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Aquaporin-9 as a molecular partner of CFTR in the Blood-Testis Barrier

Bernardo Coutinho Rodrigues

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Orientador: Prof. Doutor Pedro Fontes Oliveira (ICBAS-UP/I3S-UP)
Co-orientadora: Prof.ª Doutora Branca Maria Silva (CICS-UBI)
Co-orientador: Doutor Marco Gouveia Alves (ICBAS-UP)
Co-orientador: Prof. Doutor Giuseppe Calamita (BBB-UBAM)

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(Bernardo Coutinho Rodrigues)

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Resumo

A falta de conhecimento face à etiologia da infertilidade masculina tem impulsionado diversas investigações focadas nas estruturas constituintes do sistema reprodutor masculino, em especial, nos testículos. Este par de órgãos é imprescindível não só para a adequada manutenção da esteroidogénese, mas também para permitir o progresso da espermatogénese, o processo através do qual as células germinativas imaturas se multiplicam e diferenciam em espermatozoides. É nos testículos que podemos encontrar as células de Sertoli, que desempenham um papel fundamental no controlo da espermatogénese através: (1) do suporte estrutural e nutricional das células germinativas; (2) da formação da barreira hemato-testicular; e (3) do controlo da composição do fluido luminal dos túbulos seminíferos. De facto, a obtenção de um ambiente homeodinâmico nos túbulos seminíferos é imprescindível para a manutenção do potencial de membrana, do balanço osmótico e do movimento de fluidos. As células de Sertoli são contribuidoras-chave para esta regulação, pois possuem inúmeras proteínas de membrana, entre as quais se podem destacar o Regulador da Condutância Transmembranar da Fibrose Cística (CFTR) e a Aquaporina-9.

O CFTR é uma glicoproteína responsável pelo transporte transepitelial de água e sais, mediando a troca de Cl^- e HCO_3^- entre as células e o meio. Alterações na função desta proteína têm sido associadas a prognósticos clínicos severos, levando muitas vezes à morte do indivíduo. É frequentemente observada uma ausência bilateral congénita dos *vas deferens* no sistema reprodutor masculino. Para além da sua função principal, o CFTR frequentemente interage com várias proteínas de membrana, incluindo a Aquaporina-9. A Aquaporina-9 é uma aquagliceroporina que, para além do seu papel como canal transportador de água, é também permeável a uma variedade de outros solutos, incluindo lactato, glicerol, ureia, adenina e uracilo. Estudos anteriores identificaram uma interação direta entre o CFTR e a Aquaporina-9 em células de Sertoli de rato. Esta interação foi comprovada como sendo importante na permeabilidade de água e no balanço iónico, sem o qual a infertilidade masculina seria uma realidade. Com esta informação-base, torna-se imprescindível a descoberta dos mecanismos moleculares que estarão por detrás da função destes canais (e sua possível interação) nas células de Sertoli do sistema reprodutor masculino humano.

Com o desenvolvimento deste trabalho pretendemos avaliar a significância fisiológica da expressão da Aquaporina-9 e do CFTR em culturas primárias de células de Sertoli humanas. Adicionalmente, também foram investigadas as repercussões da inibição de cada uma destas proteínas, recorrendo à Floretina (inibidor da Aquaporina-9) e ao $CFTR_{(inh)}$-172 (inibidor do CFTR), na viabilidade e função das células de Sertoli humanas.

Os resultados por nós obtidos identificaram, pela primeira vez, que a Aquaporina-9 é altamente expressa nas células de Sertoli humanas. Provámos também que a Floretina reduz significativamente a expressão do mRNA do GATA4 e do SOX9 o que, associado à redução observada no consumo de glutamina, sugere que este inibidor da Aquaporina-9 despoleta um processo de desdiferenciação nas células de Sertoli humanas. Este resultado implicaria o desenvolvimento de um perfil infértil num modelo *in vivo*, destacando-se assim o papel vital da Aquaporina-9 na reprodução masculina humana. A nossa investigação também permitiu descobrir que o mRNA do CFTR se encontra expresso nas células de Sertoli humanas, revelando, ainda assim, um peso molecular diferente do presente em outros órgão/organismos previamente estudados. Este resultado permitiu-nos concluir que, muito possivelmente, estaremos na presença de uma nova isoforma deste transportador, específica das células de Sertoli humanas. A inibição da proteína pelo $CFTR_{(inh)}$-172 diminuiu significativamente a expressão do mRNA do GATA4 e do SOX9, em semelhança com o grupo tratado com Floretina, o que reforça a possibilidade de uma interação entre a Aquaporina-9 e o CFTR no sistema reprodutor masculino.

Podemos assim concluir que esta inovadora investigação permitiu a descoberta de informação imprescindível relativamente à implicação da Aquaporina-9 e do CFTR na manutenção homeostática das células de Sertoli humanas. Deve ainda acrescentar-se que várias questões foram levantadas com este estudo e que as respetivas respostas deverão ser reveladas em estudos futuros.

Palavras-chave

Aquaporina-9; Barreira hemato-testicular; Células de Sertoli; CFTR; Espermatogénese; Fertilidade Masculina; GATA4; SOX9.

Resumo Alargado

O número de casos patológicos associados a subfertilidade e infertilidade masculinas tem aumentado de forma exponencial ao longo dos últimos anos. A realidade demonstra que, apesar da esperança média de vida ter aumentado consideravelmente, a qualidade espermática tem vindo a decair drasticamente. O grande problema que se coloca atualmente é o facto de a etiologia da maioria destes casos patológicos ser totalmente desconhecida. Partindo deste pressuposto, diversos estudos têm sido desenvolvidos no sentido de interpretar as complexas interações que ocorrem no sistema reprodutor masculino. Estes mesmos estudos têm dado grande destaque aos testículos, como órgãos primordiais deste sistema. Para além de apresentarem uma função esteroidogénica, os testículos são responsáveis pela produção de espermatozoides, através de um processo designado espermatogénese. A espermatogénese corresponde a um complexo conjunto de eventos biológicos responsáveis pela maturação e desenvolvimento de células germinativas imaturas, culminando na formação de espermatozoides. Este processo ocorre nos túbulos seminíferos e depende da intima relação entre as células germinativas e um conjunto de células somáticas: as células de Sertoli.

As células de Sertoli têm funções imprescindíveis na manutenção da fertilidade masculina. A sua influência inicia-se na fase de formação do embrião, altura em que estas células são responsáveis pela regressão dos ductos Müllerianos. Mais tarde, as células de Sertoli têm também importantes funções relacionadas com: (1) o suporte estrutural e nutritivo das células germinativas; (2) a formação da barreira hemato-testicular, que confere proteção imunológica, anatómica e fisiológica às células germinativas; (3) e o balanço da composição do fluido dos túbulos seminíferos. De facto, as células germinativas dependem do estabelecimento de um ambiente altamente especializado, contendo os componentes certos e balanceados para o seu crescimento e diferenciação. Especificamente, um conteúdo iónico adequado no meio luminal é imperativo para manter os potenciais de membrana, o balanço osmótico e o movimento de fluidos. Um dos iões mais importantes neste processo é o bicarbonato, um tampão móvel que protege as células de rápidas (e, consequentemente, fatais) variações no seu pH. As células de Sertoli desenvolvem todas estas importantes funções recorrendo ao auxílio de diversos transportadores proteicos, entre os quais, o CFTR e a Aquaporina-9.

O CFTR é uma glicoproteína expressa essencialmente nas membranas das células epiteliais de vários tecidos, incluindo o reprodutor. Esta proteína atua como um canal transportador de Cl^-/HCO_3^-, sendo responsável pela permuta transepitelial de sais e água. Mutações que levam à sua hipofunção estão associadas ao desenvolvimento de uma doença designada Fibrose Cística, enquanto que a sua hiperfunção resulta numa outra doença designada Diarreia Secretória. No que ao sistema reprodutor diz respeito, pacientes com Fibrose Cística

frequentemente apresentam a ausência bilateral congénita dos *vas deferens* o que, em último caso, resulta em infertilidade. Como o CFTR demonstrou ter uma ampla distribuição ao longo do sistema reprodutor masculino, destacando-se a sua já identificada expressão nas células de Sertoli e nas células germinativas, é compreensível que mutações no gene que o codifica causem doenças bastante graves a nível reprodutor. Ainda assim, deve salientar-se que o CFTR não atua de forma isolada, interagindo frequentemente com uma variedade de canais iónicos, transportadores proteicos, recetores e quinases. A interação entre esta proteína e a Aquaporina-9 deve ser destacada.

A Aquaporina-9 pertence a uma família de 13 Aquaporinas conhecidas pelo seu papel no transporte de água através das membranas plasmáticas. Esta família está dividida em três subfamílias: (1) as Aquaporinas clássicas, que são exclusivamente transportadoras de água; (2) as Aquagliceroporinas que, para além da sua permeabilidade à água, são também permeáveis a vários outros solutos; (3) e as Aquaporinas "não-ortodoxas" que, pela sua baixa homologia com as restantes Aquaporinas, pouco se conhece sobre as suas funções. A Aquaporina-9 pertence à subfamília das Aquagliceroporinas pelo que, para além da sua permeabilidade à água, também transporta outros solutos, como lactato, glicerol, ureia, adenina e uracilo. Esta é uma das isoformas mais predominantes no sistema reprodutivo masculino, tendo sido identificada na maioria das células presentes no testículo. Mais concretamente, estudos já realizados identificaram a interação direta entre o CFTR e a Aquaporina-9 em células de Sertoli de rato. Esta interação foi comprovada como sendo imprescindível na permeabilidade de água e no balanço iónico e, consequentemente, indispensável também para a fertilidade masculina.

Partindo desta informação-base, a nossa investigação teve como principal objetivo avaliar a significância fisiológica da expressão da Aquaporina-9 e do CFTR (e sua possível interação) em culturas primárias de células de Sertoli humanas. Adicionalmente, também foram investigadas as repercussões da inibição de cada uma destas proteínas, recorrendo à Floretina (inibidora da Aquaporina-9) e ao $CFTR_{(inh)}$-172 (inibidor do CFTR). Inicialmente avaliámos a viabilidade celular após exposição das culturas celulares a várias concentrações dos inibidores referidos, tendo sido selecionada uma dose para cada inibidor. Paralelamente, determinámos o perfil metabólico das culturas celulares e avaliámos possíveis alterações na expressão do mRNA de GATA4 e SOX9 e na expressão proteica da Aquaporina-9 e do CFTR, como marcadores de funcionalidade e diferenciação das células de Sertoli.

Os resultados por nós obtidos demonstraram, pela primeira vez, que a Aquaporina-9 é expressa nas células de Sertoli humanas. Seguindo o estudo e após análise dos efeitos citotóxicos da Floretina em células de Sertoli expostas a 3 diferentes concentrações (0.05 mM; 0.01 mM e 0.005 mM), selecionámos, para as etapas posteriores do nosso trabalho, a concentração de Floretina mais elevada (0.05 mM). Os resultados que se seguiram demonstraram que este inibidor da Aquaporina-9 reduz significativamente a expressão do

mRNA do GATA4 e do SOX9. Concomitantemente, a avaliação do perfil metabólico demonstrou uma redução significativa do consumo de glutamina por parte das células tratadas. Estes resultados sugerem que a exposição ao inibidor da Aquaporina-9 despoleta um processo de desdiferenciação nas células de Sertoli humanas, destacando-se assim o papel vital da Aquaporina-9 na manutenção da funcionalidade destas células e, consequentemente, no potencial reprodutivo masculino humano.

