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Review artilcle

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IN SEARCH OF NEW POTENTIAL MARKERS FOR MALE FERTILITY AND SEMEN QUALITY CONTROL. AQUAPORINS IN REPRODUCTIVE SYSTEM AND METABOLOMIC PROFILING OF SEMEN

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Male infertility is one of the many problems currently faced by science and medicine. Despite intensive research in this area conducted in recent years, the reasons for the lack of the desired pregnancy are often unrecognized. The current standards and general recommendations, including the World Health Organization (WHO) guidelines for diagnostic testing of male reproductive organs and sperm quality analysis, seem to be insufficient. Hence, it has been postulated for years that it is necessary to search for and identify new, unknown factors that significantly affect male fertility, and to define modern indicators/biomarkers that would enable precise determination of male reproductive potential. Among the many interesting recently published data, the information on the identification and expression analysis of aquaporins (AQPs) in the male reproductive system and metabolomic semen analysis is of particular interest. In this review, we will try to solve the question whether AQPs and metabolomic sperm analysis can be the answer to the current needs and whether their measurements may become a useful parameter in the future for determining male reproductive potential.

Key words: *male infertility, water channels, metabolome analysis, spermatozoa, sperm plasma*

INTRODUCTION

The authors of many recently published papers have alarmed about the global increase in fertility disorders observed in recent years. According to the latest data, the problem of infertility currently affects approximately 70 million people (1). Until recently, the barely noticeable and often neglected "male factor" is now responsible for almost 50% of the lack of the expected pregnancy. Many different factors lie at the root of male fertility disorders, ranging from genetic determinants, anatomical abnormalities, medical illnesses, infections, oxidative stress, trauma to lifestyle choices (1-4). Despite many studies and significant advances in knowledge in this field observed in recent years, the causes and mechanism of 30 – 40% of male infertility have not been explained till today (5). Determining the male reproductive potential and starting appropriate therapy, if necessary, is one of the key elements of preventing and treating infertility. Many authors have indicated that the current standards and general recommendations, including the World Health Organization (WHO) guidelines for diagnostic testing of male reproductive organs and sperm quality analysis, seem to be insufficient (1, 6). It happens often in clinical practice that fertilization does not occur despite the lack of anatomical abnormalities and dysfunction of male reproductive system and normal sperm parameters, even with the use of modern procedures such as *in vitro* fertilization and intracytoplasmic sperm injection (6). Hence, it has been postulated for years that it is necessary to search for and identify new, unknown factors that significantly affect male fertility, and to define modern indicators/biomarkers that would enable precise determination of male reproductive potential.

Among the existing evidence-based medicine, the information on the identification and expression analysis of aquaporins (AQPs) in the male reproductive system and metabolomic semen analysis is of particular interest. The recent studies published in this field not only seem to be particularly promising, but also appear to set out a new direction in male infertility research.

AQUAPORINS AND THEIR FUNCTION IN THE MALE REPRODUCTIVE TRACT

More than 30 years have passed since the "birth" of water channel proteins in Romania and the beginnings of the great discovery crowned with the identification of the first aquaporin in the erythrocyte cell membrane, awarded in 2003 with the Nobel Prize in chemistry (7-11). During this time, a number of studies were carried out, as a result of which the structure and role of these "unusual" small proteins were elucidated in detail and extensively described. Currently it is widely accepted that aquaporins belong to a family of integral, transmembrane proteins that form channels selectively permeable to water and a number of small uncharged molecules. AQPs are tetrameric proteins with the molecular weight

form 27 kDa to 37 kDa. In mammals thirteen AQPs have been identified (AQP0-AQP12), which are located in various cell types (12, 13). Until recently, the literature provides a nomenclature according to which AQPs were organized into three groups: classical aquaporins, aquaglyceroporins and unorthodox aquaporins (12). New information regarding phylogenetic distribution and functional properties resulted in supplementing the present nomenclature with one more group now known as Aqp8-type aquaammoniaporins (14) (*Fig. 1*). The so-called classical or orthodox AQPs (AQP0, AQP1, AQP2, AQP4, AQP5 and AQP6) belong to the first group. These aquaporins are described as to be most selective for water, although may also transport other small molecules. The exception is AQP2 that is selective permeable only for water. The second group known as aquaglyceroporins, is composed of four members: AQP3, AQP7, AQP9 and AQP10, which, apart from water, allow the flow of other small molecules, i.e. glycerol, ammonia or urea. Aquaglyceroporins also transport trivalent arsenic and antimony compounds (15). The third group is composed only by AQP8 due to its unique and different phylogenetics from other AQPs (14). This AQP is permeable for water, ammonia, urea and hydrogen peroxide (16, 17). The last group known as unorthodox or superaquaporins include AQP11 and AQP12, which differ in homology from other proteins in this family, and their transport role is still not fully understood. According to some authors these AQPs are responsible for the flow of water and neutral solutions in intracellular compartments (18, 19). It should be noted that the classifications of AQPs presented in this publication is rather conventional and depends on the point of view of researchers. For instance, Ribeiro *et al.* (19) emphasized that AQP3, AQP4, AQP6, AQP8 and AQP9 are known as ammoniaporins because they enable ammonia transport. AQP3, AQP5, AQP8, AQP9 and AQP11 that also facilitate the transport of hydrogen peroxide are often referred to as peroxiporins (19).

