M	[89,90]
	AQP4	M	[92]
	AQP5	M, P	[92,94]
	AQP8	M	[95]
	AQP9	P, R	[94,96]
Oviduct	AQP1	P, R	[97,98]
	AQP2	H	[89]
	AQP3	H	[89]
	AQP5	M	[99]
	AQP8	R	[100]
	AQP9	H	[100]
Ovary	AQP1	H	[103]
	AQP2	H	[103]
	AQP3	H	[103]
	AQP4	H, M	[93,103]
	AQP6	H[a]	[105]
	AQP7	H[a], R	[105,106]
	AQP8	H[a], R	[13,105]
	AQP9	R	[13]
Oocyte	AQP3	H, M	[108,111]
	AQP7	H, M	[108,111]
Blastocyst	AQP3	H, M	[108,113]
	AQP7	H	[108]
	AQP6	M	[115]
	AQP8	M	[113]
	AQP9	M	[113]

Legend: H-Human, M-Mice, R-Rat, P-Pig.
[a] Expression reported in non-healthy individuals.

5.1. Aquaporins in the vagina

The vagina is an organ with fluid secretion abilities, especially during sexual activities, that increase lubrification, but also that hosts the spermatozoa after male ejaculation. It is suggested that cells of vaginal epithelium express multiple AQPs, which assist in the creation of the vaginal fluid. Data available for AQP0 concerns only its presence in rodent vagina, this AQP is abundantly expressed in rat vaginal epithelium [80]. With regard to AQP1, and similarly to what reported for the male reproductive tract, this protein is mostly expressed in the vascular endothelial cells of the small capillaries of human vagina [81]. In a study conducted with female rats, a similar AQP1 tissue expression pattern was noticed in the vagina. Pelvic nerve stimulation led to a translocation of AQP1 and AQP2 from intracellular membranes to the plasma membrane of the endothelial cells of rat vagina [82]. This data evidences a role of both AQPs on vaginal lubrification. With the increase in blood flow characteristic of sexual arousal, the authors suggested that AQP1 and AQP2 are involved in the transportation of fluid from the capillaries to the subepithelial layer [82]. Authors also reported that vaginal epithelial cells expressed AQP2 in humans [81]. Human and rat vaginal epithelial cells also present AQP3 expression. Contrastingly, its expression is constitutively located in the plasma membrane [81,82]. The involvement of an aquaglyceroporin in vaginal lubrification is plausible taking into consideration the physical properties of the vaginal fluid

[83]. Thus, these studies strongly suggest that AQP1, AQP2, and AQP3 have roles in vaginal lubrification composition.

The same study that described the expression of AQP1-3 in human vagina also noticed that epithelial cells express AQP5 and AQP6 throughout the cytoplasm [81]. The author hypothesized that both these AQPs were involved in assisting vaginal lubrification by facilitating transepithelial fluid transportation, but more studies are needed to clarify the roles of these specific AQPs. Rat vaginal epithelium also expresses AQP10, AQP11, and AQP12 [80]. However, and to the best of our knowledge, the expression of these AQPs on human vaginal tissue has not yet been confirmed. In rat vaginal epithelium, AQP10-12 expression decreased after ovariectomy, probably due to the consequent decrease in estrogens (E2) secretion. Yet, no specific function to these AQPs was described [80].

