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ORIGINAL ARTICLE

CFTR modulates aquaporin-mediated glycerol permeability in mouse Sertoli cells

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Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel that is crucial for fluid homeodynamics throughout the male reproductive tract. Previous evidence shed light on a potential molecular partnership between this channel and aquaporins (AQPs). Herein, we explore the role of CFTR on AQPs-mediated glycerol permeability in mouse Sertoli cells (mSCs). We were able to identify the expression of CFTR, AQP3, AQP7, and AQP9 in mSCs by RT-PCR, Western blot, and immunofluorescence techniques. Cells were then treated with CFTRinh-172, a specific CFTR inhibitor, and its glycerol permeability was evaluated by stopped-flow light scattering. We observed that CFTR inhibition decreased glycerol permeability in mSCs by 30.6% when compared to the control group. A DUOLINK proximity ligation assay was used to evaluate the endogenous protein–protein interactions between CFTR and the various aquaglyceroporins we identified. We positively detected that CFTR is in close proximity with AQP3, AQP7, and AQP9 and that, through a possible physical interaction, CFTR can modulate AQP-mediated glycerol permeability in mSCs. As glycerol is essential for the control of the blood-testis barrier and elevated concentration in testis results in the disruption of spermatogenesis, we suggest that the malfunction of CFTR and the consequent alteration in glycerol permeability is a potential link between male infertility and cystic fibrosis.

Keywords Aquaglyceroporin · CFTR · Glycerol · Sertoli cell · Male infertility

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a transmembrane channel that is responsible for the transport of HCO_3^- and Cl^-. CFTR absence or malfunction is the main cause of cystic fibrosis, one of the most prevalent and lethal monogenic disorders among Caucasians that is estimated to affect nearly 1 in 2500 newborns in Europe and 1 in 3500 newborns in the US [1]. The absence or malfunction of CFTR leads to impaired transport of Cl^- and HCO_3^- and, consequently, impaired fluid and ionic regulation in the affected tissues. Of particular importance, cystic fibrosis greatly affects the male reproductive tract. For instance, CFTR mutations are the main cause of the congenital absence of the vas deferens [2]. In addition and due to the wide expression of CFTR in the male reproductive tract, cystic fibrosis results in a debilitated pH regulation and fluid homeodynamics, which culminates in male subfertility or even infertility [3, 4].

CFTR has been shown to directly impact water movement between the intra and extracellular space, which is

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especially evident in epithelial cells, but CFTR itself is not the primal rout for water across cell membranes [5, 6]. Water diffusion is driven by the establishment of solute gradients that are transported by CFTR, being mostly facilitated by another family of transmembrane proteins, aquaporins (AQPs). AQPs are a family of channel proteins that facilitate the transport of water and small uncharged solutes across biological membranes [7]. Like CFTR, AQPs are also widely expressed in the male reproductive tract, where they assist in the fluid homeostasis essential for spermatogenesis [8]. Alterations in the expression or activity of AQPs are linked with male subfertility or infertility scenarios. Among the AQPs family, aquaglyceroporins are one of the three subgroups essential for the testis function due to their capacity to mediate the movement of glycerol across membrane [9]. Mammals possess four distinct aquaglyceroporins–AQP3, AQP7, AQP9, and AQP10. Glycerol is reported as necessary for spermatogenesis, although only at specific concentrations [9]. Acute exposition to high concentrations of glycerol in the testis can lead to a temporarily arrest of spermatogenesis, while chronic exposure to high concentrations of glycerol may lead to definitive damage in the blood-testis barrier (BTB), death of germ cells, and, consequently, permanent oligospermia or even azoospermia [10].

Compelling evidence suggests that CFTR acts as a molecular partner of AQPs, regulating their activity [3]. For instance, our group described a molecular interaction between CFTR and both AQP4 and AQP9 in primary cultured rat Sertoli cells [11, 12]. Sertoli cells are responsible for the subsistence and regulation of the BTB, conferring crucial mechanical and nutritional support to germ cells [13]. Since AQP9 is reported as crucial for water and solute homeostasis in several tissues, it is also expectable that this AQP regulates water homeostasis in Sertoli cells and consequently inside seminiferous tubules [14–16]. Recently, our group identified for the first time the expression of AQP3 and AQP9 in mouse Sertoli cells (mSCs) [17]. In that study, we showed that both these aquaglyceroporins are responsible for the glycerol entry into Sertoli cells, whereas AQP9 was observed as the main glycerol transmembrane channel. Since CFTR modulates AQP-mediated water transport [18], we hypothesize that CFTR would also modulate the transport of other AQP-mediated solutes. Following these results, we hypothesized that CFTR physically interacts with aquaglyceroporins in mouse testis, specifically in mSCs, where it may directly modulate aquaglyceroporins-mediated glycerol transport. Thus, this study aimed to evaluate the effect of CFTR on the membrane glycerol permeability of mSCs. We also aimed to determine a possible physical interaction of CFTR with mice aquaglyceroporins AQP3, AQP7, or AQP9 (*Aqp10* gene is a pseudogene in mice) [19].

Materials and methods

Chemicals

NZY Total RNA Isolation kit, NZY M-MuLV Reverse Transcriptase, and NZYSpeedy qPCR Green Master Mix were purchased from NZYtech (Lisboa, Portugal), fetal bovine serum (FBS) from Biochrom AG (Berlin, German), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Cell culture and experimental groups

Mouse Sertoli cell line TM4 (mSCs) was purchased from ATCC (Manassas, VA, USA) and cultured as previously described [17]. Briefly, cells were seeded in 75 cm^2 flasks (SPL7005, SPL Life Sciences, Gyeonggi, South Korea) in Dulbecco's modified Eagle medium and Ham's nutrient mixture F12 (DMEM:F12) (1:1) supplemented with 10% fetal bovine serum (FBS), 1.2 g/L sodium bicarbonate, 15 mM 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES), 50 U/mL penicillin, 50 mg/mL streptomycin sulphate, 0.5 mg/mL fungizone, and 50 ug/mL gentamicin. Cells were handled in a laminar flow chamber and maintained in an atmosphere of 5% CO_2 at 37 ºC. Experiments were carried out with n = 6.

Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative Real Time-PCR (qPCR)

The extraction of total RNA from mSCs and mouse testis was conducted using the NZY Total RNA Isolation Kit according to the manufacturer's instructions. The extracted RNA was quantified using a Nanodrop 2000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). Then, RNA was reversely transcribed using the NZY M-MuLV Reverse Transcriptase. The resultant complementary DNA (cDNA) was used with primer sets designed to amplify cDNA fragments of interest. Conventional reverse transcriptase PCR (RT-PCR) was executed to identify *Aqp3*, *Aqp7*, and *Aqp9* mRNA in mSCs and mouse testis. Quantitative Real-Time PCR (qPCR) was performed to evaluate the *Aqp3, Aqp7, and Aqp9* mRNA abundance in mSCs, as previously described [20]. Specific primers were designed for the amplification of the *Aqp3*, *Aqp7*, *Aqp9*, and β-2-microglobulin transcripts (Table 1). qPCR conditions were previously optimized and the specificity of the amplicons was determined by melting curves. Primers' efficiency was set to 90–110%. Amplification conditions: 5 min at 95 °C, followed by 30 or 40 runs of a 3 steps cycle: 10 s at 95 °C; 30 s with a specific temperature for each set of primers, and

Table 1 Oligonucleotides and cycling conditions for PCR amplification of Aquaporin-3 (AQP3), Aquaporin-7 (AQP7), Aquaporin-9 (AQP9), and β-2-microglobulin. C: Number of cycles

Gene	Sequence 5′-3′	Annealing T°	C
Aqp3	FWD: GGACCCTCATCCTTGTGATGTT	63 °C	40
(NM_016689.2)	RVS: TCGTAGTACAGCCCAAAAACAA		
Aqp7	FWD: CCTTGTTACCGTCCTTGGGG	65 °C	37
(NM_001378639.1)	RVS: ATGAAAGTGAACAACCGGGGA		
Aqp9	FWD: CTTTGACTCCAGAAACCTGGG	60 °C	40
(NM_022026.3)	RVS: ACCAGAGTTGAGTCCGAGAGA		
β-2-microglobulin	FWD: GCTTCAGTCGTCAGCATGGC	58 °C	30
(NM_009735.3)	RVS: GGATTTCAATGTGAGGCGGGT		

10 s at 72 °C. β-2-microglobulin transcript levels were used to normalize gene expression levels. Fold variation of gene expression levels was calculated following the model proposed by Pfaffl [21], using the formula $2^{-\Delta\Delta Ct}$.

Western blotting

Proteins from mSCs and mouse testis were extracted using radioimmunoprecipitation assay (RIPA) buffer and then quantified using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA). Western blotting was performed using standard methods [22]. Protein samples (20 µg) were denatured for 10 min at 55 °C. Then, protein samples were fractionated on a 10% polyacrylamide gel from TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad, Hemel Hempstead, UK) and transferred to a low fluorescence polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hemel Hempstead, UK) using a Trans-Blot® Turbo™ System (Bio-Rad, Hemel Hempstead, UK). Membranes were blocked with a solution of 5% bovine serum albumin (BSA) solution in Tris-buffered saline solution. Then, membranes were incubated overnight at 4 °C separately with primary antibodies rabbit anti-AQP3 (1:1000, ab125219, Abcam, Cambridge, UK), rabbit anti-AQP7 (1:1000, NBP1-30,862, Novus Biologicals, CO, USA), mouse anti-AQP9 (1:500, sc-74409, Santa Cruz Biotechnology, Heidelberg, Germany), or mouse anti-CFTR (1:500, sc-376683, Santa Cruz Biotechnology, Heidelberg, Germany). Proteins were separately detected upon incubation with goat anti-mouse (1:5000, AP308P, Merck Millipore, Burlington, MA, EUA) or goat anti-rabbit (1:5000, AP307P, Merck Millipore, Burlington, MA, USA) and reaction with Clarity™ Western ECL Substrate (Bio-Rad, Hemel Hempstead, UK) following manufacturer's instructions. Membranes were read using a Bio-Rad ChemiDoc XR (Bio-Rad, Hemel Hempstead, UK).

Immunofluorescence

Immunofluorescence was performed to further observe the cellular localization of CFTR, AQP3, AQP7, and AQP9. Cells were cultured on glass coverslips inside 12-wells plates until reaching 70% confluence. Then, mSCs were fixed with 4% paraformaldehyde for 60 min and permeabilized by incubation with a solution of 0.1% Triton X-100 in PBS, both at room temperature. Cells were blocked in 0.1% gelatine in PBS for 60 min at 37 °C and incubated with primary antibodies rabbit anti-AQP3 (1:50), rabbit anti-AQP7 (1:50), mouse anti-AQP9 (1:50), or rabbit anti-CFTR (1:50, ACL-006, Alomone, Jerusalem, Israel), overnight at 4 °C. Cells were incubated without primary antibodies as the negative control. Afterward, cells were washed and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:1500, A-11001, Thermo Fischer Scientific, Waltham, MA, USA) or Alexa Fluor 488 goat anti-rabbit IgG (1:1500, A-11008, Thermo Fischer Scientific, Waltham, MA, USA) for 1 h at room temperature. Coverslips were mounted and nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). For observation by confocal microscopy, all preparations were also co-incubated with anti- Na^+/K^+ ATPase antibody (1:50, [EP1845Y]—Plasma Membrane Marker—Alexa Fluor 647, ab198367, Abcam, Cambridge, UK), overnight at 4 °C. Fluorescence was observed in a Zeiss LSM 510 META confocal microscope. Images were obtained with Zen Black Software (Carl Zeiss, Jena, Germany). For visualization of the proximity ligation assay slides, fluorescence was observed in a Nikon Eclipse E400 microscope equipped with a Y-FL Epi-Fluorescence Attachment and HB-10103AF Super High-Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan). Images were obtained with NIS-Elements Imaging Software (Nikon, Shinagawa, Tokyo, Japan).

Stopped-flow light scattering

Stopped-flow light scattering was performed to measure the membrane permeability of mSCs to glycerol, following a previously described method [17]. Cultured mSCs were detached with trypsin and centrifuged at 300 ×*g* to obtain a pellet. Then, cells were resuspended in an isotonic medium (300 mOsm, pH 7.4, constituted by 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, and 1 mM PMSF) and left for 10 min to reach the equilibrium. Cells

were checked under light microscopy for homogeneity and spherical shape in suspension. The diameter of cells was measured with pictures obtained by light microscopy using the ImageJ software.