Maintaining proper fluid homeostasis within the male reproductive system is one of the key elements conditioning proper development, maturation and function of vertebrate male germ cells (20, 21). Water flow in the mammalian testis from Sertoli cells of the germinal epithelium and from germ cells is necessary to maintain proper environment of the lumen of the seminiferous tubules for spermatogenesis (22). Membrane transport of water is also necessary during the transformation of round spermatids into spermatozoa, which is accompanied by drastic reduction of the cytoplasm and chromatin condensation. Control of fluid composition is essential for transport, maturation and concentration of spermatozoa in the efferent duct and epididymis (23). Finally, appropriate concentration of water is crucial in the regulation of sperm volume, when after ejaculation it enters the uterus and the oviduct (20). The effective flow of water during the formation and maturation of spermatozoa and during their journey from testis and epididymis to the female womb, when they move through media of different osmolality, undoubtedly involves AQPs (24). Hence, the analysis of the

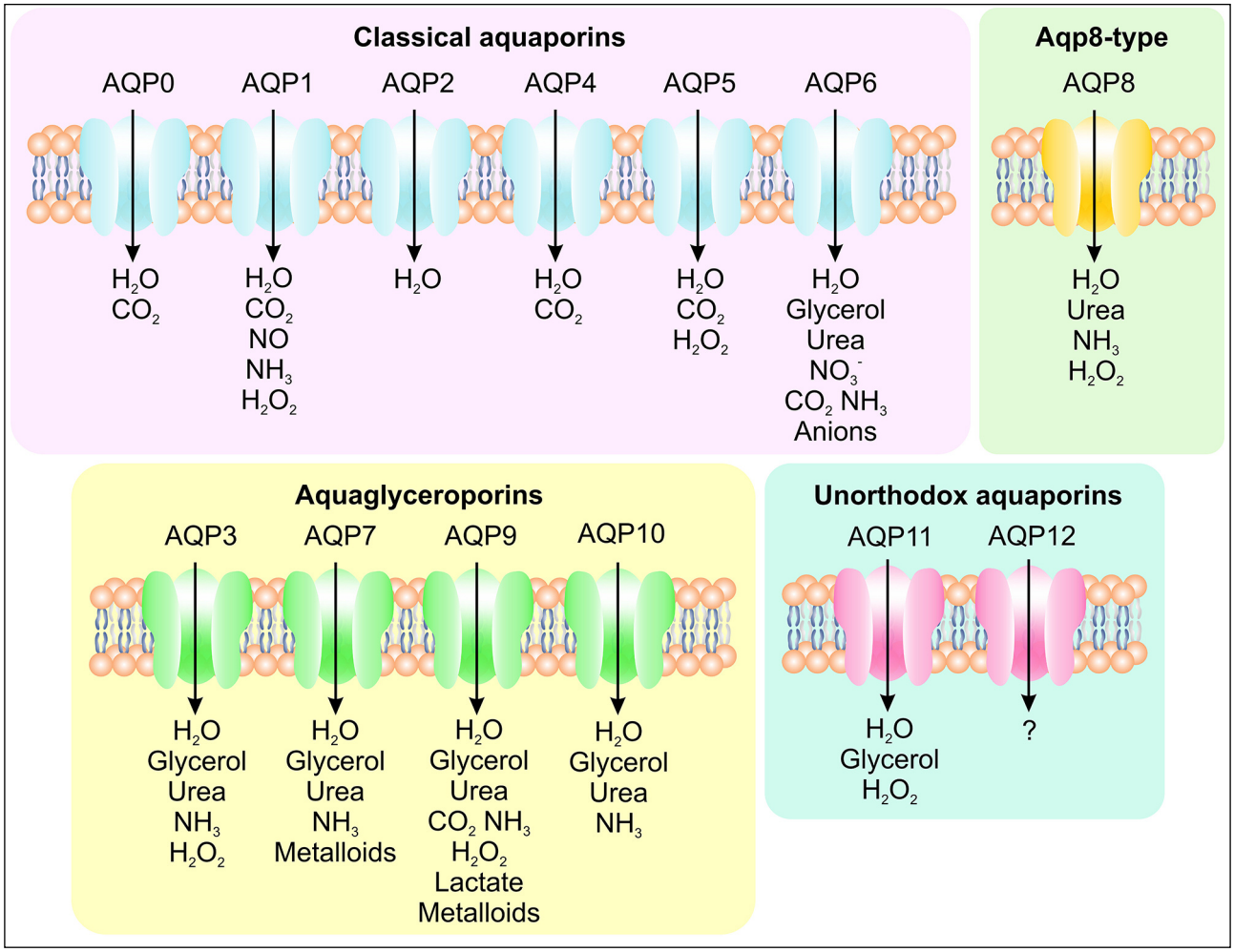

Fig. 1. Aquaporins classification. In mammals 13 aquaporins have been identified and presently they are divided into four groups: classical (orthodox) aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5 and AQP6); Aqp8-type aquaammoniaporins (AQP8); aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10); unorthodox (superaquaporins) aquaporins (AQP11 and AQP12).

location and expression of these proteins within the male reproductive system and sperm, in the context of the increase in fertility disorders observed in recent years, not only arouses huge interest, but also gives great hope for finding new possibilities to increase fertility and treat the causes of male infertility.