5.2. Aquaporins in the cervix

Upstream of the vagina, sperm cells will encounter the cervix. This organ undergoes massive changes during both, gestation and delivery. These changes include a significant increase in water content assisted by AQPs [84]. To assist in water accumulation, cervix cells express multiple AQP. Human studies are scarce in this matter, with most of the information available on AQP expression in this tissue having been obtained from women with unhealthy cervixes. The expression of AQP1, AQP3, and AQP8 was confirmed in the cervix of women with mild cervicitis, cervical intraepithelial neoplasia, and cervical cancer. While AQP1 was expressed in vascular endothelial cells, AQP3 and AQP8 were localized in the epithelial membranes of mild cervicitis and cancerous cells [85]. In fact, these specific AQPs were overexpressed in cancer cells, indicating their possible role in facilitating the infiltration and metastasis of cervical carcinoma cells [85]. In mice, cervix cells also express AQP3 and AQP8, as well as AQP4 and AQP5 [86]. Interestingly, the localization pattern of AQP3 and AQP8 rely upon cell type, and gene expression also changes during mice pregnancy. AQP3 is situated in the basal cells of cervical epithelium [86] and its gene expression is almost non-detectable on nongravid mice but peaks before parturition [87].

Regarding their function, AQP3 seems to be responsible for the increase in water content of cervical tissue and facilitate cervical ripening in pregnant mice [87]. AQP4, AQP5, and AQP8 are expressed in apical cell layers of mice cervix [86]. A study assessing the gene expression of AQPs during pregnancy noticed that, while *Aqp*4 gene expression was low before and throughout pregnancy, expression of *Aqp*5 and *Aqp*8 gene increased significantly around middle gestation time (12 days) and decreased to the levels seen in nonpregnant mice before delivery [86]. The authors of this study suggested that the enhanced *Aqp*5 and *Aqp*8 gene expression at mid-point gestation underlies a possible role of these AQPs in cervical softening and extensibility, which are characteristic of this pregnancy phase [86].

5.3. Aquaporins in the uterus

Further up the female reproductive tract is situated the uterus. This highly vascular and epithelial rich tissue has highly expressed AQPs, with expression patterns that changes throughout the different phases of cycle that this organ goes through. In fact, AQP1 is expressed throughout the endothelium of small vessels in the uterus and its expression levels are higher during the secretory phase, when compared to the proliferative phase in premenopausal women [14]. This may evidence the role of AQP1 in the growth of new blood vessels (angiogenesis) representative of the secretory phase in the uterine cycle [14]. Like AQP1, AQP2 expression is more accentuated during the secretory phase. Still, it is mainly expressed in the luminal and glandular epithelium and it has been suggested to have a different function [14]. The enhanced AQP2 expression levels coincide with the increased formation of endometrial stromal edema [14]. This event, together with the enhanced blood circulation, is known to optimize uterine growth by draining or diminishing the concentration of inhibitory factors [88]. In the case of AQP3, its expression is located in the smooth muscle, endometrial glands, and myometrium tissue of women uterus [89]. In mice, AQP3 is involved in the efflux and influx of water in the uterine cavity during the estrous cycle, probably complementing AQP2 in the creation of stromal edema [90]. It is worth mentioning that mouse uterus has a similar AQP3 expression pattern to the one described in humans [89,91], evidencing that the role described in the murine model could be homologous to the humans, despite humans presenting menstrual cycles instead of estrous cycles. The expression of AQP4 in the uterine tissue has only been confirmed in mice. In mouse, AQP4 expression is found with great intensity on the luminal epithelium [92]. Interestingly, *Aqp4*-knockout mice developed with normal uterine compartmentalization. Still, these knockout mice had decreased uterine hypertrophy and endometrial thickness when comparing to the ones reported in the wild type mice, which could explain the subfertility displayed by these animals [93]. The authors of this study pointed that this subfertility and decreased endometrial development were probably more related to the inhibited levels of progesterone and estrogens, than to the lack of AQP4 expression, since these two hormones are essential for endometrium development [93].

AQP5 expression has not been observed in human uterine tissue but has been described in other mammals. In mouse, AQP5 expression was noticed in the basolateral region of the glandular cells of the uterine epithelium [92]. In pigs, AQP5 expression was described on the cells of the myometrium and in the uterine epithelia [94]. Functionally, AQP5 expression levels during pregnancy point to its possible involvement in fluid homeostasis, following the process of implantation and during placentation in mice [92].