Paralelamente, a investigação do papel do CFTR nas células de Sertoli humanas apresentou, também ela, resultados importantes. Foi-nos possível a identificação do CFTR, demonstrando-se assim presente nas células de Sertoli humanas. Contudo, a banda correspondente a esta descoberta apresentou um peso molecular superior ao expectável. Este resultado permitiu-nos inferir que, muito possivelmente, poderemos estar na presença de uma nova isoforma deste transportador, específica das células de Sertoli humanas. A inibição da proteína pelo $CFTR_{(inh)}$-172 foi também realizada, após a seleção da concentração ideal, de entre as três inicialmente utilizadas (3 μM, 300 nM e 30 nM) nos estudos de citotoxicidade. Nas condições do tratamento selecionado ($CFTR_{(inh)}$-172, 3 μM), as células de Sertoli humanas apresentaram uma diminuição significativa da expressão do mRNA do GATA4 e do SOX9, em semelhança ao resultado observado no grupo de células tratadas com Floretina. Teorizámos assim a hipótese de este resultado ter origem na interação entre a Aquaporina-9 e o CFTR no sistema reprodutor masculino, que já havia sido descrita em células de Sertoli de rato.

Concluindo, pode dizer-se que este estudo representa apenas um início de uma longa caminhada, mas que poderá ser crucial para despertar o interesse por parte de outros grupos de investigação, relativamente à implicação da Aquaporina-9 e do CFTR na manutenção homeostática das células de Sertoli no sistema reprodutor masculino. Inúmeras pontas de um emaranhado novelo foram encontradas e o investimento no estudo aprofundado do envolvimento destes transportadores no estabelecimento e manutenção do potencial reprodutivo masculino poderá ser a chave do sucesso no combate de grande parte dos casos de infertilidade observados atualmente.

Abstract

The lack of knowledge regarding the etiology of male infertility boosted several investigations focused on male reproductive structures, particularly on male gonads - the testes. These paired organs are pivotal not only for the correct maintenance of steroidogenesis, but also to allow the occurrence of spermatogenesis, the process through which immature germ cells grow and differentiate into spermatozoa. Within testes, Sertoli cells perform an essential role in the control of spermatogenesis by: (1) developing a structural and nutritional support; (2) establishing a protective barrier to germ cells, the blood-testis barrier; and (3) controlling the balance of the seminiferous tubular fluid composition. To achieve this balanced environment, which is critical to the maintenance of membrane potential, osmotic balance and to allow the fluid movement, Sertoli cells are supported by several membrane proteins, including Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and Aquaporin-9.

CFTR is a glycoprotein responsible for transepithelial salt and water transport, regulating Cl^- /HCO_3^- exchange. Malfunctions in this protein have been associated with severe clinical outcomes, such as Congenital Bilateral Absence of *Vas Deferens* in male reproductive tract. In addition to its role, CFTR often interacts with several other plasma-membrane proteins, including Aquaporin-9. Aquaporin-9 is an Aquaglyceroporin permeable to water and to a variety of other solutes, including lactate, glycerol, urea, adenine and uracil. Several studies identified the close interaction between CFTR and Aquaporin-9 in the male reproductive tract, including in rat Sertoli cells. This specific interaction was further proved to be pivotal in water permeability and ionic balance, without which male infertility may be a reality. With that said, the full enlightenment of the molecular mechanisms behind these channels function in the human reproductive system is crucial.

Herein, we evaluated the physiological significance of Aquaporin-9 and CFTR mRNA and protein expression in human Sertoli cells primary cultures. We also investigated the repercussions of inhibiting each one of the proteins with specific inhibitors for Aquaporin-9 (Phloretin) and CFTR ($CFTR_{(inh)}$-172) in human Sertoli cells viability and function.

Our results showed, for the first time, that Aquaporin-9 is highly expressed in human Sertoli cells. We were also able to demonstrate that Phloretin, at a concentration of 0.05 mM, significantly reduces GATA4 and SOX9 mRNA expression which, in accordance with a reduction on glutamine consumption, suggest that this inhibitor triggers an undifferentiation process of human Sertoli cells, highlighting the vital role of Aquaporin-9 in human male reproductive tract. Further investigation unveiled that CFTR is present in human Sertoli cells and that it may exist in a different isoform from the ones previously described. Protein inhibition by $CFTR_{(inh)}$-172 (3 μM) also decreased significantly GATA4 and SOX9, similarly to what happened

with the Phloretin-treated cells, which uncovers the possibility of a crucial interaction between Aquaporin-9 and CFTR, as it has been described in rat Sertoli cells.

We can conclude that this innovative investigation allowed the discovery of pivotal information regarding Aquaporin-9 and CFTR implications in human Sertoli cells. A lot of questions were raised with this study and the respective answers should be unveiled with future investigation.

Keywords

Aquaporin-9; Blood-Testis Barrier; CFTR; Sertoli cells; GATA4; Male Fertility; SOX9; Spermatogenesis.

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Abbreviations

^{1}H-NMR	Proton Nuclear Magnetic Resonance
ABC	ATP-Binding Cassette
AQP	Aquaporin
ATP	Adenosine Triphosphate
B2M	B2-Microgloguline
BTB	Blood-Testis Barrier
cAMP	Cyclic Adenosine Monophosphate
CBAVD	Congenital Bilateral Absence of *Vas Deferens*
cDNA	Complementary Deoxyribonucleic Acid
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylenediamine Tetraacetic Acid
F12	Ham's Nutrient Mixture F12
FBS	Fetal Bovine Serum
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
$HBSS_f$	Hank's Balanced Salt Solution without calcium or magnesium
LH	Luteinizing Hormone
M-PER	Mammalian Protein Extraction Reagent
mRNA	messenger Ribonucleic Acid
MSD	Membrane-Spanning Domain
MTT	Thiazoyl blue tetrazoliumbromide
NBD	Nucleotide Binding Domain
NMR	Nuclear Magnetic Resonance
NPA	Asparagine-Proline-Alanine
PBS	Phosphate-Buffered Saline
Pen-Strep	Penicillin-Streptomycin
PVDF	Polyvinylidene Difluoride
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCs	Sertoli cells
SD	Secretory Diarrhea
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SOX9	SRY-box 9

Introduction

The decrease on male fertility has been progressing alarmingly over the last few decades, with several studies evidencing a negative correlation: while life expectancy increases, sperm quality tends to decrease. Indeed, infertility is one of the most blatantly visible, yet somewhat overlooked health problems that affects millions of couples worldwide. Nearly 50% of the cases are exclusively attributed to the male factor [1]. However, the etiology of this adverse trend in male reproductive health remains a matter of debate, becoming difficult to establish an accurate diagnosis for the observed anomalies. Indeed, the individuals are often apparently clinical healthy-looking but with severe defects in sperm quality. In these cases, the treatment is usually related with assisted reproductive technologies which are applied in order to improve the odds of conception. As male infertility grows to epidemic proportions, a huge investment in reproductive healthcare have been applied. This originated a demographic pressure that is already bringing subfertility and infertility topics into the spotlight.

It is easily understandable that the key to human survival throughout time relies on a proper reproductive function. In men, this presupposes, among others, the success of two testicular functions: the synthesis of steroid hormones (mostly testosterone) and the production of spermatozoa.

Testicular Anatomy and Physiology

The male reproductive system presents different structures that can be divided into primary and secondary sex organs. The secondary sex organs have mainly sustenance and nourishment functions, supporting testes, the primary sexual organs, in the achievement of their primordial functions [2]. The mammalian testes, or male gonads, are paired complex organs covered by two tunics: the tunica vaginalis, the outer one, and the tunica albuginea, the inner one (Figure 1) [3]. Fibrous inner extensions of the tunica albuginea form the septum, which divides the testis into lobules. In humans, each testis consists of 250-300 lobules, containing long and highly coiled seminiferous tubules [4]. It is inside these tubules, considered the functional units of the testis, that Sertoli cells (SCs) and germ cells (in different development stages) can be found [5]. The interstitial spaces between these numerous tubules contain all the blood and lymphatic vessels that allow the transport of nutrients and hormones into the testes [6]. Besides, the interstitial space is also the home of Leydig cells, which are responsible for the synthesis of sex steroid hormones [6].

Testes perform two main functions: the synthesis of steroid hormones and the production of the male gametes [7]. These two distinct processes are hormonal regulated by the hypothalamic-pituitary-gonadal axis, which involves the interaction between the hypothalamus, pituitary and testes [8]. In brief, gonadotropin-releasing hormone (GnRH) is synthesized by the hypothalamus and stimulates the gonadotrophic cells of the pituitary to produce the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

LH acts on membrane receptors in Leydig cells, stimulating testosterone production, while FSH acts on membrane receptors in SCs, stimulating 17β-estradiol, activin and inhibin B production [9]. Concomitantly, there are also complex local interactions among the diverse testicular cell types, including germ cells, SCs, Leydig cells and peritubular cells that are involved in spermatogenesis control [10, 11].