AQUAPORINS LOCATION IN MALE REPRODUCTIVE SYSTEM AT A GLANCE

Studies conducted in animals and humans have identified as many as 11 out of 13 mammalian aquaporins (AQP0, AQP1, AQP2, AQP3, AQP4, AQP5, AQP7, AQP8, AQP9, AQP10 and AQP11) in the male reproductive tract. Available data on humans and rodents will be presented and discussed in this chapter. As Carrageta and co-workers (22) note, mouse and rats share some similarities with human, which has proven to be helpful, as the majority of functional and knockout studies have been conducted in these species. Information on the expression of AQPs in male organs of livestock and poultry is included in a separate paper (25).

Aquaporins are ubiquitously expressed in the testis. The cellular localization of AQP0, AQP1, AQP3, AQP4, AQP7, AQP8, AQP9, AQP10 and AQP11 has been identified and described within this portion of the male reproductive tract. In rat

Table 1. Summary of studies illustrating distribution of AQPs in sperm cells and their possible role in maintenance of sperm quality.

Isoform	Localization	Species	Origin/Source	References	Suggested function
AQP1	Not present	Human	Ejaculated sperm	(35, 57, 58)	N/A
	Not present	Mouse	Testis and efferent ducts	(41)	
AQP3	Principal piece of the sperm tail	Human	Ejaculated sperm	(24, 49)	Transport of glycerol into sperm cells for energy production, ROS elimination, regulation of sperm volume and osmoadaptation during the male-female tract transition
	Principal piece of the sperm tail	Mouse	Cauda epididymal sperm	(24)	
AQP7	Sperm head, pericentriolar area of the neck, mid-piece and tail	Human	Ejaculated sperm	(35, 45, 49, 59)	Transport of glycerol into sperm cells for energy production, ROS elimination, controlling sperm motility and reduction of cytoplasm during spermiogenesis
	Round and elongated spermatids and spermatozoa	Human	Testis	(35, 45)	
	Tail of elongated spermatids and spermatozoa	Mouse	Testis	(50)	
	Sperm tail	Mouse	Epididymis	(50)	
	Round and elongated, spermatids, spermatozoa and residual bodies	Rat	Testis	(43, 44, 46-48)	
	Sperm mid-piece	Rat	Epididymis	(48)	
AQP8	Sperm mid-piece and tail	Human	Ejaculated sperm	(35, 49)	ROS elimination, regulation of sperm volume and osmoadaptation during the male-female tract transition, reduction of cytoplasm during spermiogenesis and sperm maturation
	Spermatogonia and elongated spermatids	Human	Testis	(35)	
	Elongated spermatids	Mouse	Testis	(51)	
	Primary and secondary spermatocytes, elongating and elongated spermatids, and residual bodies	Rat	Testis	(46, 47, 51)	
	Cytoplasmic droplet and sperm tail	Rat	Epididymis	(26, 51)	
AQP9	Not present	Human	Ejaculated sperm	(35)	?
	Cytoplasmic components of spermatocytes	Human	Testis	(35)	
	Primary spermatocytes	Rat	Testis	(52)	
	Not present	Rat	Epididymis	(48)	
AQP11	Intracellular structures of the head and sperm tail	Human	Ejaculated sperm	(49)	Transport of glycerol into sperm cells for energy production, ROS elimination, and cytoplasm and organelle elimination during sperm maturation
	Elongated spermatids and residual bodies	Mouse Rat	Testis	(31)	
	End piece of the sperm tail	Rat	Cauda epididymal sperm	(31)	

Abbreviations: N/A - not applicable, ROS - reactive oxygen species.

testis, Sertoli cells express AQP0, AQP3, AQP4, AQP8, AQP9 and AQP11 (26-33). It is noteworthy that the distribution of AQP0 was restricted to a specific region and development stage of Sertoli cells (29). To date, AQP3 and AQP9 have been shown to be located in mice Sertoli cells, while AQP4, AQP8 and AQP9 are present in these sustentacular cells in humans (32, 34-36). In rat Leydig cells, immunolocalization studies revealed the presence of AQP0 and AQP9 (29, 36, 37). AQP1 was present in the rat testis while its expression in humans was limited to vascular endothelial cells (38, 39). Immunolabeling of this protein was also found in epithelial cells of the efferent ducts in humans, rats and mice (38-41). AQP9 was also detected in the efferent ducts of humans and rats (42). Aquaporin 10 was observed in efferent duct epithelium in rats (21). AQP7, AQP8, AQP9 and AQP11 are located in the epithelium of the seminiferous tubule. AQP7 was found in human and rats round and elongated spermatids (43-49) (*Table 1*). In mice, AQP7 expression was restricted to the tails of elongated spermatids (50). AQP8 immunolabeling was observed in human spermatogonia and elongated spermatids (35). In rats, this protein was detected in the primary and secondary spermatocytes, elongating and elongated spermatids and residual bodies (46, 47, 51). AQP8 was also observed in elongated spermatids in mice (51). In rats, AQP9 expression was identified in the primary spermatocytes (52). AQP9 has been rarely found in the germinal epithelium as cytoplasmic components of several spermatocytes in humans (35). AQP11 in rats and mice was found in elongated spermatids and residual bodies (31). To date, AQP1, AQP2, AQP3, AQP5, AQP7, AQP9, AQP10 and AQP11 have been observed in the human, rats or mouse epididymis. The expression of AQP1 was found in nonciliated epithelial cells of the human and rats epididymis (38, 39, 42, 53). AQP2 is expressed in the cauda of the epididymis of young but not adult rats (42). AQP3 was identified in ciliated epithelial cells of the human and rats epididymis (29, 54). In rats, AQP5 is present in the apical plasma membrane of principal cells in the corpus and cauda of the epididymis (30). In the latter species of animals, AQP7, AQP9 were also detected in nonciliated cells and principal cells, AQP10 in endothelial cells of vascular channels, while AQP11 in the microvilli of principal cells in the epididymis (29, 30, 38, 48). In rats, AQP1 expression was found in the plasma membranes of epithelial cells in the ampulla of the vas deferens (38). Immunolabeling of AQP2 was also observed in this segment of reproductive organs. This protein has been reported to be present in the apical and subapical regions of the principal cells (55). In rats, AQP2 was expressed in the apical plasma membrane of principal cells in the ampulla of the vas deferens (56). AQP9 expression was observed, in the proximal vas deferens in humans and rats (28, 37).