In mice uterine tissue, AQP8 was found to be present in the stromal cells of the endometrium and myometrium [95]. This AQP is believed to regulate water distribution throughout these tissues and participate in the uterine edema development in the stromal layer [95]. Uterine AQP9 was only studied in pigs and in pregnant rats. Its expression was found in the epithelial cells and in the apical region of the glandular epithelial cells, respectively [94,96]. In the latter, it was reported that AQP9 expression enhances at the time of implantation, possibly to assist in the decrease of uterine volume and the closing of the uterine lumen, specific traits of this phase of pregnancy [96]. Taking this information into consideration, AQPs seem to have important roles in processes like cervical tissue preparation for delivery and in regulating uterine cycle and even in the events that lead to embryo implantation.

5.4. Aquaporins in the oviduct

The passage from the ovaries to the uterus is known as the oviduct. In humans this structure is known as the Fallopian (or uterine) tube. This organ can be divided into three different sections: isthmus (section closer to the uterus), ampulla (middle section), and infundibulum (section closer to the ovary). Within these sections, oviduct secretions assist gametes transportation and fertilization.

Several studies support the role of AQPs in the creation of oviductal secretions and other roles to ensure a proper tissue function of this tubular structure. In the case of AQP1, as seen in other tissues, it is expressed in pig and rat oviductal vessels [97,98]. However, in rats, its expression was also confirmed in the mesothelial cells of the outer surface of the tube and in the membrane of the smooth muscle cells of the muscular myosalpinx [98]. There, it was reported that AQP1 assists in volume regulation of smooth muscle cells, thus, assisting in the modulation of lumen diameter. This process is suggested to be important for the controlled transportation of fertilized eggs throughout the tube into the uterus [98]. In humans, cells of the epithelial lining of the oviduct express AQP2 and AQP3 [89]. The latter is encountered in non-ciliated secretory cells as in ciliated cells, which are known to be responsible for nutrient secretion into the oviduct that also reaches the uterine lumen [89]. Being an aquaglyceroporin, AQP3 is able to assist in the secretion

of glycerol and other neutral nutrients probably required for lubrification and as an energy source for the spermatozoa and fertilized egg. Concerning AQP5, its expression was described in the apical and basolateral membrane of mice secretory non-ciliated cells, as well as in the cytoplasm of these cells, with more intensity in the infundibulum and ampulla sections of the oviduct [99]. It was reported that this AQP is responsible for assisting in the secretion and reabsorption of the oviductal fluid before and after ovulation, respectively, taking into consideration the pattern of *Aqp*5 gene expression throughout these processes [99]. In addition, AQP5 expression in the non-ciliated cells highlights the participation in the development of secretory granules and fluid homeostasis in the endocytic vesicles characteristic of this cell type [99]. In rat oviduct, AQP8 expression is present the epithelial cells throughout the ampulla and isthmic sections [100]. AQP9 expression was observed on these same cells of rat oviduct [100]. Similar to what is seen in the male reproductive tract, the orthodox AQP8 and the aquaglyceroporin AQP9 are probably responsible for the homeodynamics of water and nutrients [100] between the tract epithelia and, in the case of the female tract, the fertilized egg. AQP9 is the only AQP whose expression was confirmed in women oviduct. In humans, as in rats, the location of AQP9 was reported to be mainly restricted to epithelial cells [101]. It is in the oviduct section of the female reproductive tract where normally egg fertilization occurs. To achieve this, spermatozoa need large amounts of energy for hypermotility that allow them to propel itself through the zona pellucida. Glycerol release facilitated by aquaglyceroporins like AQP9 could be important for the conclusion of this process and assist spermatozoa to reach the oocyte and achieve fertilization [102].