Glycerol permeability was measured in mSCs after 15 min incubation with a specific CFTR inhibitor (1 μM CFTRinh-172), a general inhibitor of mediated transport of glycerol (0.7 mM phloretin) or the vehicle (DMSO) [23, 24]. Stopped-flow light scattering experiments were performed on a Stopped-Flow SX20 apparatus (Applied Photophysics, Leatherhead, Surrey, UK), which has a 1 ms dead time and temperature controlled at 25 °C. The osmotic shock was performed with a hyperosmotic glycerol solution (isotonic medium supplemented with 500 mM glycerol). A minimum of seven runs were analyzed in each experimental condition. In each run, 100 μL of cellular suspension was mixed with an equal amount of hyperosmotic glycerol solution to reach inwardly directed gradients of solute. In the first instance, the hyperosmotic solution leads to fast cell shrinkage due to water outflow. Then, a glycerol influx occurs in response to its chemical gradient, which is followed by water influx and subsequent cell re-swelling. The tracing of the curves obtained agree with the specifications of the equipment used (SX20). The kinetics of cell re-swelling were measured from the time course of 90° scattered light intensity at 450 nm until a stable light scatter signal was attained. Glycerol permeability (P_{gly}) was calculated as:

$$P_{gly} = \frac{1}{\left(\frac{A_0}{V_0} * \tau\right)}$$

where τ is equal to $1/k$ which is the exponential rate coefficient (s^{-1}) obtained by fitting the light scattering signal of glycerol influx; A_0/V_0 is the initial cell area to volume ratio. Isotonic solution osmolarity was determined from freezing point depression on an Osmometer Basic (Löser, Berlin, Germany) using standards of 300 and 900 mOsm.

Proximity ligation assay

Proximity ligation assay (PLA) was carried out to study a potential physical interaction of CFTR with AQP3, AQP7, and AQP9. To do so, it was used a Duolink® In Situ Red Starter Kit Mouse/Rabbit (DUO92101) and the technique was performed using the manufacturer's instructions. Briefly, cells were cultured on glass coverslips inside 12-wells plates until reaching 70% confluence. Then, mSCs were fixed with 4% paraformaldehyde for 60 min and permeabilized by incubation with a solution of 0.1% Triton X-100 in PBS, both at room temperature. Cells were incubated with the kit's Blocking Solution for 60 min at 37 °C and then with antibodies from two different species (mouse and rabbit) overnight at 4 °C. For CFTR and AQP3 interaction, the antibodies used were rabbit anti-AQP3 (1:50) and mouse anti-CFTR (1:50). CFTR and AQP7 interaction was studied with mouse anti-CFTR (1:50) and rabbit anti-AQP7 (1:50) whereas CFTR and AQP9 interaction was studied with mouse anti-AQP9 (1:50) and rabbit anti-CFTR (1:50). Then, cells were incubated with the Duolink PLUS and MINUS probes for 1 h at 37 ºC. Ligation of the probes was conducted by incubating the cells with a ligase for 30 min and with a polymerase for 100 min, both at 37 °C. Finally, cells were covered with Duolink® In Situ Mounting Media with DAPI and analyzed in a Nikon Eclipse E400 microscope equipped with a Y-FL Epi-Fluorescence Attachment and HB-10103AF Super High-Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan). Images were obtained with NIS-Elements Imaging Software (Nikon, Shinagawa, Tokyo, Japan).

Statistical analysis

Experimental results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 8 (GraphPad software, USA). The statistical significance of the mRNA abundance was assessed with the Kruskal–Wallis test followed by Dunn's multiple comparison test since the data do not follow a Gaussian distribution according to the Shapiro–Wilk normality test. The statistical significance of the glycerol permeability groups was assessed by one-way ANOVA, followed by Tukey's multiple comparisons test, with single pooled variance. $p < 0.05$ was considered significantly different.

Results

Aqp3, *Aqp7*, and *Aqp9* mRNA and proteins are expressed in mouse testis and mSCs

The presence of aquaglyceroporins in the male reproductive tract and their role in male fertility remains to be fully elucidated. In our previous study [17], we were able to identify, for the first time, the expression of *Aqp3* and *Aqp9* in mSCs. Herein, we were able to identify the expression of *Aqp3*, *Aqp7,* and *Aqp9* in mouse testis (as a positive control) and in mSCs by RT-PCR (Fig. 1a). Then, the relative abundance of mRNA expression of the different AQPs was accessed by qPCR. In mSCs, the relative abundance of *Aqp3* mRNA (0.0024 ± 0.0014 arbitrary units) was similar to the one of *Aqp7* mRNA (0.0039 ± 0.0013 arbitrary units). However, *Aqp9* mRNA relative abundance (0.018 ± 0.0018 arbitrary units) was approximately sixfold greater than both the other AQPs (Fig. 1b). Protein expression of AQP3, AQP7, AQP9, and CFTR was also confirmed in mSCs and mouse testis