Studies carried out to date have demonstrated that spermatozoa express AQP3, AQP7, AQP8 and AQP11, however, cellular localization of these proteins differs between species. It is also worth noting that the presence of AQP1 in the human and mice sperm was excluded (*Table 1*) (35, 41, 57, 58). AQP3 is located in the principal piece of human and mouse sperm tail (24, 49). Other studies have reported that AQP7 in humans is present in the sperm head, pericentriolar area of the neck, midpiece and tail (35, 45, 49, 59). In contrast, this protein in rats was observed in the sperm mid-piece, and in the sperm tail in mice (43, 44, 46-48, 50). AQP8 was found in the human sperm mid-piece, mitochondria and tail (35, 49). Expression of this protein in rats was observed in the sperm tail and cytoplasmic droplet (26, 46, 47, 51). AQP11 is present in intracellular structures of the head and in human sperm tail (49). In rats, AQP11 was observed on the end piece of the sperm tail (31).

The information provided above still does not allow to define a characteristic, repeatable and common location pattern of individual AQPs in the male reproductive tract. It is known, based on many studies carried out to date, that the distribution of aquaporins in organs or systems is similar in mammals. Therefore, it can be assumed that despite the lack of the convincing evidence, the location of these proteins in male reproductive organs will also be similar. Hence, conducting further research in this area on all AQPs, not only seems to be highly justified, but even necessary to fully understand the role of these proteins in shaping male fertility.

AQUAPORINS: A DOOR TO MALE FERTILITY

Despite the fact that more and more authors indicate the wide possibilities related to the analysis of AQPs in the male reproductive tract and sperm, unfortunately the perspective of using the measurements of these proteins in everyday clinical practice still seems to be quite distant. Thus far, very promising, but often fragmentary information on disorders associated with abnormal expression of aquaporins in the male reproductive system, still does not allow for specific management and indication of appropriate therapy. The results of certain mouse knockout studies also raise serious doubts. No impairment of fertility was frequently observed in mice lacking the gene for a particular AQPs and consequently, absence of this protein in the male reproductive system.

In the face of the growing number of men struggling with infertility problems, conducting extensive research concerning the location and influence of various factors on changes in AQP expression seems to be one of the most important and critical steps, leading eventually to increased male fertility. There are still too few original works in the literature that directly focus on changes in AQPs expression in the male reproductive system under the influence of specific factors or during the course of reproductive disorders in humans and rodents. It is noteworthy that many extremely interesting reviews have been published recently, which confirm the belief that extensive research in this area is the right direction of actions taken to “save” male fertility.

Studies on changes in AQPs expression were carried out, among others in varicocele patients. It is widely known that varicocele testes are one of the common causes of decreased fertility in young men. These features include among others exceeding endotubular fluid (ETF) and extracellular matrix (ECM), with subsequent germ cell sloughing and hypospermatogenesis. Progressive damages in varicocele have detrimental effects on semen quality and sperm function (60, 61). A study on 13-18 years old boys suffering from idiopathic varicocele showed that both the location and expression of AQP1 and AQP9 were changing. Compared to the control group, where, as mentioned in the previous chapter, the expression of AQP1 in men is limited to microvessel endothelial cells, this protein in varicocele patients was also observed in the membrane of Sertoli, diploid and haploid germ cells (38). Downregulation of AQP9 was found in boys suffering from varicocele, which in healthy men occurs in primary spermatocytes (60). Therefore, Yeste and co-workers (20) rightly suggested that AQP1 and AQP9 were the basis of the altered water flow in the tubular and interstitial compartments in varicocele patients.

The undescended testis is one of the most common congenital anomalies of the male genitalia, occurring in 1% of boys by the age of one year (62). Cryptorchidism is accompanied by a number of disorders, including changes in the expression and location of AQPs. Studies conducted so far have demonstrated that the distribution and expression of AQP7, AQP8 and AQP9 in cryptic testis are altered only in dogs (63). Will these changes also be observed in humans and other animal species? To date, no research has been carried out in this area.

Increasingly common obesity and metabolic syndrome (MetS) in human population, caused i.a. by improper nutrition

and high-fat diet can also negatively affect aquaporins in the male reproductive system. A study by Marchiani *et al.* (64) on a well-established high fat diet (HFD) rabbit model resembling human MetS showed decreased AQP1 and increased AQP9 expression in the epididymis. The effect of a diet on the expression of AQPs in male genital tract was also observed by Arrighi *et al.* (42). These authors found that underfeeding pregnant rats and its male pups up to 25 days of life, results in a reduction of AQP9 expression in the microvilli of principal and clear cells of the cauda epididymis. It is well known that chronic ethanol consumption is toxic to the entire body, including the male reproductive system. As it turns out, alcohol consumption by men may also affect the expression of aquaporins in genital organs. It has been shown that in adult rats, undergoing a chronic exposure to ethanol, a decrease in AQP9 expression was observed in the microvilli of epithelial cells of the cauda epididymis (65).