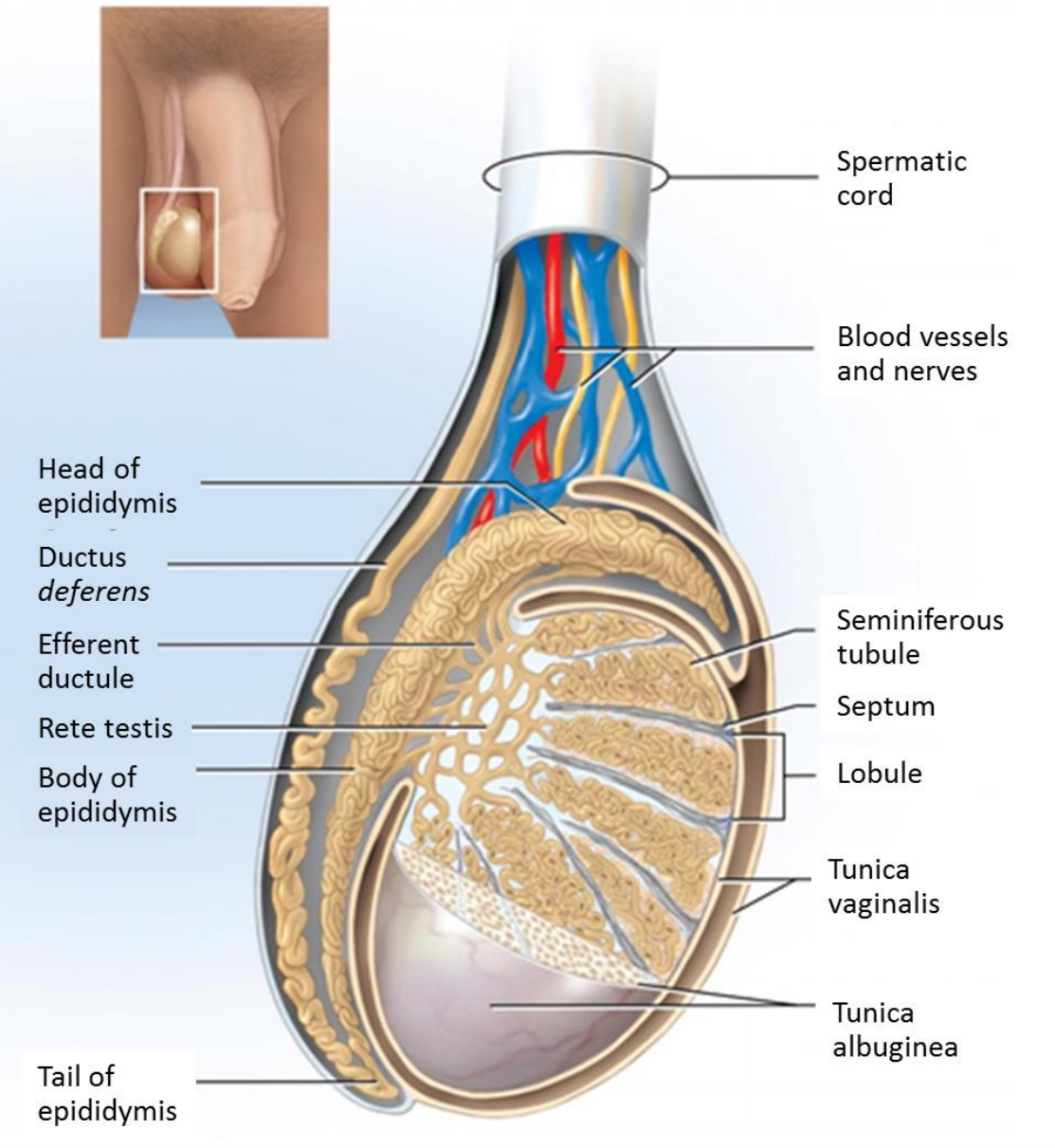

Figure 1: **Schematic representation of the mammalian testis and epididymis**. The mammalian testis is covered by two tunics: tunica vaginalis (the outer one) and tunica albuginea (the inner one). Fibrous extensions from tunica albuginea (septum) divide testis into lobules containing the seminiferous tubules. Seminiferous tubules converge into the rete testis, which is linked to the efferent ducts. The head of the epididymis is connected to the testis by several efferent ducts. Adapted from [3].

Spermatogenesis is a complex biological process that begins at puberty and continues throughout the entire adult life. It represents the maturation and development of immature germ cells to originate haploid spermatozoa [12]. Spermatogenesis occurs in seminiferous tubules by close association of germ cells with SCs, without which germ cell differentiation, meiosis and transformation into spermatozoa would be impossible to occur.

The Sertoli cell

Seminiferous tubules, the most important functional units of the testes, are constituted by a diversity of cells, in which SCs must be highlighted [13]. SCs are irregularly shaped, columnar and highly polarized epithelial cells that extend from the basement membrane of the seminiferous tubule to the lumen [14]. SCs occupy a volume of approximately 17–20% of the seminiferous epithelium of adult men [15], keeping close associations with germ cells at different stages of their development and during all the steps throughout spermatogenesis [16]. In rats, SCs cease to divide at approximately 15 days postpartum [17], while, in humans, it is soon at birth [18]. At the adulthood, rats present about 40 million SCs, while men can reach 3700 million [17, 19]. SCs allow several important functions with pivotal relevance to male fertility. Indeed, these cells influence testis formation already in the embryo, since they ensure the regression of the Müllerian ducts via secretion of the anti-Müllerian hormone [20]. SCs also sustain other crucial roles like the control of the correct nutritional and structural development of germ cells, the establishment of the blood-testis barrier (BTB) and the balance of the composition of the seminiferous tubular fluid [21, 22].

It was Enrico Sertoli, in 1865, who came out with the concept that SCs work as “nurse cells” [23], providing nutrients and regulatory factors for the nourishment of germ cells [24]. Indeed, SCs produce lactate, mainly via the glycolytic metabolism, one of the most important metabolic requirements that allows developing germ cells to produce adenosine triphosphate (ATP) [25]. SCs also ensure the nutritional support of germ cells by secreting amino acids, carbohydrates, lipids, vitamins, and metal ions [14, 26]. SCs columnar shape and large volume allows them to support alone a huge number of developing germ cells. Their cytoplasmic extensions surround germ cells, which is crucial for germ cell movement and, thus, for spermatogenesis [14]. Remarkably, each Sertoli cell can support up to 30 to 50 germ cells at different stages of development [5].

Another important function of SCs is the generation and maintenance of the BTB [27]. The BTB is formed by the specialized junctions between adjacent SCs, which include tight junctions, basal ectoplasmic specializations, basal tubulobulbar complex gap junctions and desmosome-like junctions [12, 28]. Any dysfunction in this structure arrests germ cell differentiation compromising, consequently, spermatogenesis [14]. BTB consists in 3 main components: an anatomical barrier, that restricts the entry of molecules into the adluminal

compartment; an immunological barrier, that limits the movement of immune cells and regulates the level of cytokines in the seminiferous epithelium; and a physiological barrier, that is highly dynamic to fulfill the needs of both germ cells and SCs [29]. The importance of this barrier highlights the essential role of SCs for the establishment of a normal spermatogenesis.

As previously mentioned, SCs also regulate the composition of the seminiferous tubular fluid [30]. In fact, all developing germ cells need a balanced environment, containing many components that allow their correct transport and maintenance in the reproductive tract. With that said, a proper ionic content in the luminal milieus is imperative for maintaining the membrane potential, osmotic balance and fluid movement. Bicarbonate (HCO_3^-) has been shown as one of the most important ions in these processes. It is a mobile physiological buffer that protects cells from fast and local changes in pH [31], which would naturally destroy them. In fact, a central feature of the luminal fluids along the reproductive tract is its pH. In mammals, critical variations in the pH of the luminal fluids of the reproductive system may have significant effects on the male reproductive potential, decreasing the production and/or maturation of spermatozoa and causing subfertility/infertility [32]. SCs are capable of controlling the seminiferous fluid pH and electrolytes composition by regulating its own intracellular pH [33, 34]. This role is achieved by several transport proteins, among which the ion channel Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and the Aquaporin (AQP) water channel family, should be highlighted [30].

Cystic Fibrosis Transmembrane Conductance Regulator

CFTR is a glycoprotein that belongs to the ATP-binding cassette (ABC) transporters superfamily [35]. ABC proteins bind ATP and use its energy to promote the transport of small ions, amino acids, sugars, drugs and proteins across cell membranes [36]. Besides metabolite transport, these proteins are also required for signal transduction, protein secretion and antigen presentation [37]. CFTR was shown to be expressed primarily at the apical or luminal membranes of epithelial cells in the respiratory tract, gut, pancreas, kidney, sweat glands and reproductive tissues [38]. This glycoprotein acts as a plasma-membrane cyclic adenosine monophosphate (cAMP)-regulated chloride (Cl^-) channel, which is responsible for transepithelial salt and water transport [39, 40]. It has also been shown to act as a HCO_3^- channel [41], regulating Cl^-/HCO_3^- exchange [42].

CFTR consists of two repeated motifs, each one containing a hydrophobic Membrane-Spanning Domain (MSD) with six α-helices and a cytosolic hydrophilic region that binds to ATP, the Nucleotide Binding Domain (NBD) [43]. MSDs are predicted to form a pore through which ions pass. When compared with other ABC transporters, CFTR reveals the particularity of presenting a regulatory (R) domain. NBD links to this domain, which contains several charged

residues and multiple phosphorylation sites for protein kinases, such as Protein Kinase A (PKA), Protein Kinase C (PKC) and Protein Kinase G (PKG) [44]. Both the amino (N) and carboxyl (C) terminal tails of CFTR are cytoplasmically oriented and mediate its interaction with a variety of other proteins [45, 46] (Figure 2).

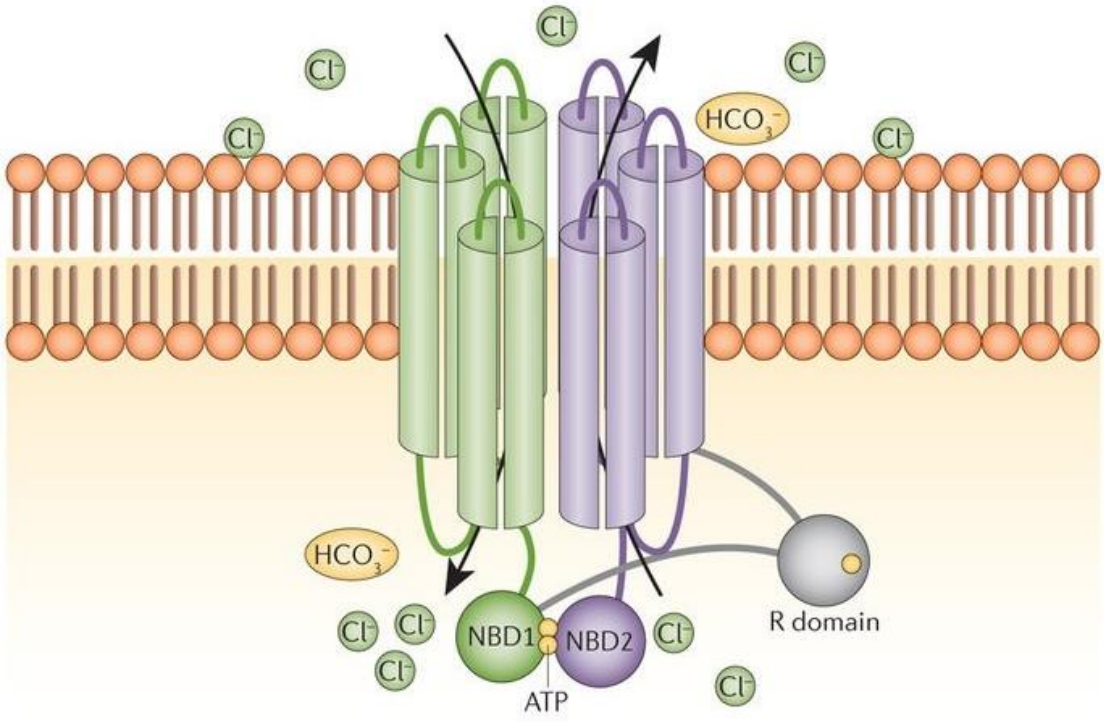

Figure 2: **The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein structure.** CFTR is comprised of two Membrane-Spanning Domains (MSDs), each one containing six α-helices. MSD connects to a respective Nuclear Binding Domain (NBD). NBDs bind ATP, as well as the regulatory (R) domain. The channel opens when the R domain is phosphorylated by Protein Kinase A (PKA) and when Adenosine Triphosphate (ATP) is bound at the NBDs. Adapted from [47].

Maintaining and regulating a dynamic balance between CFTR activation and inactivation is an important mechanism to ensure body homeostasis. Once this balance is broken, two major problems may occur: hypofunction of CFTR, which may lead to Cystic Fibrosis (CF); or hyperfunction of CFTR, which induces Secretory Diarrhea (SD).