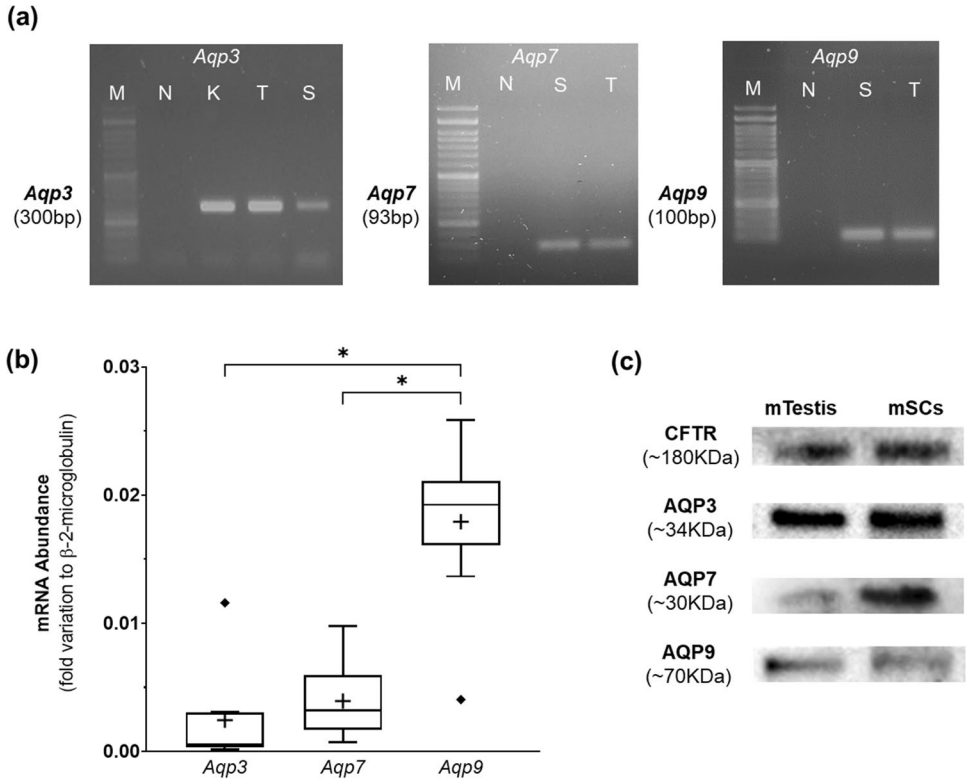

Fig. 1 Identification and quantification of mRNA abundance of *Aqp3*, *Aqp7* and *Aqp9* and assessment of AQP3, AQP7, and AQP9 protein expression in mouse Sertoli cells (mSCs). **a** Representative RT-PCR experiment for identification of *Aqp3, Aqp7, Aqp9* mRNA on mouse testis and mSCs. Mouse testis and kidney cDNA was used as positive control. **b** Relative abundance of *Aqp3*, *Aqp7*, and *Aqp9* mRNA in mSCs. Significantly different results ($p < 0.05$) are indicated as: *. **c** Representative blots of AQP3, AQP7, and AQP9 protein expression in mSCs from Western blot technique. Results from mRNA abundance experiment are expressed as Tukey boxplot ($n = 6$) with 3 repetitions for each gene studied. *N* negative control, *K* kidney, *T* testis, *S* Sertoli cells

through the Western blot technique (Fig. 1c). The blot band representative of AQP9 expression had an expected molecular weight of ~ 33 kDa. Our results show a band at a higher molecular weight (~ 70 kDa) that is due to AQP9 dimerization, process that is in common with AQP9 [25].

CFTR, AQP3, AQP7, and AQP9 are expressed in the plasma membrane of mSCs

The localization of the different AQPs and CFTR in mSCs was assessed by immunofluorescence through confocal microscopy. CFTR (Fig. 2a), AQP7 (Fig. 2c), and AQP9 (Fig. 2d) presented a wide expression in all the cell's frame. AQP3 also presented diffuse expression throughout the cells frame, however, together with CFTR, also presented expression in the cytoplasmatic perinuclear region; yet this information does not allow their specific localization (Fig. 2a, b).

Images resulting from the z-stacks from confocal microscopy further support the presence of CFTR (Fig. 3a), AQP3 (Fig. 3b), AQP7 (Fig. 3c), and AQP9 (Fig. 3d) in the plasma membrane by co-localizing with specific plasma membrane protein Na^+/K^+ ATPase (Fig. 3) although, to some extent, some expression was observed in the cell's cytoplasm, particularly as observed for CFTR and AQP3, which show a signal in the perinuclear region of mSCs. No labeling was observed in negative control preparations where the primary antibodies were omitted (data not shown).

CFTR inhibition decreases glycerol permeability in mSCs

It is known that CFTR can modulate water movement across the plasma membrane by creating a gradient through Cl^- and HCO_3^- transport [26]. To study if CFTR malfunction can affect AQPs glycerol permeability, mSCs were treated with a specific CFTR inhibitor (CFTRinh-172) and then exposed to an osmotic stress created by a hyperosmotic solution of glycerol as described in Materials and Methods. Cells were also incubated with phloretin, a non-specific inhibitor of glycerol facilitated transport across membranes known to decrease cellular glycerol permeability [27]. Glycerol permeability was obtained by analyzing the variation in light scattering intensity associated with the osmotic influx of water following the entry of glycerol into cells that moved based on its chemical gradient. Before the permeability experiment, cells were suspended in osmotic equilibrium in a 300 mOsm solution and treated with CFTRinh-172, phloretin, or the vehicle (DMSO). Then, the diameter of cells was measured by optical microscopy (no difference was found between the treatments, data not shown). The mean diameter of mSCs was 21.50 ± 0.32 µm. Scattered light intensity representative curves of mSCs from the control, CFTRinh-172-treated, and phloretin-treated groups are illustrated in Fig. 4a, b, c, respectively). We further observed that the inhibition of CFTR by CFTRinh-172 led to a 30.6% reduction in glycerol permeability when