Hormones should also be mentioned among many factors that influence AQPs in the male reproductive tract. It is known from the data published so far that estrogens can modulate AQP1 and AQP9 expression, although information available in this regard is contradictory. According to some authors, estrogens can reduced AQP1 and AQP9 expression in the efferent ducts and epididymis and decreased AQP9 level in Sertoli cells in mice (36, 66, 67). On the other hand, AQP1 expression did not change under the influence of estrogens, and AQP9 level decreased only in the efferent ductule in rats (53). Other study indicated that estrogens could increase AQP9 expression in the rat efferent duct even by 300% (68). Actually, there are only few information in the literature regarding the effect of the main male sex hormone - testosterone. The data from works published by Pastor-Soler's group (69) and Hermo *et al.* (29) show that testosterone stimulated the expression of AQP3 and AQP9 in the epididymis. In another study, Ramli *et al.* (70) found out that testosterone stimulate an increase in the expression levels of AQP1, AQP2 and AQP9, mainly at the apical membrane of vas deferens epithelium. Hormonal regulation of AQPs in males is still unclear and requires further research in this area. According to Badran and Hermo (28), both the expression of AQP1 and AQP9 were not modulated by androgens in the adult rat efferent ducts and epididymis.

It is noteworthy that some AQPs also appear to be involved in the proper development of postnatal reproductive organs. Da Silva *et al.* (30) found that AQP2 was absent in the epididymis at birth and during the first and second week of life. However, significant apical AQP2 labeling was observed in epithelial cells of the cauda epididymis in the third and fourth week. Interestingly, Da Silva's groups found that this protein did not occur in the adult rat epididymis. AQP2 was not detected at birth also in the vas deferens. AQP2 was in turn detected in the apical pole of epithelial cells during the first and second postnatal week, and its expression increased in the third and fourth week (30). Changes in AQP7 and AQP8 expression were also observed along with growth and development (46, 47). According to Calamita *et al.* (46), first expression of AQP7 in rat testis was recorded on day 45 after birth, and its level was substantially increasing up to 90 days of life. On the other hand, Kageyama and coworkers (47) found that weak AQP7 expression could be observed already on the 25th day of life in developing rat testis. Data on postnatal changes of AQP8 expression in rat testis indicate that the protein starts to be expressed at 15 or 20 days of life and its expression increases with growth and development (46, 47).

The condition for fertilization is that the sperm produced in testis reaches the egg in the mother's womb. As mentioned in the 2nd section of this manuscript, traveling such a "long" path involves osmolality changes of the surrounding fluid in both the male and female tract (35, 51). Maintaining the appropriate volume, shape and mobility of the spermatozoa in response to changing osmolality of the environment is necessary to retain the proper sperm function. AQPs located in sperm ensure proper transport of water and other small molecules, and thus efficient cell volume regulation during germ cell development and sperm movement (23). Therefore, the proper distribution and expression of these proteins within spermatozoa is a necessary condition for successful fertilization. Using currently available techniques, it is possible to perform a non-invasive detailed assessment of both the expression and location of aquaporins in sperm. Although there is still too little data on AQPs in sperm, a pattern that can be referred to abnormal sperm parameters already emerges from the works published so far (*Fig. 2*). Chen