CF is an autosomal human genetic disease, being most common among caucasians [48]. Although the life expectancy of individuals with CF has increased dramatically in the past three decades, the average age of death is 37 years old [49]. CF is caused by mutations in CFTR alleles, which lead to the loss or dysfunction of the CFTR Cl^- channel activity and, consequently, decreasing protein biosynthesis and/or function [50, 51]. The CFTR malfunction disrupts ion and fluid homeostasis at epithelial surfaces in the organs in which it is present [52]. CF is related with the accumulation of viscous mucus in the epithelial surfaces of several

organs, such as lungs [53], pancreas [54], gut [55] and testes [56]. Such accumulation may result in blockage, infection, inflammation and, ultimately, organs failure [57, 58]. Although CF is clinically dominated by chronic lung disease [59], the past decades brought cumulative evidence regarding the pivotal role of CFTR in male fertility. Studies suggest that an average of 97% of male CF patients are infertile. This is related not only with the well-known role of CFTR in regulating electrolyte and fluid transport in the luminal microenvironment of the male excurrent duct, but also with several other implications present in men with reduced sperm quality [60, 61]. In fact, most CF patients show Congenital Bilateral Absence of *Vas Deferens* (CBAVD) [62], which results in obstructive azoospermia. However, the mechanisms through which CFTR mutations lead to male infertility remain unsolved.

In other hand, SD is a disease caused by excessive activation of CFTR in the gut, being associated to an intestinal colonization of pathogenic microorganisms, such as *Escherichia coli* and *Vibrio cholera* [63]. The toxins released by these pathogenic bacteria activate membrane associated secondary messengers that lead to the phosphorylation of CFTR and thus to its excessive activity [64]. This induces massive Cl^- secretion across the epithelium and concomitant sodium (Na^+) and water loss through the gut lumen. The result is a dehydration process, which can be fatal if untreated [65]. With that in mind, it seems reasonable to propose that blocking luminal CFTR channels would be the appropriate treatment for these patients.

Once CFTR mutations are suggested as the cause of severe diseases, several studies regarding its role were performed. In what male reproductive tract is concerned, CFTR importance is evidenced in its wide distribution going from *vas deferens* epithelia to epididymis [66]. In the seminiferous tubules, CFTR was shown to have a wide expression going from SCs to all germ cell types [67-69]. However, CFTR does not act alone, interacting with several other proteins that help to preserve the homeostasis of the reproductive system. In addition to its role as a Cl^-/HCO_3^- channel, CFTR often interacts (spatially and temporally) with a variety of other ion channels, protein transporters, receptors and kinases [70]. These dynamic interactions influence CFTR channel (and its ligands) functions, as well as its location and processing within cells. In this aspect, the interaction between CFTR and AQPs must be especially highlighted.

Aquaporins

Water is one of the main elements of living organisms, presenting unique features that allow the existence of life on the planet. On the body, it reveals three main functions: (1) establishing blood plasma and mammalian cells; (2) regulating physiological and biochemical processes in the organism (like body temperature and toxins removal) and (3) allowing the movement of solutes into different cells, to keep the osmotic pressure [71]. Indeed, the

water flow across biological membranes is a fundamental process of cell physiology. During a long time, it was assumed that this transport was only achieved through simple diffusion [72]. However, further observations showed that there are tissues that require a rapid and massive water movement across the cell membrane, where the diffusion is simplified by a specific class of membrane channels: AQPs [73].

AQPs belong to a family of small (25-35 kDa), hydrophobic, integral membrane channel proteins that permeate water transport across biological membranes [74]. Since the diffusional movements of water across biological barriers are relatively slow, the presence of AQP channels is mandatory to increase the capacity of water permeation. In epithelial cells, this capacity can be enhanced from 10- to 100- fold [73]. Since the first AQP was discovered, subsequent studies have described the existence of several other proteins with similar characteristics [75].

The primary structure of an AQP consists in six transmembrane domains (1-6) arranged in a helical bundle that provides the basic support for the channel [76]. Five loops (A-E) connect the six helices [76]. The protein also presents an N- and C-termini, which, together with loops B and D are oriented to the intracellular space. In an opposite way, loops A, C and E are extracellularly oriented [77]. The two highly conserved Asparagine-Proline-Alanine (NPA) motifs in loops B and E should also be highlighted in the AQP structure (Figure 3) [78]. AQPs are presented in a homo-tetramer conformation, in which each monomer forms an independent hole for solutes movement [79]. AQP's monomer is folded in the "hourglass model", in which loops B and E dip into the monomer like short α-helices, allowing the monomer to be fully functional [80].

Thirteen members of the AQP family have been described so far (from AQP0 to AQP12), and their expression has already been identified in mammalian cells of kidney, lung, pancreas, brain, gastrointestinal tract, eye, ear, immune system, skin, adipose tissue, muscles, uterus and testis [81]. These proteins can be grouped in three major subclasses, based on their biophysical transport properties: (1) the classical AQPs (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, AQP8); (2) the Aquaglyceroporins (AQP3, AQP7, AQP9, AQP10); (3) and the unorthodox AQPs (AQP11, AQP12) [82]. Classical AQPs are exclusively water channels, while Aquaglyceroporins are non-selective water channels that, beyond water, permeate membranes to glycerol, urea and/or other small nonelectrolytes [83]. On the other hand, unorthodox AQPs show distinct evolutionary pathway, with little homology (around 20%) with the other family members [84].

Fluid secretion and absorption are vital processes in the physiology of the male reproductive tract, so it is not surprising that AQPs had been pointed out as pivotal proteins on these tissues. Water and ionic movement affects not only the composition of the ductal luminal fluids, but also the maturation, concentration and storage of spermatozoa in seminiferous

vessels [85-87]. The secretion of the luminal fluid is mostly performed by SCs, with a slight contribution from differentiating germ cells [88], being crucial for a proper development of spermatogenesis. Concomitantly, the maturation of spermatozoa and related sperm concentration and storage are associated with considerable fluid movement [86, 87]. Specially, in the metamorphosis of round spermatids into elongated spermatids, one of the most distinct morphological changes is a striking reduction of germ cell volume, largely because of the osmotically driven fluid efflux [89], becoming expectable the intervention of the AQPs in this process. Such movement of fluid is consistent with the presence of multiple AQPs in the male reproductive tract. Indeed, apart from AQP12, the presence of all the other known isoforms has already been reported in the testis, epididymis, and/or other excurrent ducts [90].

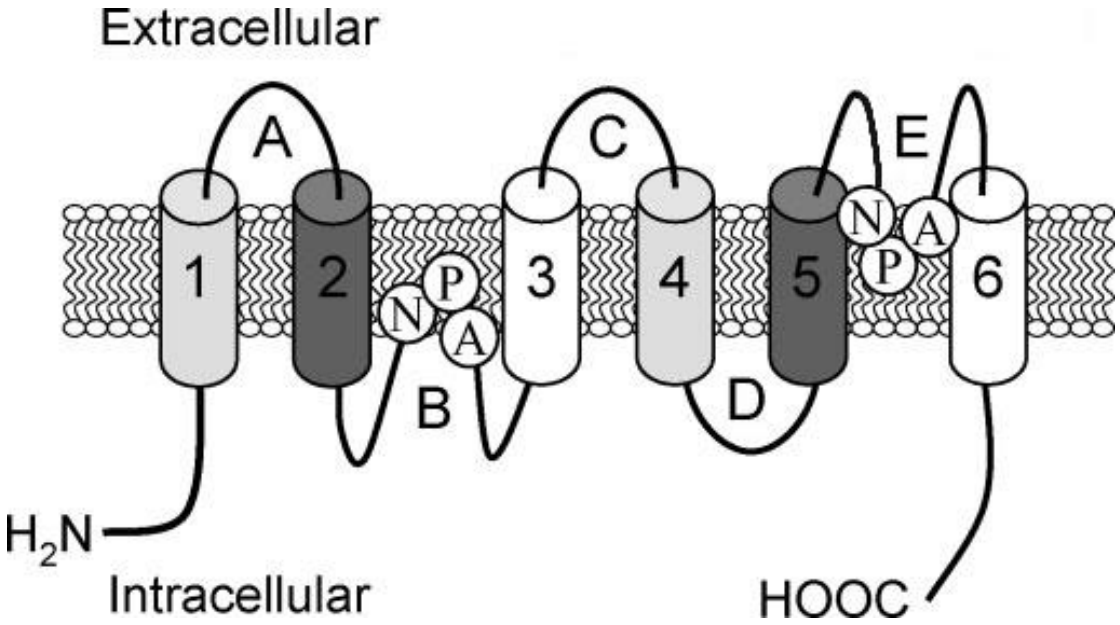

Figure 3: Structure of the aquaporin (AQP) monomer. Each AQP monomer contains six transmembrane domains (1–6) connected by five loops (A–E). Connecting loops B (intracellular) and E (extracellular) there are asparagine–proline–alanine (NPA) motifs. The protein also presents an amino (N)- and carboxyl (C)-termini, oriented to the intracellular space. Adapted from [89].

There are several studies identifying the association between AQPs and CFTR [91]. In fact, it was shown that a failure in the interaction between these two proteins can lead to CF and respective fluid accumulation [92]. Initially, these interactomes were described only in human airway epithelial cells and in Xenopus oocytes [93, 94]. However, subsequent research found that they are also present in the reproductive system, including the epididymal cells [95] and SCs [96]. The research of the last few years brought to the spotlight the specific and crucial role of AQP9.

Aquaporin-9

AQP9 belongs to the subclass of Aquaglyceroporins, being, in addition to water, also permeable to a variety of solutes, including monocarboxylates (e.g. lactate), polyols (e.g. glycerol), carbamides (e.g. urea), purines (e.g. adenine) and pyrimidines (e.g. uracil) [97]. This protein was already found to be expressed in the liver, leukocytes, ovary, brain and testis of several mammals [98-100]. Its high expression in the brain, mostly in astrocytes [101], the main responsible for solute transport regulation, should be highlighted. Astrocytes establish the blood-brain barrier, revealing a similar role to the one of SCs in the constitution of the BTB [102]. This information is helpful to understand the role of AQP9 in the male reproductive tract.

AQP9 is one of the most predominant isoforms along male reproductive system [103, 104]. In fact, this protein was already identified in testes, efferent ducts and epididymis [103]. It is mainly expressed in rodent germ cells, particularly in early developmental stages of spermatocytes [86]. It is also expressed in the plasma and intracellular membranes of Leydig cells [105] and in the plasma membrane of SCs [68]. On the other hand, there is still no evidence supporting the existence of AQP9 in spermatozoa, so far. Although no functional studies were performed regarding the role of AQP9 in SCs, its well-studied role in water balance and ion homeostasis in several other tissues [81], allows to expect that it also participates in water homeodynamics in the seminiferous tubules.

The specific association of CFTR and AQP9 in the luminal membrane of rat and human epididymal principal cells shows the pivotal role of this AQP in the establishment of the luminal fluid, especially in the cauda epididymis [95], which is crucial for sperm maturation and storage. Indeed, animal studies regarding the inhibition of any of these channels show a significant decrease in water permeability, which may lead to male infertility [95]. It has also been previously proved that AQP9, in similarity to AQP4 [96], interacts directly with CFTR in rat SCs [68]. This strongly suggests that CFTR might act as a regulator of AQPs and water homeodynamics in SCs. The full enlightenment of these molecular mechanisms may point towards possible pharmacological targets to counteract male subfertility/infertility in men with CFTR and/or AQP9 malfunctions. Altogether, this information highlights the need of further research regarding the significance of the CFTR-AQP9 interaction in human SCs, and if it is as vital in human reproductive tract as it is in rats.