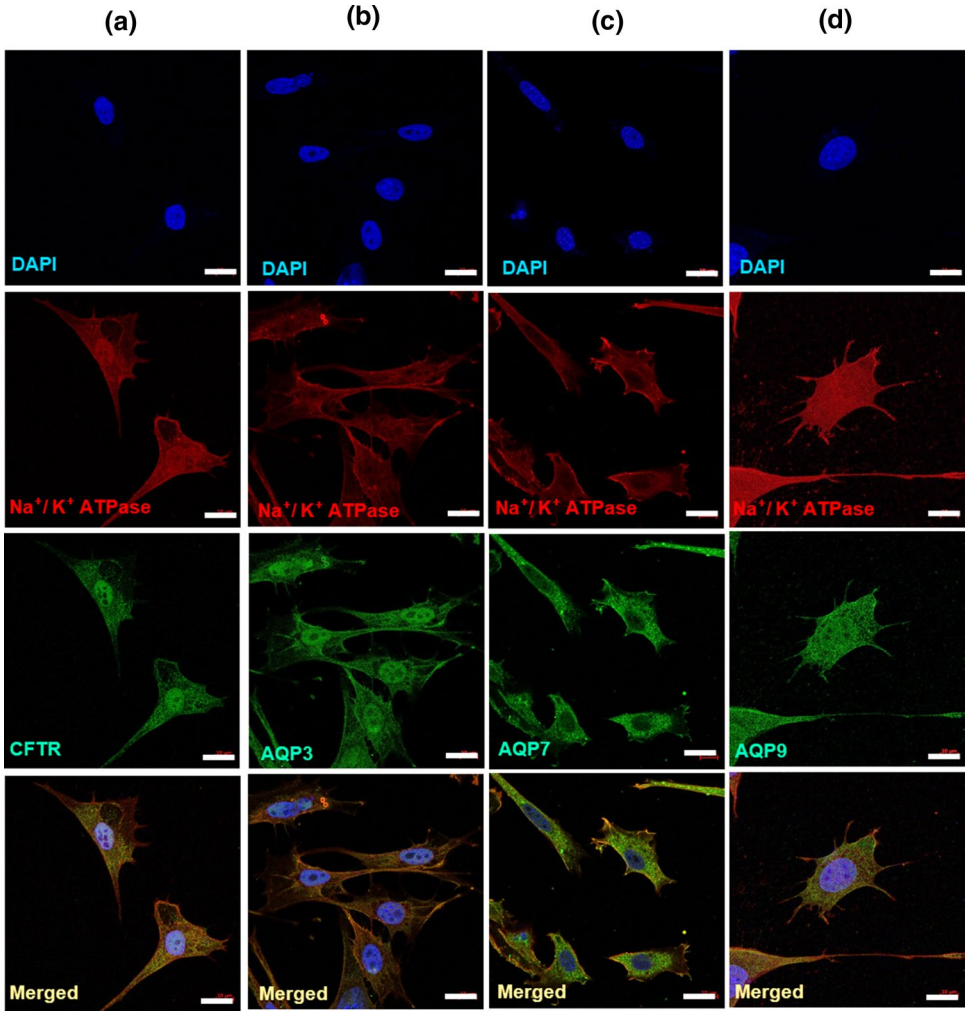

Fig. 2 Immunofluorescence analysis of CFTR, AQP3, AQP7, AQP9 and membrane marker Na^+/K^+ATPase, in mouse Sertoli cells (mSCs) by confocal microscopy. Immunolabeling of Na^+/K^+ATPase (red) CFTR (**a**), AQP3 (**b**), AQP7 (**c**), and AQP9 (**d**) (green) is seen in mSCs. Negative controls were carried out without primary antibody for each protein. The nuclei of cells were stained blue with VECTASHIELD® Antifade Mounting Medium with DAPI. White bar, 25 µm

compared to the control group ($5.04\times10^{-6}\pm4.9\times10^{-7}$ cm/s and $7.26\times10^{-6}\pm5.9\times10^{-7}$ cm/s, respectively, $p<0.05$) (Fig. 4d). In addition, phloretin reduced glycerol permeability in mSCs ($3.96\times10^{-6}\pm4.8\times10^{-7}$ cm/s, $p<0.01$) by 54.5%, when compared with the cells of the control group.

AQP3, AQP7, and AQP9 are in close proximity with CFTR in mSCs

CFTR can modulate the function of various proteins [28] and even physically interact with and control the function of other membrane transporters [29]. To shed light on the mechanism by which the noted decrease in glycerol permeability after CFTR inhibition was caused by physical modulation, we used a protein ligation assay (Duolink Proximity Ligation Assay). The results obtained show that CFTR is adjacent to AQP3, AQP7, and AQP9 in mSCs (Fig. 5). The vast majority of CFTR and AQP3 interactions was localized above the DAPI-marked nucleus but few were also present in other zones of the cell's membrane (Fig. 5a). CFTR and AQP7 or CFTR and AQP9 interactions are evident throughout the mSCs membrane (Fig. 5b, c, respectively). These results are in concordance with the ones obtained from the immunofluorescence preparations that indicated an expression of CFTR, AQP7, and AQP9 in the plasmatic membrane of mSCs. On the other hand, CFTR interaction with AQP3 in the perinuclear region also indicates the presence of CFTR in intracellular membranes. Not only the results herein obtained from confocal microscopy point into that, but it also has already described in previous works [30, 31]. No labeling was observed in negative control preparations where the primary antibodies were omitted (Fig. 5d).

Discussion

Solute and fluid regulation in the male reproductive tract is essential for the production, movement, and maturation of competent spermatozoa [4]. CFTR is regarded as one of the major regulators of fluid dynamics and ion homeostasis in the male reproductive tract due to its direct and indirect activity on the modulation of the function of multiple