5.5. Aquaporins in the ovary

The ovaries are the female gonads, where the maturation of follicles and ovulation occurs. This highly complex organ and the cells that constitute it are also responsible for the secretion of hormones that are responsible for the ovarian and uterine cycle. The follicular phase of the ovarian cycle is known to require large amounts of water movement to allow the occurrence of ovulation. AQPs involvement in these processes has already been described and will be discussed below.

In humans, AQP1 expression is present in the vascular cells surrounding the theca layer, in theca cells, and in granulosa cells [103]. In the cells of the theca layer, mainly responsible for androgen synthesis, *Aqp*1 mRNA levels were reported to be constant throughout the ovulation phases. However, in the granulosa cells that surround the oocyte, *Aqp*1 transcript expression increases after the follicular rupture in the postovulatory phase. This suggests that AQP1 may have a role in the process of transformation of the follicle into the *corpus luteum* [103]. Expression of AQP2 and AQP3 have also been described in human theca and granulosa cells, with their expression increasing prior to ovulation, suggesting a role of these AQPs in follicular rupture by increasing the follicular antrum fluid content [103]. The same study also confirmed the protein expression of AQP4 on the theca and granulosa cells, although with lower intensity when compared to AQPs previously mentioned. AQP4 mRNA expression increased in the early ovulatory phase and taking this into consideration, the authors suggested an involvement of this AQP in the creation of the follicular antrum to be complemented by AQP2 and AQP3 in later stages of ovulation [103]. In AQP4 knockout mice, a decrease in the number of antral follicles was reported, solidifying the hypothesis made in the human study. In fact, AQP4 knockout mice exhibited impaired ovulation, resulting in a decreased pregnancy success when compared to the wild type animals [93].

Most of the published information regarding AQPs expression in the ovary concerns the data obtained in the granulosa cells. In fact, this cell type is essential for the ovary cycle, and as we described, AQPs are involved in the follicular phase as well as in the ovulation, thus, having an important role in female fertility. A study performed in infertile women, described the mRNA expression of the *Apq*1-4 and also of *Aqp*5-7, *Aqp*9, and *Aqp*11 in luteinized granulosa cells [104]. However, protein expression of AQP6 [105] and AQP7 [106] have been confirmed in human cytoplasm and plasmatic membrane in the epithelial ovary tissue and in mural granulosa cells, respectively. Interestingly, expression of *Aqp7* in granulosa cells was positively correlated with the fertility rate of women and to proper folliculogenesis [104].

In the case of AQP8, its expression was reported in rodent granulosa cells but was not found in healthy women ovary tissue [13] being only present in epithelial tumor tissue [105]. Nevertheless, in rat granulosa cells, AQP7, AQP8, and AQP9 were described as responsible for the creation and osmotic regulation of the follicular antrum [13]. Like AQP7, AQP9 is expressed in human granulosa mural cells [105]. The presence of these aquaglyceroporins in the plasmatic membrane of mural granulosa cells might be responsible for the transportation of neutral solutes in follicle and oocyte development [84,107].

5.6. Aquaporins in the oocyte and blastocyst

After ovulation, the oocyte is still covered with a layer of follicular cells that allow the passage of water and nutrients into the oocyte, as described in the previous section. In addition, the oocyte itself should also express several AQPs to facilitate the exchange of water and nutrients with the extracellular medium. Unfortunately, the information available regarding the expression of AQPs in human oocytes is still scarce. In rodents, however, some information describing the expression of AQP in oocytes and posterior phases of embryo development is already available. The first study on this subject was performed by Edashige and colleagues, who examined the gene expression pattern of the *Aqp*1-9 transcripts in mice oocytes. AQP1 does not seem to be expressed in mammalian oocytes. In contrast, both AQP3 and AQP7 are expressed in human and mouse oocytes [108]. The results of the Edashige study further showed that mice oocytes express the mRNA of *Aqp*3 and *Aqp7* at low quantities [109]. The low expression of these AQPs was justified by the minor role that AQPs have in mediating water transport in this cell type, justified by the low water permeability characterizing the oocyte cellular membrane [109]. This study, together with the subsequent ones suggested dynamic expression for AQPs in oocytes. In fact, *Aqp*3 gene expression increased from immature to mature oocytes, where it achieved its peak expression [110]. After maturation, *Aqp*3 mRNA levels decreased again, highlighting the possible role of this AQP homologue on oocyte maturation process [110]. *Aqp*3 mRNA expression is no longer needed in mature oocytes and the presence of AQP7 seems to be sufficient for proper cell functioning. Oocyte AQP7 expression studies evidence its importance in cryopreservation processes [111]. However, this topic will be further discussed later in this review. In addition to this, it was also reported that AQP7 translocates from the cytoplasm to the plasmatic membrane to respond to osmotic stress in mice oocytes [111]. Moreover, being that these two AQPs are indeed aquaglyceroporins, the available data also evidence a role for glycerol in oocyte metabolism and development.