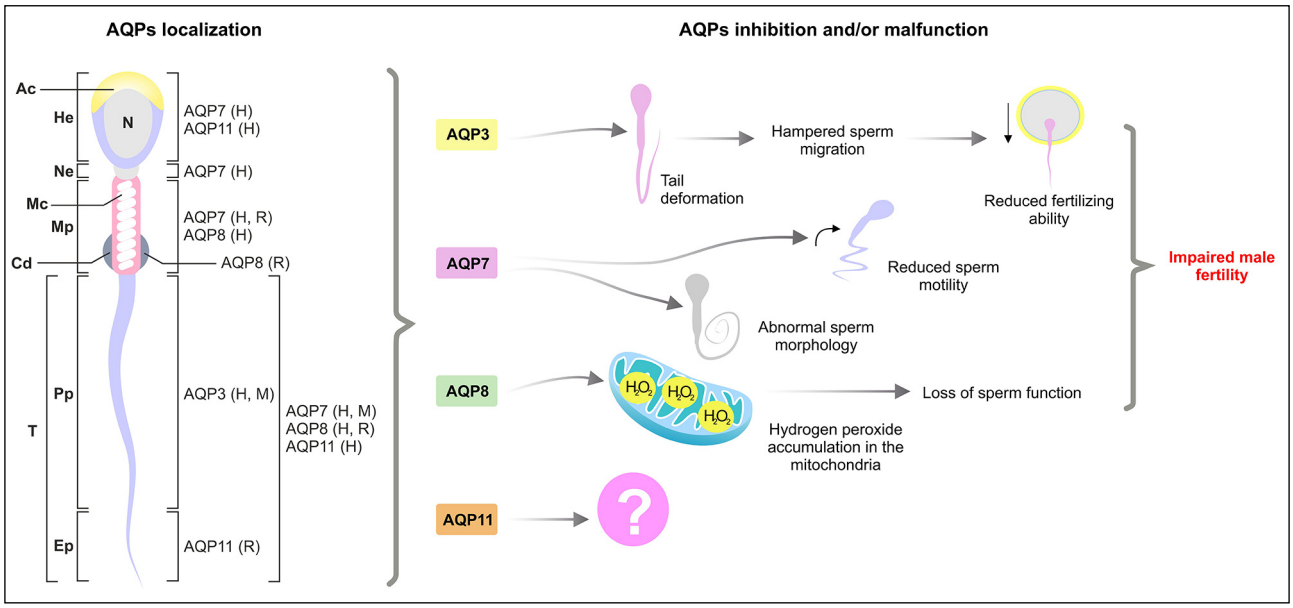

Fig. 2. Diagram showing the distribution of aquaporins (AQP3, AQP7, AQP8 and AQP11) in sperm (modified from 23) and changes in semen quality related to their abnormal expression. *Abbreviations:* H, human; M, mouse; R, rat; Ac, acrosome; Cd, cytoplasmic droplet; Ep, end piece; He, head; Mc, mitochondria; Mp, mid-piece; Ne, neck; N, nucleus; Pp, principal piece; T, tail.

et al. (24) showed nearly a decade ago that AQP3 localized in principal piece of the sperm tail was essential for postcopulatory sperm osmoadaptation and migration. According to these authors (24), lowered expression of this protein did not affect motility activation in response to hypertonicity, but caused increased vulnerability to hypotonic cell swelling, characterized by increased tail bending after entering the uterus. The sperm defect is a result of impaired sperm volume regulation and progressive cell swelling in response to physiological hypotonic stress during male-female reproductive tract transition. The role of AQP3 in maintaining the proper migration was also confirmed by other authors (49, 71, 72). Data on the localization of AQP7 in human sperm vary, although the authors agree that lower expression of this protein is accompanied by decreased sperm quality. A study by Saito *et al.* (45) showed that some infertile patient lacked AQP7 expression in ejaculated sperm, although all fertile men expressed this protein in the tail and mid-piece. According to these authors, the lack of AQP7 expression could be a case of infertility, as decreased sperm motility was observed in men with no expression of this protein. Changes in AQP7 expression and the associated low sperm parameters were also observed by Moretti *et al.* (59). In men with normal semen parameters, AQP7 expression was observed in the pericentriolar area, mid-piece and tail. In men with reduced sperm quality, the expression of AQP7 was weaker and more diffuse in comparison to normal semen. According to these authors, reduced AQP7 expression was accompanied by serious morphological alternations, such as dysplasia of the fibrous sheath combined with abnormalities of the head-neck attachment, because AQP7 observed in the mid-piece and in the cytoplasmic droplet was involved in spermatid volume reduction during the spermatogenesis, and thus in shaping normal sperm morphology (44, 59). AQP3 and AQP7, permeable not only to water, but also other small molecules, enable the transport of glycerol into sperm cells, essential for energy production (35). The reduction in expression or complete absence of one or two of these proteins, and the associated insufficient supply of glycerol, is undoubtedly an additional factor reducing sperm motility and migration. AQP8 was intensively labeled in the mid-piece of the spermatozoa, and apparently was involved in reactive oxygen species (ROS) elimination in the mitochondria (49). High ROS levels in seminal plasma, observed at nearly 30 – 40% infertile objects, determines the negative effects on spermatozoa, such as decreased viability and motility, and more morphological defects in the mid-piece (49, 73). According to Laforenza *et al.* (49), AQP8 deficiency in mitochondrial membranes could lead to impaired efflux of ROS from sperm and loss of its functionality. Interesting information has been provided by the results of the latest research conducted by Pellavio *et al.* (74). These authors showed that human papillomavirus (HPV) infection directly inhibited AQP8 functionality and probably made spermatozoa more sensitive to oxidant stress. According to the latter authors, AQP3, AQP7 and AQP11 could also be involved in ROS elimination from spermatozoa (49). The role of AQP11 in maintaining high semen quality has not been clarified to this day, although Yeung and Cooper (31) reported that this protein could be involved in the end stage of cytoplasm and organelle elimination during sperm maturation. AQP11, also permeable to glycerol, is probably involved in providing the energy required to maintain the proper motility of spermatozoa.

Despite the large amount of evidence that AQPs are necessary for the proper course of many processes in the male reproductive tract and the maintenance of normal sperm parameters, some results from knockout animals studies seem to contradict this. For instance, animal models with a deletion of the AQP1, AQP2, AQP4, AQP7, AQP8 or AQP9 gene do not exhibit severe abnormalities and are still fertile. How to explain this phenomenon? According to Yang *et al.* (75), rarely observed fertility disorders in transgenic animals were most likely associated with a number of processes compensating for the lack of a specific AQP, including changes in the expression of other water and solute transporters.

One of the many important procedures used in human reproduction is semen cryopreservation. Despite the many benefits associated with semen cryopreservation, it is generally recognized that the freeze-thawing process negatively affects the quality of sperm (76). In order to limit cryoinjuries, the cryoprotective agents are applied, such as glycerol. Hence, aquaglyceroporins have generated a lot of interest for a long time as that they are located in the spermatozoa, and facilitate the transport of water and glycerol across the plasma membrane. According to Fujii *et al.* (77), to avoid damage to the membranes and organelles that leads to reduced mortality and fertility after cryopreservation, spermatozoa must control the flow of water and glycerol *via* the plasma membrane and adapt to dramatic osmotic changes during cryopreservation process. This controlled flow of water as well as glycerol seems to be relevant for the survival of cryopreserved cells (20). The results of studies carried out so far have shown that AQP3, AQP7 and AQP11 located in the sperm, are probably a key element in this field, and may be a useful biomarker to predict tolerance to freeze-thawing in spermatozoa (77, 78).