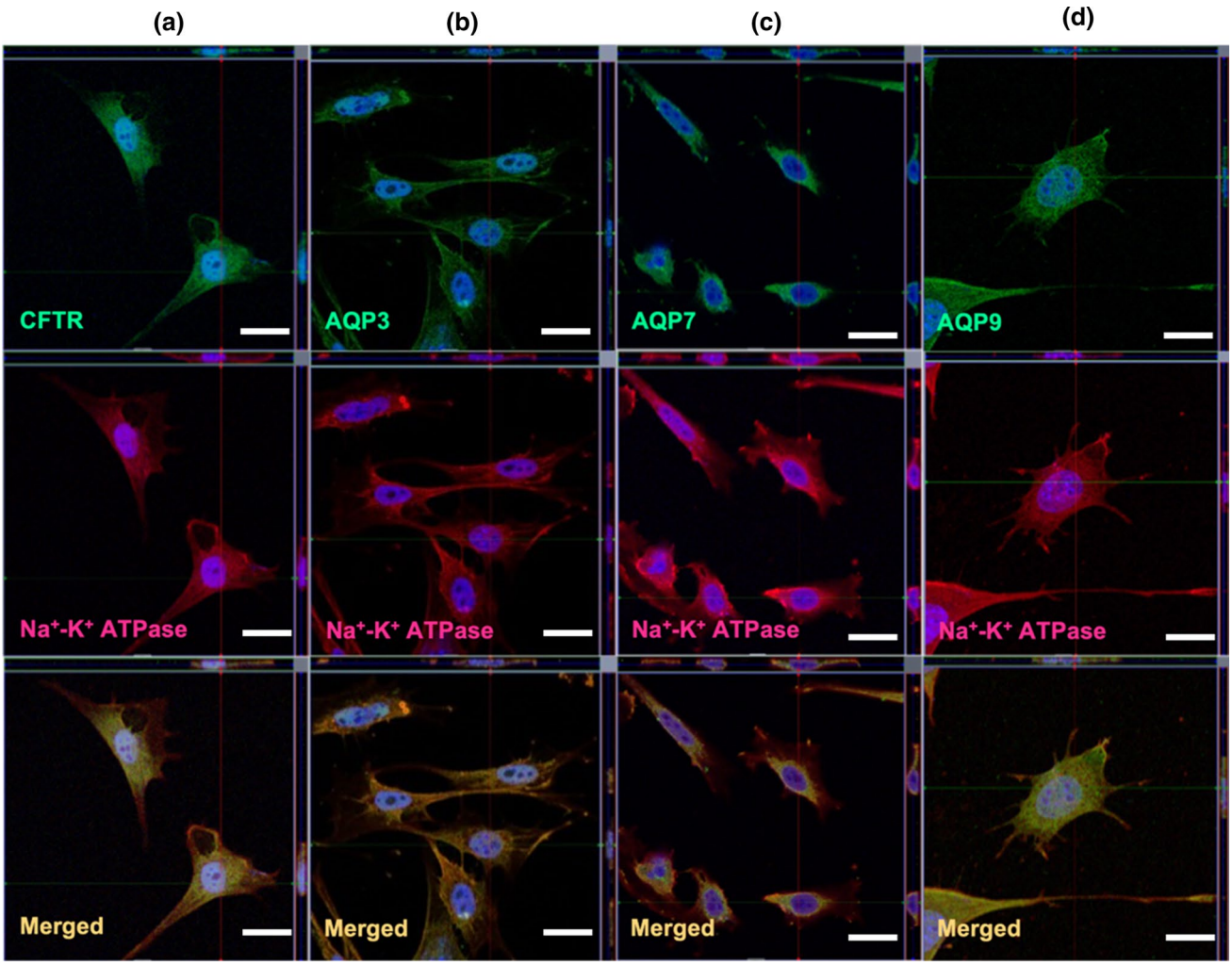

Fig. 3 Immunofluorescence analysis of (co-)localization of CFTR, AQP3, AQP7, and AQP9 with membrane marker Na^+/K^+ ATPase in mouse Sertoli cells (mSCs) plasma membrane by confocal microscopy Ztacks. Immunolabeling of Na^+/K^+ ATPase (red) and CFTR, AQP3, AQP7, and AQP9 (green) is seen in mSCs as well as, its co-localization (yellow) is also represented in z-stacks. The nuclei of cells were stained blue with VECTASHIELD® Antifade Mounting Medium with DAPI. White bar, 25 µm

proteins [28], including other membrane transporters [29]. Aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10) are a subgroup among the AQPs family with the specific biophysical property of allowing facilitated transport of glycerol in addition to other solutes and water. Glycerol is usually described as a non-toxic molecule, which is important for the structure of several lipids and, at adequate levels, for healthy spermatogenesis [9]. Although glycerol may be used as a metabolic substrate, high concentrations and its overaccumulation in the testis have adverse effects which could lead to definitive oligozoospermia and even azoospermia [10]. Taking this into consideration, this work intended to unravel if CFTR can modulate aquaglyceroporin glycerol permeability and if a physical interaction between CFTR and aquaglyceroporins occurs in mSCs. This would shed light on the direct involvement of CFTR in aquaglyceroporin-mediated solute permeability and on the potential importance of this interaction in male fertility.

To accomplish the objective of this work, CFTR, AQP3, AQP7, and AQP9 gene and protein expressions were evaluated in mSCs. To the best of our knowledge, the expression of AQP7 in mSCs is herein described for the first time. A previous report had already described the presence of AQP7 in rat's seminiferous tubules [32] but no information was available on mSCs. Moreover, qPCR results indicate that *Aqp9* seems to be the most predominantly expressed gene of all aquaglyceroporins found in mSCs. That is in concordance with a previous work from our group that suggested that AQP9 is the most important aquaglyceroporin in mSCs [17]. CFTR was also previously reported to be expressed in testis, where it was mainly identified in SCs [33]. In humans with SC-only syndrome, CFTR is present throughout the