After fertilization, the embryo goes through a cleavage stage by performing a series of mitotic divisions to give origin to the blastocyst, and then initiate the uterine implantation. During this phase, embryos present high rates of gene expression. In human embryos in the 2–8 cells stage, mRNA expression of *Aqp*1-4, *Aqp*7, *Aqp*9, *Aqp*11, and *Aqp*12 has been reported [108]. However, only the presence of AQP3 and AQP7 proteins has been confirmed at these stages. Moreover, AQP3 and AQP7 proteins were the only ones that are present throughout all the preimplantation stages. These AQPs are expressed in human and mice oocyte, zygote, 2–8 cell embryo, morula, and blastocyst [108]. Throughout these stages of development, several other AQPs start being expressed and their expression levels vary during the different phases. For example, in mice, embryos in the 4-cell stage start presenting expression mRNA of *Aqp*8, which is even more prevalent in the 8-cell stage [112]. Moreover, 8-cell mice embryos start to express AQP9 and in the compacted morula phase, AQP8 protein presence was confirmed [113]. In

mice blastocyst, protein expression of AQP3, AQP6, AQP8, and AQP9 was also confirmed [113,114]. AQP3 is expressed in the trophectoderm (cells from the outer layer of the blastocyst) and inner mass cells basolateral membranes, AQP8 and AQP9 are located in the apical and basolateral cells membrane of the trophectoderm [113]. In mice blastocysts, the presence of *Aqp7* and *Aqp*11 mRNA was also reported, together with the confirmation of that of *Aqp*3, *Aqp*8, and *Aqp*9 [115]. In regards to AQP6, while in some studies this AQP was found in the cytoplasm of mice trophectoderm, others using the same animal model reported no expression [109,114].

In functional studies, mice blastocysts incubated in a hyperosmotic medium with glycerol were able to maintain their normal development compared to incubation with a highly concentrated solution of a non-diffusible solute such as sucrose [115]. The inability of AQPs in transporting sucrose resulted in the failure to reach osmotic equilibrium. However, thanks to the presence of aquaglyceroporins the cells of the blastocyst were able to diffuse glycerol and attain equilibrium, thus, maintaining the normal development process [115]. Interestingly, mice blastocyst under hyperosmotic conditions presented decreased levels of *Aqp*8 mRNA expression, indicating a dynamic expression probably to prevent excessive water transportation and avoid blastocoele shrinkage [115]. These results were consolidated in studies reporting that AQP3, AQP8, and AQP9 are crucial in the fluid accumulation for the process of blastocyst cavitation and in the response to osmotic stress, which are important for the maintenance of the blastocoele in mice blastocyst [113]. In *Aqp3* and *Aqp7* knockdown mice, a decrease in preimplantation embryo development was observed [108]. AQP3 presence in mice embryos is crucial for proper water and glycerol diffusion [116] and studies on mice oocyte cryopreservation have already reported AQP7 as important in allowing glycerol transport [111]. These findings highlight the fact that energy production through glycerol metabolization can be an important pathway to embryo development, as it is in sperm cells, suggesting importance for aquaglyceroporins in facilitating glycerol uptake.