Aims of the project

The male reproductive tract consists of a huge amount of different cell types with specific roles and highly complex relations between them. The proper cooperation between these cells allows the maintenance of the reproductive functions, being pivotal for male fertility. Within testes, the somatic SCs are required for the nutritional and structural support of the developing germ cells and, consequently, to allow an appropriate spermatogenesis. SCs are also responsible for the establishment of the BTB, which reveals crucial anatomical, immunological and physiological roles. Finally, SCs present an important function in regulating the composition of the seminiferous tubular fluid. Indeed, these cells regulate the luminal pH and ionic content through the specific role of several transport membranes, among which CFTR and AQP9 seem to be essential. Not only the expression of CFTR and AQP9, but also the cooperation between them, were shown to be critical factors for the reproductive activity in male rats. This remarkable discovery uncovered the question whether these channels are also expressed in the human male reproductive tract and if their role is also determinant for male reproductive health in men.

The general aim of the present research was to deepen the physiological significance of the interaction between AQP9 and CFTR in human SCs and its importance for male fertility. This study proposed to be the first step in unveiling new paths to understand the indispensable interaction between these two proteins in human SCs, and, subsequently, to improve the knowledge about the diseases that result from their malfunctions.

To achieve our main goal, we firstly aimed to evaluate messenger Ribonucleic Acid (mRNA) and protein expression of both AQP9 and CFTR in human SCs. We also intended to define the suitable concentration of Phloretin (AQP9 inhibitor) and $CFTR_{(inh)}$-172 (CFTR inhibitor) to our study by evaluating the toxicity/viability of the cell cultures after the exposure to different concentrations of these inhibitors. Besides, we determined the metabolic profile of the culture medium of the treated cells (and the respective control). To disclose the impact of the inhibitors in SCs metabolism and function, SRY-Box9 (SOX9) and GATA4 mRNA expressions were also evaluated. Finally, we further intended to analyze the effects of the selected treatments in AQP9 and CFTR respective expressions.

Materials and methods

Chemicals

Hank's Balanced Salt Solution without calcium or magnesium ($HBSS_f$), HEPES buffer solution, Mammalian Protein Extraction Reagent (M-PER), "BCA Protein Assay Kit" and "Pierce ECL Plus Western Blotting Substrate" were obtained from Thermo Fisher, United States of America. Dulbecco's Modified Eagle Medium (DMEM), Ham's Nutrient Mixture F12 (F12), gentamicin, amphotericin B, trypsin-Ethylenediamine Tetraacetic Acid (EDTA) solution, Phloretin and $CFTR_{(inh)}$-172 were obtained from Sigma-Aldrich, United States of America. Fetal Bovine Serum (FBS) was obtained from Biochrom, Germany. Penicillin-Streptomycin (Pen-Strep) solution was obtained from Corning, United States of America. "GRS Total RNA kit - Blood & cultured cells" was obtained from GRISP, Portugal. Random hexamer mix, Deoxynucleotide Triphosphates (dNTPs), "NZY M-MuLV Reverse Transcriptase", "NZYTaq 2x Green Master Mix", "NZY qPCR Green Master Mix" and Ponceau S staining solution were obtained from NZYTech, Portugal. Human AQP9, CFTR, SOX9, GATA4 and ß2-Microglobulin (ß2M) primers were obtained from Stab Vida, Portugal. The antibodies against human AQP9, CFTR and glutaminase were obtained from LsBio, Cell Signaling/Santa Cruz Biotechnology and RabMAb, respectively. Thiazoyl blue Tetrazoliumbromide (MTT) solution was obtained from Amresco, United States of America.

Patient selection and testicle tissue preparations

Patients' clinical studies and testicle tissue processing was performed at the Centre for Reproductive Genetics Alberto Barros (Porto, Portugal) according to the guidelines of the local, national and European Ethical Committees. The studies have been performed according to the Declaration of Helsinki. Testicular biopsies were obtained from patients under treatment and used after informed written consent. Only cells left in the tissue culture plates after patient's treatment were used. SCs were isolated from six testicular biopsies of men with anejaculation, vasectomy or traumatic section of the *vas deferens*, but with conserved spermatogenesis.

Human Sertoli cells primary culture

Testicle biopsies were washed twice in $HBSS_f$ through centrifugation at 500-600 g, for 5 minutes each and at room temperature, as described by Oliveira and coworkers [106]. Following, human SCs were isolated, according to a routine method [33]. The resulting cellular pellet was suspended in 10/12 mL of culture medium (DMEM:F-12 in a 1:1 ratio, supplemented with 10% FBS, 1% Pen-Strep, 15 mM HEPES, 14 mM $NaHCO_3$, 2.5 µg/mL amphotericin B and 50 g/mL gentamicin), plated on 75 cm^2 culture flasks (Sarstedt, Germany). T-flasks were incubated at 37ºC, in a 5% carbon dioxide (CO_2) humidified

atmosphere, until re-feed, 72 hours later. When cells reached 70/75% confluence, they were transferred and incubated with a 0.05% trypsin – 0.02% EDTA solution. Re-feed and passage processes were handled in a laminar flow chamber, with sterile equipment and reagents.

Experimental design

After culture cells exhibit a confluence of 80/90%, they were divided into seven groups: a control group, developed in a culture medium with 0.05% Dimethyl Sulfoxide (DMSO); three groups with different Phloretin concentrations (0.05 mM, 0.01 mM, 0.005 mM) and three groups with different $CFTR_{(inh)}$-172 concentrations (3 μM, 300 nM, 30 nM). Inhibitors concentrations were defined based on previous studies regarding their successful use [97, 107, 108]. Then, upper and lower concentrations were chosen for the present study. Phloretin and $CFTR_{(inh)}$-172 were purchased in powder form, subsequently prepared in pure DMSO to a stock solution and diluted to the intended concentrations. It was maintained a 0.05% percentage of DMSO throughout all the groups in study. At the beginning and after 24 hours of treatment, 1 mL of each culture medium was collected for metabolite analysis. Cells of the seven described groups were detached from the flasks, counted in a Neubauer chamber, and collected for further investigation.

Cytotoxic profile assay

Inhibitors' effect on human SCs viability was determined by the colorimetric MTT assay, as previously described [109]. This procedure allows viable cells (with active metabolism) to reduce MTT into purple colored formazan crystals, with a maximum absorbance near 570 nm. It is associated with the measurement of mitochondrial dehydrogenases activity [110], since the conversion of MTT to formazan is related with a concomitant oxidation of NADH to NAD^+. Dead cells lose the ability to convert MTT, so the purple color is not observed. A reference wavelength at 655 nm was also obtained. This measurement is associated with the non-specific reduction of MTT, which must be removed from the actual value of interest (at 570 nm). About 50 μL of MTT 5 mg/mL, dissolved in Phosphate-Buffered Saline (PBS) solution, were used in each well, containing 500 μL of fresh culture medium (without serum). After 2 hours of incubation, at 37ºC, in the dark, the medium was removed and cells were re-suspended in 500 μL of DMSO. Finally, 100 μL of each well were transferred to a 96-well plaque for further absorbance measurement using an "Anthos 2010 microplate" reader (Biochrom, Germany).

Total Ribonucleic Acid (RNA) Extraction

Total RNA was extracted from isolated SCs using the "GRS Total RNA kit - Blood & cultured cells", according to the manufacturer's instructions. Briefly, the cellular pellet was re-suspended in Lysis Buffer which, when added "Buffer R1" and ß-mercaptoethanol, triggered the lysis of cell membranes. Then, after the addition of ethanol 70% to the sample lysate (intended to help solubilize cell components), the solution was placed in a "RNA minispin column" and the respective collection tube. Following this step, the solution was centrifugated to eliminate most of unwanted cell fragments. Then, the column suffered a series of washing steps, in which Wash Buffers 1 or 2 were added, intended to remove all non-RNA elements from the column. A centrifugation was performed after each one of the washing steps. Finally, the spin column was transferred to a new collection tube and "RNase-free Water" was placed on the column in order to elute the resulting purified total RNA, after a final centrifugation. RNA concentration and absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}) were determined by spectrophotometry using a nanophotometer (Implen, Germany).

Complementary deoxyribonucleic acid (cDNA) synthesis

cDNA synthesis was performed using the "NZY M-MuLV Reverse Transcriptase Kit", according to manufacturer's instructions. Briefly, the reverse transcriptase reaction was performed in a final volume of 20 µL, including 1 µg of total RNA, 2.5 µL of random hexamer primers (50 ng/µL), 1 µL of dNTPS Mix (10 mM), 2 µL of Reaction Buffer 10x, 1 µL of "NZY M-MuLV Reverse Transcriptase" and a proper volume of Diethyl Pyrocarbonate (DEPC)-treated water to fulfil 20 µL. The final solution was first incubated at 25°C, for 10 minutes, and then at 37°C, for 50 minutes. After that, the reaction was inactivated by heating at 70°C for 15 minutes and cDNA product was stored at 4°C, until use.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Agarose Gel Electrophoresis

The resulting cDNA was used to analyze AQP9 and CFTR mRNA expression. RT-PCR was performed using the "NZYTaq 2× Green Master Mix", according to manufacturer's instructions. In brief, each reaction included 6.5 µL of Green Master Mix (x2) (containing Taq DNA polymerase, reaction buffer, dNTPS and $MgCl_2$), 0.1 µL of forward primer (50 µM), 0.1 µL of reverse primer (50 µM), 1 µL of synthesized cDNA and a proper volume of DEPC-treated water, up to 12.5 µL. Primer sequences, number of cycles and optimal annealing temperatures used for RT-PCR are mentioned in Table 1. Human liver mRNA was used as positive control and cDNA-free sample as negative control.

At the end of the experiment, 10 µL of RT-PCR products were used in each well and one well with 4 µL of ladder solution. Samples were run in 1% agarose gel electrophoresis, for 30 minutes, at 120 volts. The bands were allowed to migrate from 40 to 60% of the gel length. After revealing proceeding, bands formation was visualized on the "Gel Doc XR+ System" (Bio-Rad, United States of America).

Table 1: Primers and cycling conditions used in Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) amplification of Aquaporin-9 (AQP9) and Cystic Fibrosis Transmembrane Conductance Regulator (CFTR).

Gene	Primer Sequences (5'-3')	Annealing Temperature	Number of Cycles	Size (base pairs)
AQP9	Forward: GTCATTCCCTGTTCGCTGCT Reverse: GGCAACACAACTGGCACATC	56ºC	35	171
CFTR	Forward: GACATCACAGCAGGTCAGAGA Reverse: ATTCCAGGCGCTGTCTGTATC	55ºC	35	218

Real-time Polymerase Chain Reaction (qPCR)

qPCR was performed in order to determine SOX9 and GATA4 mRNA expression levels. Specific primers were designed for the amplification of the target genes and also for ß2M, which was used as housekeeping gene (Table 2). qPCR amplifications were performed in a volume of 20 µL, containing 1 µL of synthesized cDNA (in a 1:15 dilution), 10 µL of "NZY qPCR Green Master Mix (2x)", 0.8 µL of forward and reverse primers (5 µM) specific for each gene and a proper volume of sterile nuclease-free water, until the volume of 20 µL. cDNA-free sample was used as negative control. The conditions were previously optimized, including an annealing temperature of 58°C. The specificity of amplifications was determined by melting curves. qPCR experiments were carried out in an "StepOne" equipment (Applied Biosystems, United States of America) and efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA, as described [111]. Samples were run in triplicate in each assay, with the expression values being normalized in relation to ß2M expression and in accordance with the mathematical model proposed by Pfaffl [112], through the formula $2^{-\Delta\Delta Ct}$.