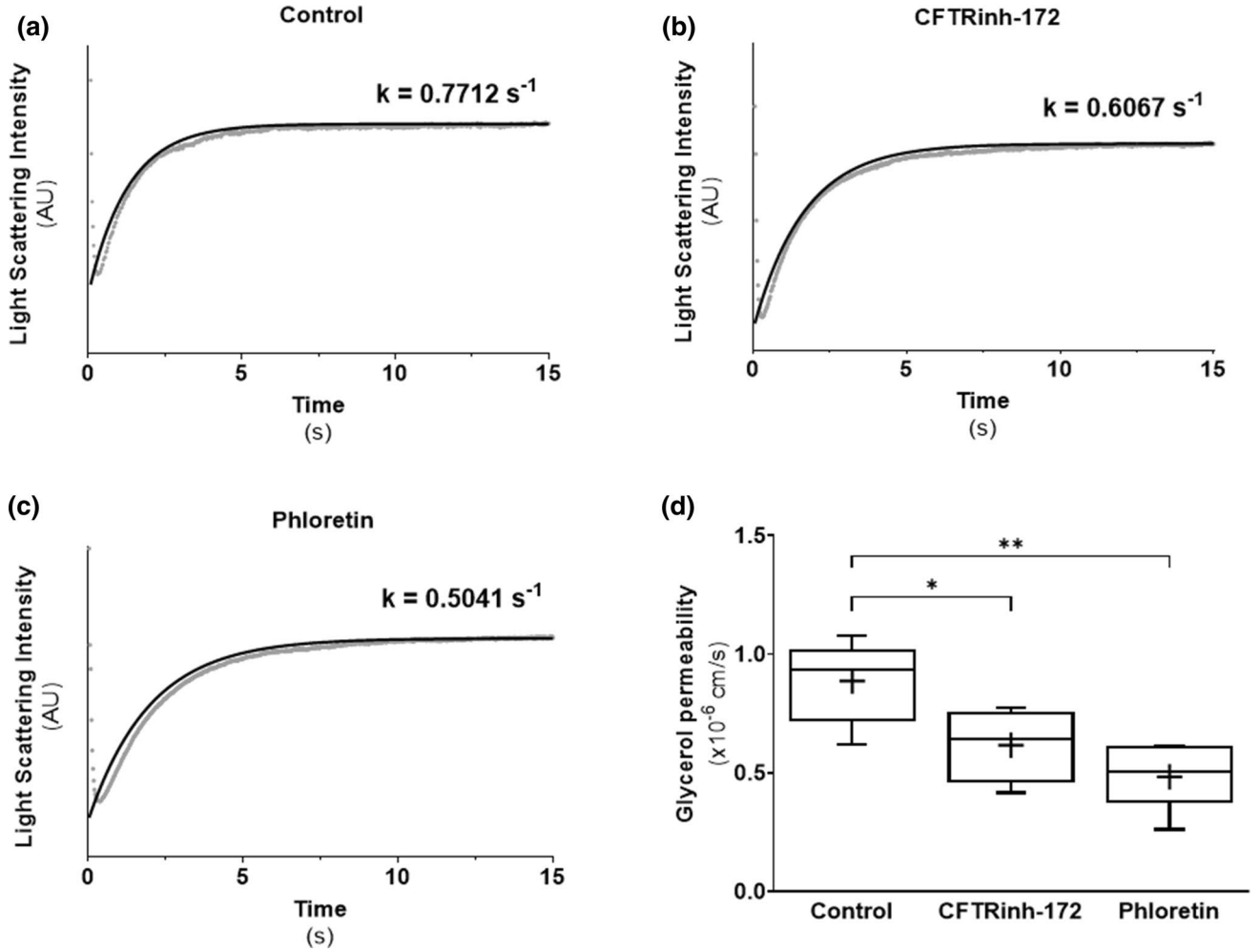

Fig. 4 Effect of 1 μM CFTRinh-172 or 0.7 mM phloretin on mouse Sertoli cells (mSCs) glycerol permeability. Representative light scattering intensity curves of control group, $k = 0.7712\ s^{-1}$ (**a**); group treated with CFTRinh-172, $k = 0.6067\ s^{-1}$ (**b**); and group treated with phloretin, $k = 0.5041\ s^{-1}$ (**c**) are represented. Data resulting from the calculation of mSCs glycerol permeability (**d**) are expressed as Tukey boxplot. The experiment was carried out with a n = 6 with a minimum of 7 repeated curves for each condition and n, presented curves are the average of the repetitions of the first n. Significantly different results are indicated as (*) when $p < 0.05$ and (**) when $p < 0.01$

SCs' plasma membrane, with increased expression in the basal region [34]. The expression pattern in healthy individuals, however, is yet to be clarified. Our results suggest that CFTR is expressed throughout the plasma membrane of mSCs but also in the membrane of cytoplasmatic organelles. Likewise, we show that AQP3, AQP7, and AQP9 expression patterns are also throughout the plasma membrane and in cytoplasmatic region. CFTR is known to be found in multiple subcellular localizations, namely in Golgi apparatus, phagosomes, lysosomes, endoplasmic reticulum (for review see: [30]). In the case of AQPs, their subcellular localization has been a topic of great interest in the past decades, with some showing the ability to move from the cytoplasm to the plasma membrane and vice-versa in response to external stimuli [35, 36]. However, this was only explored in orthodox AQPs (AQP2 and AQP4) and not in aquaglyceroporins. Our results also show the presence of CFTR and AQP3 in the cytoplasmatic perinuclear region. Human CFTR expression was previously described in perinuclear regions [31]. In the case of AQP3, this interesting expression pattern was already described by Mirabella et al. in dog testis [37]. In gonadotrophs of the tree frog, an AQP3 homolog (AQP-h3BL) was also found in the nuclear membrane and cytoplasm. The authors of this study hypothesized that these subcellular localizations were a "storage area" of the AQP3 homolog, which can be translocated as a response to volume regulation and osmotic adaptation of secretory granules [38]. Taken together, we hypothesize that AQP3 could be involved in the secretion of vesicles since SCs are known for secreting many substrates and exosomes