6. Aquaporins and conception in mammals

The oviduct is the place where fertilization and early embryonic development occurs. However, the mechanism through which the oviduct transports the ovum towards the uterus remains elusive. Under the effects of ovarian steroid hormones, the oviduct microenvironment undergoes significant morphological, physiological and biochemical changes [117,118]. The oviduct microenvironment is settled by variations in composition and volume of the oviductal fluid in response to fluctuations of the hormonal plasma levels during the estrous cycle [119]. The embryo transport is an interactive process between embryos and oviduct and is mostly regulated by the beating of the ciliated epithelial cells, oviductal fluid flow and oviductal muscle contraction. Estrogens increase embryo transport by accelerating the oviduct muscle contraction, fluid secretion and ciliary beat frequency whereas progesterone acts in the opposite way by reducing the rate of the embryo transport (for review see [120]). In addition to estrogen and progesterone, prolactin stimulates fluid production in the oviduct [120]. The oviduct tissues express several AQPs (Table 2). In the porcine oviduct, AQP1, 5 and 9 have been reported in the different stages of the cycle and early pregnancy under physiological conditions where a role in regulating ovum transport in the fallopian tube has been suggested [94,97,121]. In pig, the same AQPs are also found in the endometrium and myometrium where their expression is suggested to be regulated according to the estrous cycle and early pregnancy [84,97,122,123]. In the oviduct, under control of estrogens and progesterone, AQP1, AQP5, and AQP9 act coordinately to maintain fluid homeostasis by influencing the fluid production and providing the physiological medium for fertilization and early embryonic development. Rapid increase of water flux through AQP1 in the innermost longitudinal and the inner cells of the circular muscle layer of rat oviduct has been suggested to lead to muscle swelling with shutdown of the lumen and 'locking' of the fallopian tube and consequent facilitation of the ovum movement towards the uterus [98]. Ovarian hormones (E2 and progesterone) control AQP expression and thus, modulate AQP-mediated water transport in the epithelium lining of the rat oviductal lumen. Ovariectomized rats had less AQP5, AQP8, and AQP9 expression levels when compared to the ones of control rats, being that the AQP9 expression levels were restored by estradiol and progesterone supplementation [100]. Involvement of AQPs in fertilization and early cleavage-stage embryonic development has also been suggested in mouse after observing the existence of a correlation between sexual maturation and estrous cycle and expression of AQP5 in the oviduct non-ciliated epithelial cells [99].

The mechanisms by which, in female, cumulus oocyte complexes acquire their developmental capacity represents a challenge for modern researchers. In most mammalian species, the oocyte matures within the ovarian follicle and during the ovulation period, mainly in the stage of metaphase II meiosis. Studies with animal models suggest that an important function in these mechanisms is exerted by connexins [124–126] and AQPs [94,122,127,128]. Gap junctions appearing in oocyte-cumulus oophorus cell complexes are composed of connexins [129] and deficiency of these proteins may result in developmental disturbances of oocytes and infertility [130–132]. Gonadotropins, prolactin and growth hormone stimulate *Aqp1* mRNA and protein expression in granulosa cells and theca cells of medium and large porcine ovarian follicles where AQP1 has been purported to be implicated in follicle development and in cell proliferation and migration [133]. Water movement into the antral cavity of rat follicles has been suggested to occur predominantly by transcellular route through AQP7, AQP8 and/or AQP9 in granulosa cells [13].

Understanding the expression and role of AQPs in the cells of ovarian follicles can improve the knowledge of the mechanisms that control the oocyte maturation and the related capacity for fertilization and normal embryo development, with positive translational repercussions (i.e., oocyte and embryo cryopreservation).