Table 2: Oligonucleotides and cycling conditions for quantitative Real-Time Polymerase Chain Reaction (qPCR) amplification of GATA4, SRY-Box9 (SOX9) and β2-microglobulin (β2M).

Gene	Primer Sequences (5'-3')	Annealing Temperature	Number of cycles
GATA4	Forward: CTAGCAGCTTCTGCGCCTGT Reverse: GTGGTTCCGGAAGCTGATGTA	58°C	40
SOX9	Forward: AGGAAGTCGGTGAAGAACGG Reverse: AAGTCGATAGGGGGCTGTCT	58°C	40
B2M	Forward: GAGGCTATCCAGCGTGAGTC Reverse: GACGCTTATCGACGCCCTAA	58°C	40

Total Protein Extraction and Quantification

Human SCs were homogenized in M-PER buffer supplemented with protease inhibitor cocktail and sodium orthovanadate. The homogenate was centrifuged for 20 minutes at room temperature and then, total protein concentration was quantified using the "Pierce BCA Protein Assay Kit", according to manufacturer's instructions. The absorbance was measured at 595 nm by a "xMark Microplate" Spectrophotometer (Bio-Rad, United States of America), versus standard solutions.

Western Blot

Total protein extracted from human SCs (50 µg/µL) was mixed with sample buffer containing Tris-HCl, 10% Glycerol, 2% Sodium Dodecyl Sulfate (SDS), 5% β-mercaptoethanol and 0.01% Bromophenol blue. Samples were prepared at 90ºC, for 15 minutes, before use. After that, samples were separated in 12.5% polyacrylamide gel at 30 mA/gel, for 90 minutes. Following the electrophoresis, proteins were transferred to Polyvinylidene Difluoride (PVDF) membranes, which were previously activated in pure methanol. The electrotransfer occurred at 100 volts, for 75 minutes, at 4ºC. Afterwards, each membrane was blocked with a 5% (m/v) non-fat milk solution at room temperature, for 90 minutes. Membranes were later incubated at 4ºC, overnight, with rabbit monoclonal primary antibody against glutaminase (1:500, ab156876, RabMAb), rabbit polyclonal primary antibody against CFTR (1:500, 2269s, Cell Signaling), goat polyclonal primary antibody against CFTR (1:100, sc8909, Santa Cruz Biotechnology) and rabbit polyclonal primary antibody against AQP9 (1:1000, ls-c20772, LsBio). The immune-reactive proteins were detected individually with polyclonal anti-rabbit IgG-HRP (1:5000) or with polyclonal anti-goat IgG-HRP (1:5000). Membranes were reacted

with “Pierce ECL Plus Western Blotting Substrate” and observed on the “Chemidoc MP Imaging System” (Bio-Rad, United States of America). Band intensities were quantified using the “Image Lab Software 5.1” (Bio-Rad, United States of America) software. Ponceau staining was performed to quantify total protein in each lane, being used as the protein loading control for human SCs samples.

Nuclear Magnetic Resonance (NMR) Spectroscopy

To evaluate the metabolic profile of human SCs after incubation with the different inhibitors, samples of 180 µL of the medium culture collected during cell culture were analyzed through Proton Nuclear Magnetic Resonance (^{1}H-NMR). For sample analysis, 45 µL of a 10 mM sodium fumarate solution was mixed with the collected medium. Then, 200 µL of each sample were transferred to a NMR tube and the ^{1}H-NMR spectroscopy was performed as previously described [113, 114]. Sodium fumarate (2 mM) was used as an internal reference (6.50 ppm) to quantify alanine (doublet, 1.45 ppm) and lactate (doublet, 1.33 ppm) production and also H1-α-glucose (doublet, 5.22 ppm), pyruvate (singlet, 2.36 ppm) and glutamine (multiplet, 2.45 ppm) consumption, during the 24 hours of cell incubation. Spectra were manually phased and baseline corrected. Chosen metabolite peaks were integrated using “Amix-viewer” software (Bruker Biospin, Germany).

Statistical analysis

Statistical significance of differences among experimental groups was assessed by one-way ANOVA, followed by Tukey test. T-student tests were also performed. All experimental data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using “GraphPad Prism 6” (GraphPad Prism version 6.00 for Windows, United States of America) software. Differences with *p-value* < 0.05 were considered statistically significant and were indicated as (*), relative to control group.

Results

Aquaporin-9 mRNA is expressed in human Sertoli cells

We evaluated the expression of AQP9 mRNA in human SCs. To the best of our knowledge, AQP9 mRNA has never been identified in human SCs. Our results clearly showed that mRNA of human AQP9 is indeed expressed in these cells, near 171 base pairs (Figure 4).

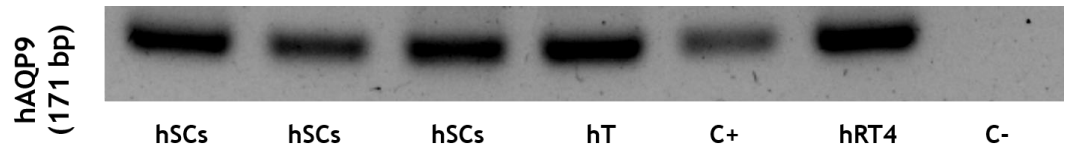

Figure 4: Identification of messenger ribonucleic acid (mRNA) expression of aquaporin-9 (hAQP9) in human Sertoli cells. Illustrative electrophoresis experiment. hSCs: human Sertoli cells; hT: human testis; C+: positive control (human liver); hRT4: human bladder cancer cell line; C-: negative control (without sample).

Aquaporin-9 protein is expressed in human Sertoli cells

Although we were able to identify AQP9 mRNA expression in human SCs, we further analyzed if that mRNA was able to codify the respective protein. Western blot assay confirmed that human SCs express, indeed, this protein. Interestingly, our result presented a two bands expression (near 63 KDa) regarding human SCs groups, in a different way to what happened with the single band expression (near 31.5 KDa) presented in the reaming groups (Figure 5). A two-band representation is thought to be associated with a glycosylation process of AQP9, which will be further discussed in this work.

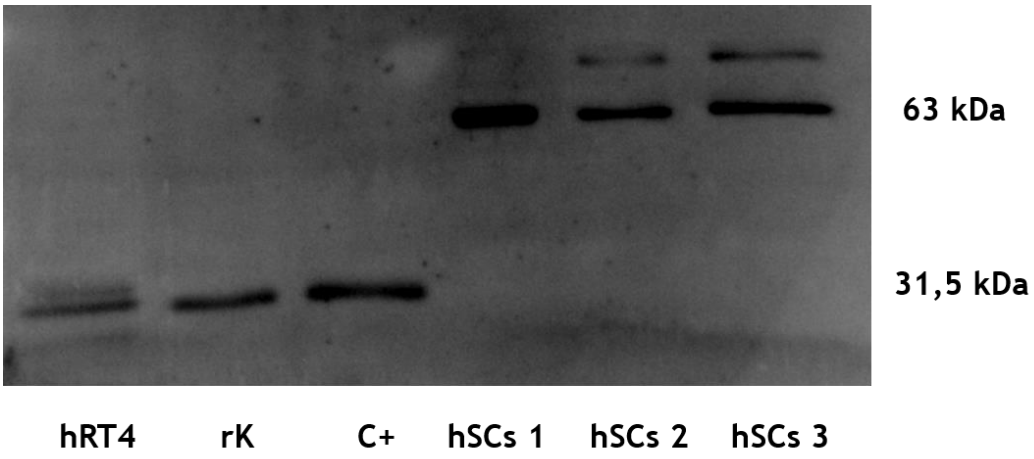

Figure 5: Identification of Aquaporin-9 protein expression in human Sertoli cells. Illustrative Western blot experiment. hRT4: human cancer cell line; rK: rat kidney; C+: positive control (rat liver); hSCs 1: human Sertoli cells, sample 1; hSCs 2: human Sertoli cells, sample 2; hSCs 3: human Sertoli cells, sample 3.

Human Sertoli cells viability remains unaffected after Phloretin treatment

To examine the effect of AQP9 inhibition on the viability of human SCs, they were exposed to different concentrations of this AQP9 inhibitor (0.05 mM; 0.01 mM and 0.005 mM), for 24 hours. After that, cellular viability was determined by the MTT assay (in fold variation to control). Our results showed that Phloretin, at the chosen doses, did not reveal cytotoxic effects on SCs, since their viability remained unaffected when compared to the control group (Figure 6). Control and treated groups (0.05 mM; 0.01 mM; 0.005 mM) presented values of 1.00 ± 0.0154; 1.12 ± 0.0557; 1.01 ± 0.0787 and 0.97 ± 0.0096, respectively. Thus, we selected the group exposed to the highest concentration of Phloretin (0.05 mM) to proceed to the remaining experiments.

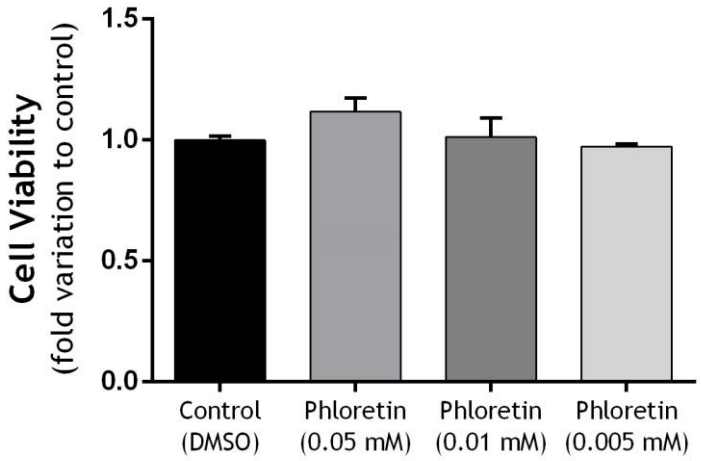

Figure 6: Evaluation of human Sertoli cells viability, after 24-hours treatment with different Phloretin concentrations. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition), in fold variation to control.

GATA4 and SOX9 mRNA expression significantly decreases after Phloretin treatment

GATA4 and SOX9 are two biomarkers which expression is associated with differentiation capability of SCs [115, 116]. To investigate the influence of Phloretin on GATA4 and SOX9 mRNA expression in human SCs, a qPCR assay was performed. The results showed that the cells treated with the aforementioned dose of Phloretin revealed a significant reduction in GATA4 (0.0011 ± 0.0003) and SOX9 (0.0003 ± 6.97e-005) mRNA expression, when compared to the respective control groups (1.0000 ± 0.2823 and 1.0000 ± 0.3378) (Figure 7).