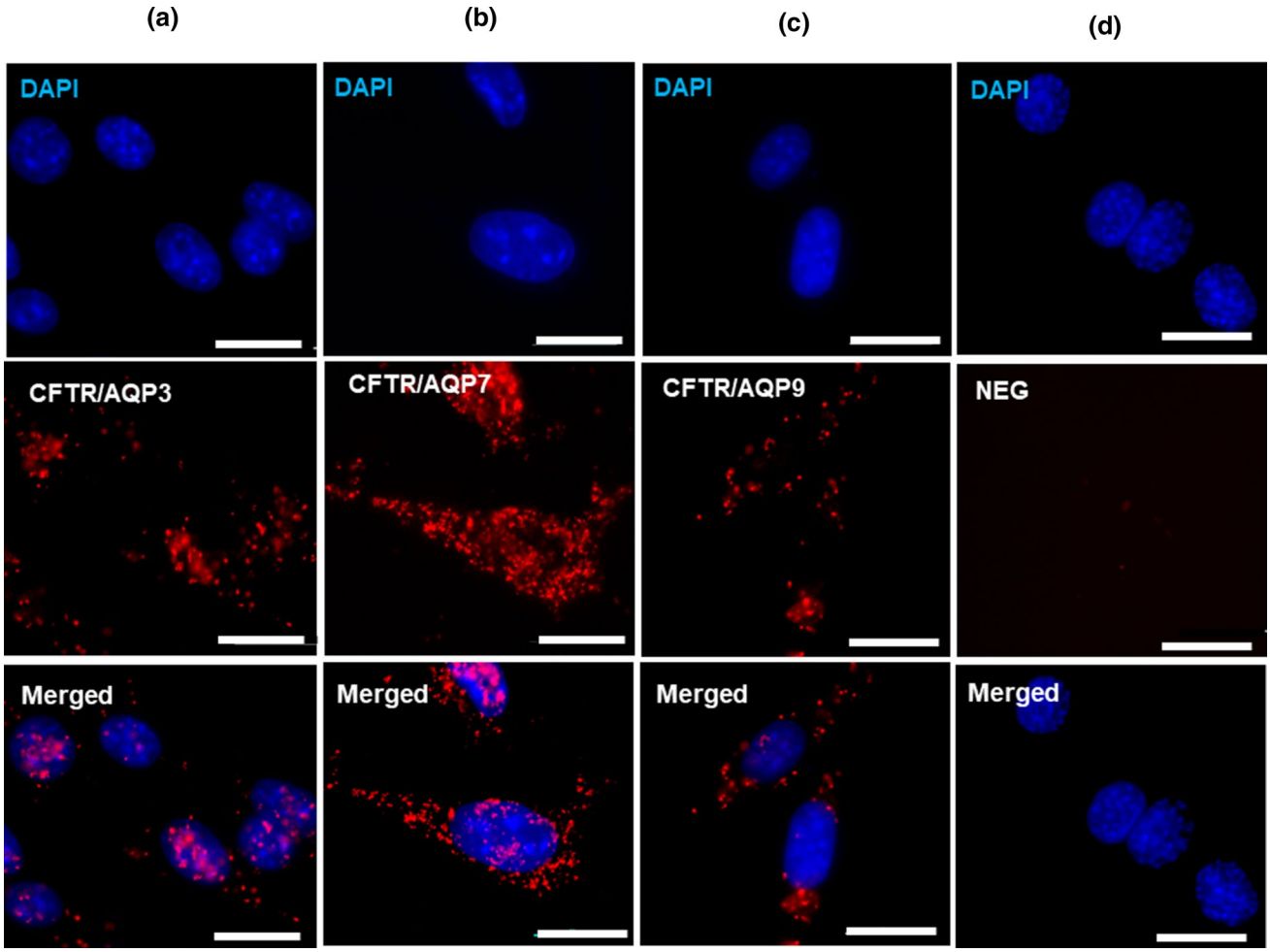

Fig. 5 Molecular interaction between AQP3, AQP7 and AQP9) and CFTR as determined by proximity ligation assay. Red signal is representative of each interaction between CFTR and AQP3 (**a**), CFTR and AQP7 (**b**), and CFTR and AQP9 (**c**). Negative control (**d**) was carried out by omitting primary antibodies. The cells nuclei were stained with Duolink® In Situ Mounting Media with DAPI. White bar, 10 µm

into the seminiferous tubule's lumen [39, 40]. Despite being an interesting proposition, more studies are needed to fully unravel the role of AQP3 in mSCs. However, this indicates that different aquaglyceroporins are specialized in different processes of water and solutes diffusion. It was described that AQP7 are specialized in the outward diffusion of glycerol in mice adipocytes [41], whereas AQP9 were found to have a greater role on the inward glycerol diffusion in murine hepatocytes [24]. The presence of the two AQPs in mSCs could indicate the importance of glycerol homeodynamics in these cells. On the other hand, it is also known that AQP7- and AQP9-*knockout* mice are fertile, demonstrating the ability of other AQPs to compensate the absence of one single aquaglyceroporin [42, 43].

In SCs, CFTR is mainly described as an HCO_3^- transporter, which highlights its function in pH regulation [44]. CFTR, however, may act as an HCO_3^-/Cl^- exchanger, which generates an osmotic driving force for water movement that occurs either paracellularly or through AQPs [45]. One of the earliest pieces of evidence concerning the molecular partnership between CFTR and AQPs was observed in the airway epithelial cells of human lungs. In this study, Schreiber et al. [46] described that CFTR promotes AQP3-mediated water transport. Herein, our results show that CFTR modulates aquaglyceroporin permeability to glycerol. CFTR inhibition by its specific inhibitor CFTRinh-172 caused a reduction of 30.6% in membrane glycerol permeability in mSCs. On the other hand, inhibition conferred by phloretin, a general aquaglyceroporins inhibitor, presented an even higher effect (-54.5%) on glycerol permeability, suggesting that CFTR is only partially able to modulate aquaglyceroporins permeability. Compelling evidence suggests that the functional modulation conferred by CFTR on AQPs could be due to the occurrence of physical interaction

in different tissues of the male reproductive tract, although the mechanisms are still unclear. For instance, both CFTR and AQP9 are co-localized in the luminal membrane of the principal cells in the epididymis of both rats and humans [18, 47]. In addition, our group previously observed that CFTR physically interacts with AQP4 and AQP9 in cultured rat primary SCs [11, 12]. In agreement with our previous results, Pietrement et al. observed that CFTR co-immunoprecipitates with AQP9 in principal cells of the epididymis and vas deferens of rats [48]. This study also demonstrated that the inhibition of CFTR decreased glycerol permeability, concluding that CFTR modulates AQP9 activity on the epididymis and vas deferens. AQP9 is the major aquaglyceroporin isoform expressed in mSCs. Herein, we demonstrate through the proximity ligation assay that, under native cellular conditions, CFTR is not only adjacent to AQP9 in mSCs, but also to AQP3 and AQP7. Taken together, these results further support the hypothesis that CFTR is a direct regulator of AQP-mediated glycerol permeability. In addition, and equally important, it highlights that CFTR can modulate the transport of other uncharged molecules that are transported by AQPs, opening the possibility for new research. One example is the already described ability for AQP9 to transport lactate. Decreased AQP9 permeability could be a factor in lactate secretion for germ cells. This hypothesis has already been raised by Arena et al. [49] in a study performed on SCs of varicocele patients. Lactate is the preferred substrate for germ cell energy production [50]. Thus, CFTR-mediated AQP9 permeability impairment could also disrupt lactate supply for germ cell development.

In conclusion, although these proteins were present in other cellular locations (apart from their presence in the plasma membrane), when we focused on the functional aspect of aquaglyceroporins (glycerol permeability through the plasma membrane) we observed that CFTR appears to be a direct functional modulator of AQP3, AQP7, and AQP9 in mSCs, being able to modulate glycerol permeability through these channels. While further studies will be needed to enlighten the relevance of the intracellular presence of these aquaglyceroporins, it is known that glycerol is essential for the control of the BTB and its accumulation results in the disruption of spermatogenesis. Thus, our results suggest that the malfunction of CFTR and the consequent decrease in glycerol permeability is a potential link between male infertility and cystic fibrosis.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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