Besides fertilization, implantation and early embryonic development AQPs have been also reported to be expressed during pregnancy participating to the control of maternal-fetal fluid homeostasis and to placental and fetal growth (for review see [134–136]). Dysregulation of AQPs is seen in pregnancy disorders such as preeclampsia, abnormal amniotic fluid volume, chorioamnionitis, and maternal under-nourished pregnancy (for review see [134]). AQPs are also expressed during parturition and roles have been suggested in the control of pregnant myometrial contractions and cervical ripening [136,137]. However, further work is needed to assess the precise role and regulation of AQPs in late gestation and parturition. Clarifying their expression and regulation in abnormal conditions may help devising novel therapeutic strategies for the treatment of gestation and parturition complications.

7. Assisted reproduction technologies and aquaporins: from gametes maintenance to embryo development

In human assisted reproduction, controlled ovarian stimulation (COS) is aimed to rescue most of the recruited follicles from their natural atresia destiny and to promote their growth. This is achieved in combination of gonadotropin and gonadothopin-releasing hormone (GnRH) analogue. The GnRH analogue, either agonist or antagonist, prevents endogenous luteinizing hormone (LH) surge during COS with gonadotropins until the adequate follicular growth is reached and the final oocyte maturation is triggered by a shot of human chorionic gonadotropin (hCG) which binds to the LH receptors to mimic the LH surge. Alternatively, trigger can be achieved by means of the GnRH analogue if the pituitary down-regulation has been induced by the GnRH antagonist. This approach is particularly useful for women with polycystic ovarian syndrome (PCOS) or for high responders in order to avoid ovarian hyperstimulation syndrome. PCOS is an endocrine disorder with LH/E2 ratio greater than 1 which often leads to anovulatory cycles as well as

prolonged menstrual cycles.

AQPs are expressed in granulosa and theca cells during folliculogenesis [13,93,94,138–141] as well as ovulation process [103], and functional relevance has been suggested in granulosa cells function and oocyte maturation. AQPs are the subject of considerable technological interest for increasing the number of retrieved oocytes and improving cryopreservation outcome of gametes and embryos in medically assisted procreation [142].

In human, two studies have investigated the differences in the expression of the different AQP homologues between women with and without PCOS considering that women with PCOS are often characterized by an exaggerated response to ovarian stimulation in IVF cycles. Lee and coworkers [104] analyzed the mRNA expression of all thirteen human AQP homologues in granulosa cells harvested from the follicular fluid of women undergoing ART. No expression of AQP0, AQP8 and AQP10 was detected in the granulosa cells of the cohort of women and, in overall, neither the number and quality of embryos nor the clinical pregnancy rate appeared to be associated with the transcript levels of any of the ten expressed AQPs. However, lower AQP11 mRNA level was found in granulosa cells from women with PCOS than high responder women without PCOS suggesting specific involvement of this AQP in PCOS mechanism. While the level of AQP7 was inversely correlated with the body mass index, positive association was found between the extent of AQP7 expression and the fertilization rate, a surrogate marker of oocyte competence, suggesting that AQP7 could be a marker for adequate folliculogenesis and healthy oocytes. As AQP1 was found to be negatively associated with the number of retrieved oocytes, it was hypothesized as one of the factors modulating individual ovarian response to exogenous gonadotropin. Since AQP1 expression in granulosa cells was found to be abruptly increased after follicular rupture [3,103] this AQP was surmised to be also involved in the transition of the follicle into a *corpus luteum*. AQP1 may therefore be a surrogate marker of *corpus luteum* function, and the decreased expression of AQP1 in high responders may reflect weaker *corpus luteum* activity in this particular case [3]. A recent work with patients with PCOS and its association with in vitro fertilization-embryo transfer outcomes [143] reported expression of *Aqp8* and *Aqp9* mRNA in ovarian tissues. The transcript level of *AQP8* was found to be higher in women with PCOS undergoing ART treatment than women undergoing ovarian biopsy for oviduct obstruction or for ovarian cysts (control group). The number of oocytes obtained in the group with high *AQP8* expression was significantly lower than that in the group with low *AQP9* expression. As suggested in mouse granulosa cells [141,144], AQP8 may be important in preventing formation of multi-oocyte follicles having a role in GC apoptosis during follicular maturation. The number of high-quality embryos in the high *AQP8* expression group was not different from that of the group with low *AQP8* expression. Interestingly, the pregnancy rate in women with high level of *AQP9* was higher than that in the low *AQP9* expression subjects and the abortion rate in the former was lower than that in the latter. The authors concluded that in patients with PCOS the expression of AQP8 is closely correlated with the occurrence and development of oocytes whereas that of AQP9 is associated with the pregnancy outcome. Although none of the AQPs seems to affect pregnancy rate in humans [104], impaired endometrial receptivity after controlled ovarian hyperstimulation (COH) has been shown to be associated with lower AQP2 levels [145] that are directly controlled by E2 [146] and low AQP2 expression is considered as a potential cause of impaired uterine receptivity [146].