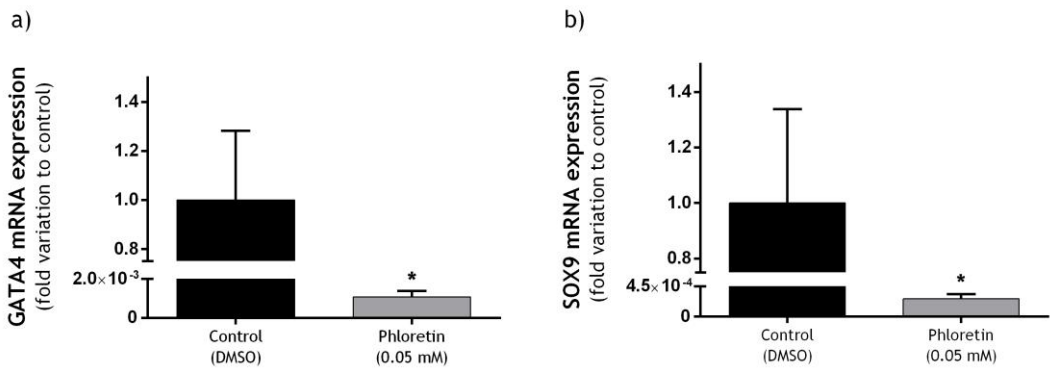

Figure 7: Evaluation of GATA4 and SRY-Box9 (SOX9) messenger ribonucleic acid (mRNA) level in human Sertoli cells, after 24-hours treatment with Phloretin (0.05 mM). Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition), in fold variation to control. Significant results relative to control (p-value < 0.05) are indicated as (*). (a) Evaluation of GATA4 mRNA expression. (b) Evaluation of SOX9 mRNA expression.

Aquaporin-9 protein expression in human Sertoli cells is not altered by Phloretin treatment

In order to analyze the influence of Phloretin in human AQP9 protein expression, a western blot assay was performed and the expression of total AQP9, glycosylated AQP9 and non-glycosylated AQP9 was analyzed (Figure 8, panels a, b and c). The results are presented in relation to control, which was acquired through the measurement of total protein expression using a Ponceau Staining. An illustrative western blot is also presented, showing clear bands near 31.5 KDa (Figure 8, panel d). We did not observe any significant variation between the control and treated groups in the expression of glycosylated AQP9 (1.000 ± 0.2682; 0.837 ± 0.0618), non-glycosylated AQP9 (1.000 ± 0.3389; 1.056 ± 0.0633) or total AQP9 (1.000 ± 0.3558; 0.877 ± 0.0800), respectively.

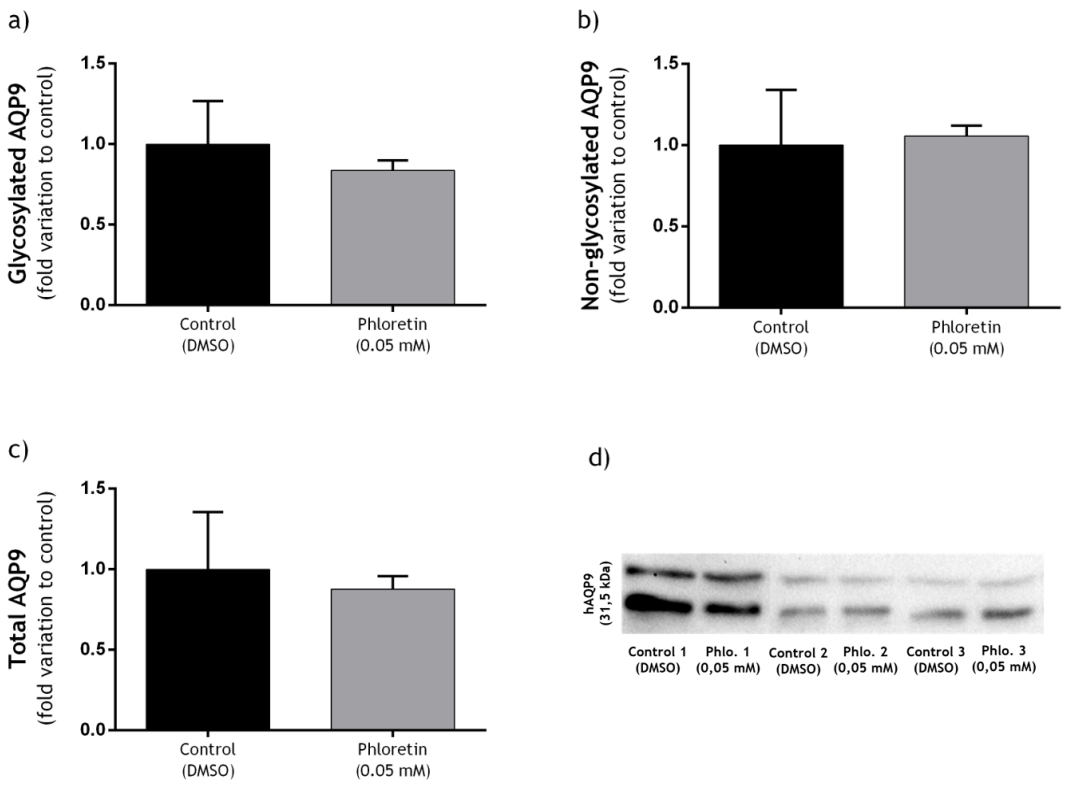

Figure 8: Effect of 24-hours treatment with Phloretin (Phlo., 0.05 mM) in human Sertoli cells Aquaporin-9 (AQP9) protein expression. Results shown on panels (a), (b) and (c) are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition), in fold variation to control. (a) Glycosylated AQP9 expression. (b) Non-glycosylated AQP9 expression. (c) Total AQP9 expression. (d) Illustrative western blot experiment, regarding three samples.

Phloretin reduces Glutamine consumption in human Sertoli cells

In order to evaluate the metabolic profile of human SCs after Phloretin treatment, an NMR assay was conducted. Glucose, glutamine and pyruvate consumption and alanine and lactate production were examined. The results are presented in μmol/10^6 cells (Figure 9). We concluded that human Sertoli cells showed no significant variation between the control and treated groups, regarding the consumption of glucose (7.329 ± 3.4060; 14.070 ± 7.1610), or pyruvate (0.726 ± 0.1588; 0.581 ± 0.1538) and the production of alanine (0.462 ± 0.1331; 0.715 ± 0.2419) and lactate (32.370 ± 4.4070; 36.380 ± 6.6430). In an opposite way, a significant reduction in glutamine consumption was observed in the treated group (0.324 ± 0.2577), when compared to the control group (2.920 ± 0.8120).

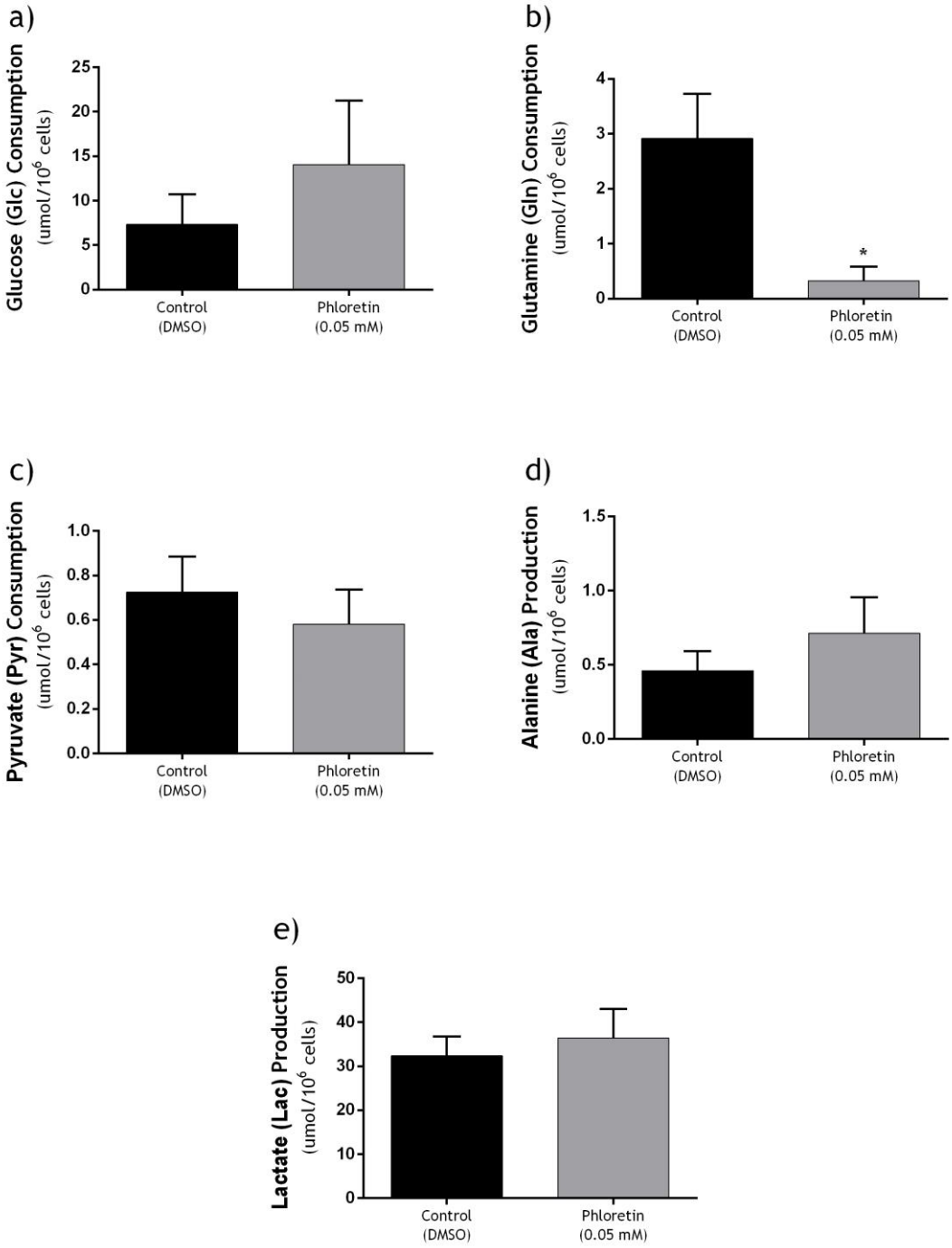

Figure 9: Effect of 24-hours treatment with Phloretin (0.05 mM) on the metabolic profile related to human Sertoli cells. All the results are presented in µmol/10^6 cells and significant results relative to control (p-value < 0.05) are indicated as (*). (a) Evaluation of glucose consumption. (b) Evaluation of glutamine consumption. (c) Evaluation of pyruvate consumption. (d) Evaluation of alanine production. (e) Evaluation of lactate production.

Phloretin does not influence glutaminase protein expression in human Sertoli cells

The significant variation in glutamine consumption observed in the NMR assay led us to examine glutaminase protein expression, since this protein is intimately associated with glutaminolysis. The result is presented in fold variation to control, which was obtained through the measurement of total protein expression using a Ponceau Staining (Figure 10, panel a). An illustrative western blot is also available, evidencing glutaminase protein expression near 73 KDa (Figure 10, panel b). The obtained result did not present any significant variation in the expression of glutaminase between control group (1.000 ± 0.3241) and treated group (1.366 ± 0.2389).