METABOLOMIC PROFILING OF SEMEN

Modern technologies and research methods, including metabolomics and related bioinformatics, as well as mathematical and analytical systems, will enable the analysis of the vast array of chemical compounds that arise in cells. Metabolomics is fairly young and one of the more difficult scientific fields, as it includes a number of molecules of low molecular weight, i.e. organic acids, lipids, carbohydrates, amino acids, nucleotides and hormones. Body fluids are mainly used in metabolomics research to often search for markers of chemical processes occurring in the cell. The assessment of the metabolome is extremely useful and has wide medical applications. One of them is the assessment of the metabolomic profile of male sperm and the search for new biomarkers of its quality. Unfortunately, thus far little information is available regarding this aspect in men, and the available data are usually derived from animal studies. There are also no data in the literature regarding the effect of aquaporins expressed throughout the epithelium of the male reproductive tract on the metabolic profile of seminal plasma. However, it can be assumed that these proteins will be involved mainly in the regulation of seminal fluid volume and concentration of individual metabolites, especially ROS. Although currently the relationship between individual AQPs in the male reproductive system and the metabolome of semen is poorly understood, the analysis of AQP expression in sperm and metabolomic profiling of semen may complement each other and shed new light on the standard assessment of sperm quality used so far.

Seminal plasma is a complex system, contains protein and lipid origin compounds and their metabolites, including amino acids (AAs) and fatty acids (FAs), monosaccharides, nucleosides, minerals, electrolytes, ions, steroid hormones etc. (79, 81). Metabolites formation depends on metabolic pathways, which are varied broadly, and they play significant roles in sperm physiology (82). Paiva *et al.* (83) identified total of 96 metabolites and more than 10 biological pathways in human sperm. Engel *et al.* (6) concluded that the relation between the total concentration of AAs, biogenic amines (BAs), acylcarnitines, lysophosphatidylcholine, phosphatidylcholines,

sphingomyelins and the sum of hexoses with standard spermiogram parameters can be very promising technique to indicate quality of sperm. It was revealed that metabolites in sperm are closely related to sperm motility, whereas those in seminal plasma are closely related to sperm concentration and morphology (6). Another metabolites - triglycerides - concentration showed positive correlation with good semen quality (84-86), as well as the concentration of polyunsaturated fatty acids (PUFAs) are a critical feature for capacitation, related with sperm motility (87, 88) and increased membrane fluidity (89). Another key lipid component is cholesterol, which influx inhibited the acrosome reaction and consequently attenuated fertilization in bovines (90). Furthermore, the cholesterol-to-phospholipid ratio is an accepted marker for semen quality, and it is known to change during capacitation, upon cholesterol efflux or increased cellular phospholipid content, from either extracellular origin or *de novo* synthesis (91). Taking into consideration that lipids plays very important role for semen quality and physiological processes, high concentration of reactive oxygen species reduces semen quality by deteriorating of membrane lipids, proteins, and nuclear/mitochondrial DNA (92). For this reason, not just FAs profile but, also, oxidation process metabolites (e.g. malondialdehyde concentration) become very important as an additional sperm quality marker. Thus, metabolic mechanisms and concentration of physicochemical markers formation in the semen and semen fluid can be directly related to membrane water transport processes, and the latter depend on AQPs and their location in male reproductive tract. Currently, some authors have already pointed out that AQPs and the related water transport are probably one of the key elements influencing the proper concentration and volume of seminal plasma (20, 22). According to Pei *et al.* (93), low semen volume may results in infertility even when other parameters are found to be normal. It is generally known that seminal fluid is mainly derived from the seminal vesicles and prostate, where the presence of AQP1, AQP3, AQP4 and AQP9 has been determined so far (34, 37, 41, 54). Classical aquaporins, which are most permeable for water, seem to be of particular importance for maintaining the correct volume of semen plasma. As mentioned in previous chapters, certain AQPs are also involved in ROS membrane transport and their elimination from spermatozoa (94). AQP8 plays a special role in this respect, although AQP3, AQP7 and AQP11 may also be involved in membrane ROS transport (49, 72). Interestingly, the aforementioned AQPs, on the one hand, protect spermatozoa by ensuring ROS efflux from them, while on the other hand, they contribute to increased ROS concentration in the seminal fluid.