Important information regarding the physio-pathological relevance of AQPs in oocyte maturation and embryo development was obtained from studies with animal models. In rat, expression of AQP7, AQP8 and AQP9 has been reported in follicular granulosa cells where roles were suggested in ovarian antral follicle water permeability [13]. *Aqp8*$^{-/-}$ knockout mice yield more oocytes and more embryos with an increased fertility in female mice [141]. Likely, this occurred through the mechanism of reducing the ovarian granulosa cells apoptosis thus preventing follicular atresia. The increase in mature follicles was associated with the decreased water permeability of ovarian granulosa cells of the AQP8-depleted mice. This observation may help to improve female fertility by reducing granulosa cells apoptosis through AQP8-inhibition. Edashige and coworkers found expression of AQPs in mouse oocytes and embryos [109]. The expression depended on the developmental stages: AQP3 and AQP7 were expressed in oocytes and embryos at all stages examined, whereas AQP8 and AQP9 were expressed only in blastocysts. AQP1, AQP2, AQP4, AQP5, and AQP6 were not detected in any of the stages examined. A study using oocytes induced by COH suggested AQP3 to play an important role in controlling mouse oocyte quality [147]. These authors observed that reduced expression and function of AQP3 in metaphase-II oocytes induced by COH was associated with subsequent low fertilization rate. While several studies suggest a role for AQPs in oocyte maturation and ovulation, further investigation is needed to fully elucidate the role and regulation of AQPs in female gametogenesis, fertilization and pregnancy and their translational value in medically assisted procreation.

8. Concluding remarks and future perspectives

Human fertility, particularly gametogenesis are intricate and highly regulated processes that rely on a variety of factors. Water and solute concentrations within the reproductive tract are pivotal to fertility, on which AQPs seem to play key roles. These membrane proteins are widely distributed throughout both male and female reproductive tracts and their expression patterns are specific to certain reproductive tissues and cells, suggesting a precise function and a complex mechanism of regulation. Indeed, the difference between AQP expression patterns in health and disease can be seen as potential biomarkers for male and female reproductive health. Moreover, several patents (for review [148]) and clinical trials for AQPs modulators and inhibitors [148,149] have been developed, highlighting the relevance of these membrane channels in both health and disease. However, mostly due to the lack of fundamental knowledge on AQPs physiology, none of those has yet been therapeutically used.

As a rule, the female reproductive system has more information available and more studies published when compared to the male reproductive tract. However, the same is not evident when addressing the role of AQPs in human gametes and embryos. This is certainly due to the ethical issues arising when working with female gametes and developing blastocyst in comparison to male gametes. Nevertheless, our knowledge is still incipient and the deepening about functions and regulation of AQPs in the human male and female reproductive tracts and gametes, and their relevance to the reproductive success must be achieved to provide better results when using ARTs and on the improvement of infertility treatments.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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