Seminal plasma of human and other species contain large numbers of AAs (6, 95). Velho *et al.* (95) identified 63 seminal plasma metabolites (of which 21 were AAs), demonstrating the relation of their profiles with low and high fertility seminal plasma of bulls. It was concluded that the AAs and peptides are the main compounds of bovine sperm and its seminal plasma (81). However, AAs concentration in semen is not stable, most of the AAs concentration in semen increases after ejaculation, and this is explained by proteolytic activities abundant in semen (81). Oxidation of the AAs in semen, leads to energy supply, causing biochemical activities and reactions in semen (97). Glutamic acid (usually with an active glutamic oxaloacetic transaminase) is the most abundant AAs in seminal plasma (95). Differences between the concentrations of L-leucine and ornithine were found between the fertility groups, as well as the concentration of fructose was correlated with glutamic acid and amino-butyrolactone content (96). It was reported about the different numbers of AAs and peptides, in seminal plasma of bull, where predominant AAs were glutamic and aspartic acid, which are associated with fertility and pregnancy rates (98). Al Ahmad *et al.* (99) reported that seminal plasma supplementation of AAs into semen extenders improved sperm viability, acrosome integrity and membrane integrity of sperm, and, according to Saravia *et al.* (100), post-thaw semen quality. Despite that AAs has an influence on physiological processes *in vivo*, it was reported that addition of glutamine to human semen as a cryoprotectant agent increased post-thaw motility in sperm (101). It was reported that supplementation of extender solutions with glutamine, glycine, and cysteine AAs, increases acrosome and membrane integrity of bualo bull semen (102), and a positive correlation between the concentrations of valine, isoleucine, leucine, and lysine with membrane integrity were established (95). In addition, AAs plays a significant role in protection against oxidative stress (81, 103). Taurine and hypotaurine antioxidant properties were explained by their mechanism of action through binding to the oxidizing agents (103). It was reported that glutamine reduced DNA fragmentation index in donkey semen (104). Also, it was published that L-glutamine and L-proline reduced lipid peroxidation and increased acrosomal integrity of ram sperm (105). Glutathione compound - glutamic acid inhibits cellular damage caused by lipid peroxidation and reactive oxygen species (106). Ugur *et al.* (81) reported that phenylalanine could have an antioxidant effect, and increased concentrations of phenylalanine in seminal plasma may reduce DNA damage caused by oxidative stress. Also, glutamine plays an important role in gene expression redox-potential, and cell integrity (107). In addition, AAs and plasma membrane phospholipids layer, on the sperm surface, protects the sperm cell from cryo-injury (108, 109). Boguenet *et al.* (110) reported about 188 metabolites analyzed, from which 110 were identified in the seminal plasma, and, that in men with severe oligoasthenospermia, there was a significant decrease in 17 phosphatidylcholines and four sphingomyelins, acylcarnitines, with free L-carnitine being the most discriminating metabolite, PUFAs, six AAs (methionine, glutamate, aspartate, tryptophan, alanine, proline), and four BAs (spermine, spermidine, alpha-aminoadipate, serotonin). Finally, despite that AAs are common compounds in seminal fluid and play an important role in the physiological process, the mechanisms of action of AAs in seminal, till now, is unclear (111). In addition to AAs, correct FA profile is very important for a normal spermatogenesis process (112). FAs has a multiple physiological actions, in both cases - as a single molecules and as a components of molecules, ranging from construction of cell membrane composition, to energy suppliers and molecules - transmitters functions (113). FAs are related with the modulation of biomembranes, and PUFAs composition plays an important role in vesiculation, lipid flip-flops, and lipid-protein interactions. By changing the MUFA/PUFA ratio it is possible to modify membrane stability. All these known lipid characteristics are related to cellular and spermatic biology (114). The lipid composition (especially phospholipids) is very important factor, related with spermatozoa functioning (115). FAs profile, especially PUFAs concentration in it, influences sperm maturation, motility, and acrosome reaction (116), and, it was reported that different reproductive pathologies (varicocele, infections, etc.) could be characterised by different FAs composition (117). Particularly, PUFAs showed activity to modulate oxidative stress and the inflammatory processes in spermatogenesis, for this reason, some research is focusing on optimal dietary FAs recommendations could help to develop personalized nutraceutical treatments to improve male reproductive efficiency (118). One from the main factor damaging male reproductive function, is oxidative stress, which can cause male infertility, because of its deleterious effects on the developing germ cells and sperm function (119). It was

reported that ROS species could change cell membranes by reacting with their compounds - PUFAs, by altering their structure, function, and permeability and lead to cell death, abnormality, and motility loss (120). Two biomarkers were suggested to monitor lipid peroxidation in sperm: malondialdehyde (121, 122) and 4-hydroxynonenal (123). It was reported that diet high in unsaturated, saturated, and trans-FAs, showed a negative influence on human and animal semen quality (124, 125). Finally, during spermatogenesis, membrane composition, permeability and fluidity is changing, and this is a normal physiological process. However, abnormal lipid metabolism can lead to spermatogenic dysfunction and cause a male infertility (126-128). Composition of the membrane lipids profile and its correlation with spermatogenesis could lead to the deeper knowledges of mechanisms of action in reproductive tract, and could provide new approaches to the diagnosis and treatment of male infertility (118). Overall, metabolism of lipids during the different phases must be analyzed, as well as optimal lipid origin biomarkers could be suggested, which are associated with the healthy reproductive function.

Localization and AQP expression measurements, both in the genital tract and sperm, as well as semen metabolomic analysis will undoubtedly become not only a useful but even indispensable tool enabling the precise determination of male reproductive potential in the future. A challenge for modern science, but also a response to great needs is filling the gaps in knowledge in this field as soon as possible and introducing new standards and parameters of male fertility assessment to everyday clinical practice.

Abbreviations: AQPs, aquaporins; ETF, endotubular fluid; ECM, extracellular matrix; MetS, metabolomic syndrome; HFD, high fat diet; ROS, reactive oxygen species; AAs, amino acids; FAs, fatty acids; BAs, biogenic amines; PUFAs, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; OS, oxidative stress.

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