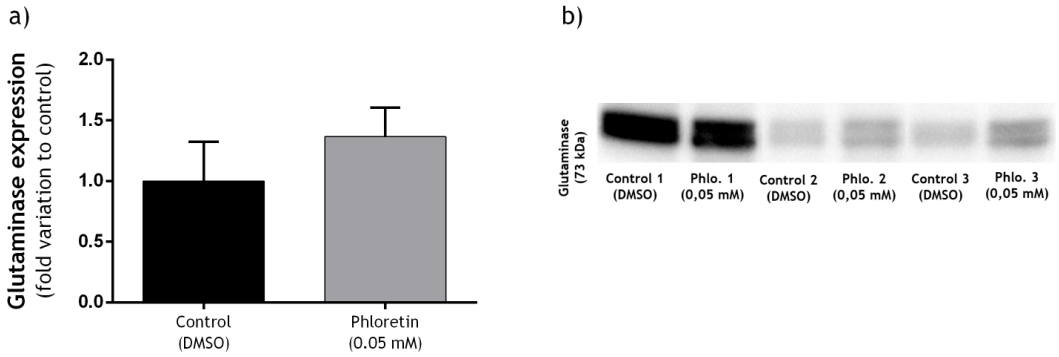

Figure 10: Effect of 24-hours treatment with Phloretin (Phlo., 0.05 mM) in human Sertoli cells glutaminase protein expression. (a) Evaluation of glutaminase expression. Result is expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition), in fold variation to control. (b) Illustrative western blot experiment, regarding three samples.

Cystic Fibrosis Transmembrane Conductance Regulator mRNA is expressed in human Sertoli cells

We evaluated the expression of CFTR mRNA in human SCs. To the best of our knowledge, CFTR mRNA has never been identified in these specific cells. Our results clearly showed that mRNA of CFTR is indeed expressed in human SCs, presenting bands with higher molecular weight (near 250 base pairs) than the positive control (human liver, near 218 base pairs) (Figure 11).

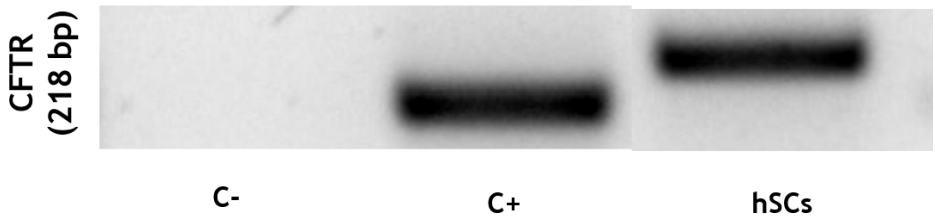

Figure 11: Identification of messenger ribonucleic acid (mRNA) expression of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in human Sertoli cells. Illustrative electrophoresis experiment. C-: negative control (without sample); C+: positive control (human testis); hSCs: human Sertoli cells.

Human Sertoli cells viability remains unaffected after $CFTR_{(inh)}$-172 treatment

To examine the effect of $CFTR_{(inh)}$-172 on the viability of human SCs, cells were exposed to different concentrations of this inhibitor (3 μM; 300 nM and 30 nM) for 24 hours. Afterwards, cellular viability was determined by the MTT assay, in fold variation to control. Our results showed that this inhibitor, at the chosen doses, did not reveal any cytotoxic effects on treated cells, since their viability remained unaffected when compared to the control group (Figure 12). Control and treated groups (3 μM; 300 nM; 30 nM) presented values of 1.00 ± 0.0154; 1.05 ± 0.0471; 0.87 ± 0.0624 and 1.07 ± 0.0285, respectively. Thus, we selected the group exposed to the highest concentration of $CFTR_{(inh)}$-172 (3 μM) to proceed the investigation.

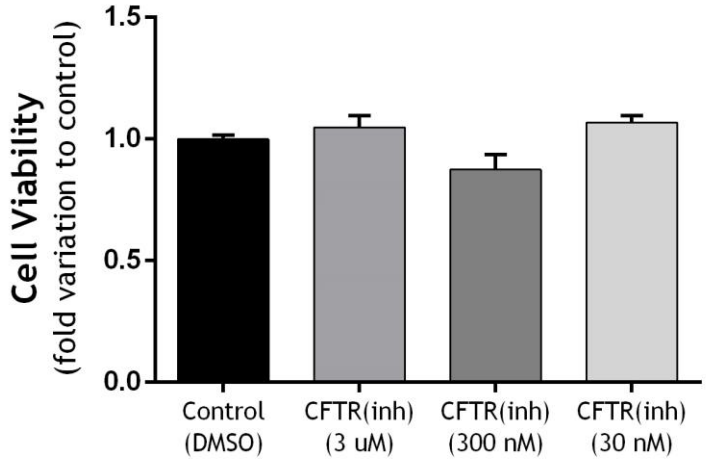

Figure 12: Evaluation of human Sertoli cells viability, after 24-hours treatment with different $CFTR_{(inh)}$-172 concentrations. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition), in fold variation to control.

GATA4 and SOX9 mRNA expression significantly decreases after $CFTR_{(inh)}$-172 treatment

To investigate the influence of $CFTR_{(inh)}$-172 on GATA4 and SOX9 mRNA expression, a qPCR assay was performed. The results showed that $CFTR_{(inh)}$-172 treated human SCs revealed a significant reduction in GATA4 (0.0011 ± 0.0002) and SOX9 (0.0003 ± 6.976e-005) mRNA expression, when compared to their respective control groups (1.0000 ± 0.2823 and 1.0000 ± 0.3378) (Figure 13). This result is similar to that obtained with the Phloretin treated group, which corroborates the possibility of it being due to the existence of a functional CFTR-AQP9 interaction.

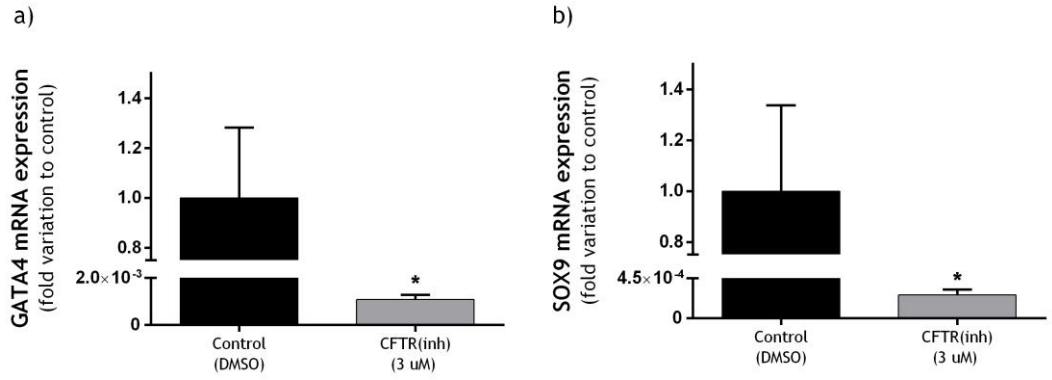

Figure 13: Evaluation of GATA4 and SRY-Box9 (SOX9) messenger ribonucleic acid (mRNA) level in human Sertoli cells, after 24-hours treatment with $CFTR_{(inh)}$-172 (3 µM). Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition), in fold variation to control. Significant results relative to control (p-value < 0.05) are indicated as (*). (a) Evaluation of GATA4 mRNA expression. (b) Evaluation of SOX9 mRNA expression.

Aquaporin-9 protein expression in human Sertoli cells is not affected by $CFTR_{(inh)}$-172 treatment

In order to evaluate the influence of $CFTR_{(inh)}$-172 in AQP9 protein expression in human SCs, a western blot assay was performed and the expressions of total AQP9, glycosylated AQP9 and non-glycosylated AQP9 were analyzed. The results are presented in fold variation to control, which was acquired through the measurement of total protein expression using a Ponceau Staining (Figure 14, panels a, b and c). An illustrative western blot is also presented, highlighting bands expression near 31.5 KDa (Figure 14, panel d). We did not observe any significant variation between the control and treated groups in the expression of glycosylated AQP9 (1.000 ± 0.2682; 0.692 ± 0.1543), non-glycosylated AQP9 (1.000 ± 0.3389; 0.615 ± 0.1526) or total AQP9 (1.000 ± 0.3558; 0.568 ± 0.1043), respectively.

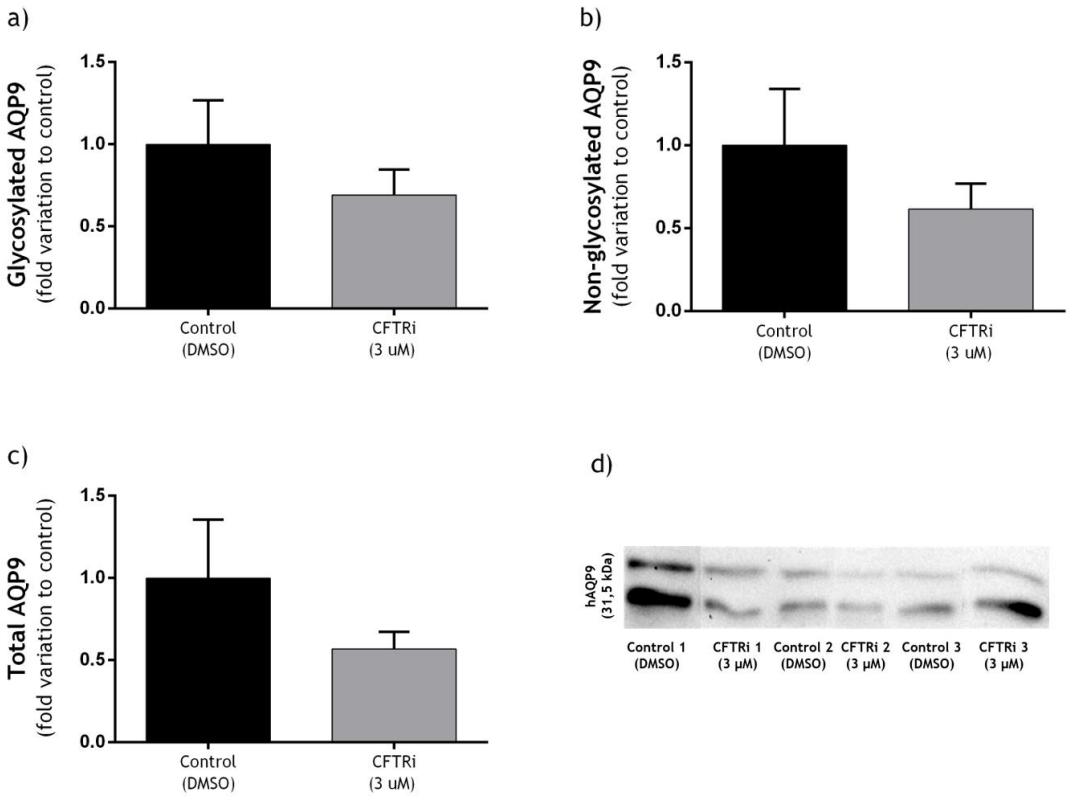

Figure 14: Effect of 24-hours treatment with $CFTR_{(inh)}$-172 (CFTRi, 3 µM) in human Sertoli cells Aquaporin-9 (AQP9) protein expression. Results shown on panels (a), (b) and (c) are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition), in fold variation to control. (a) Glycosylated AQP9 expression. (b) Non-glycosylated AQP9 expression. (c) Total AQP9 expression. (d) Illustrative western blot experiment, regarding three samples.

$CFTR_{(inh)}$-172 does not influence human Sertoli cells metabolic profile

In order to evaluate the metabolic profile of human SCs after $CFTR_{(inh)}$-172 treatment, a NMR assay was conducted. Glucose, glutamine and pyruvate consumption and alanine and lactate production possible variations were analyzed. The results are presented in µmol/10^6 cells (Figure 15). We concluded that human Sertoli cells showed no significant variation between the control and treated groups, regarding the consumption of glucose (7.329 ± 3.406; 16.320 ± 4.413), glutamine (2.920 ± 0.812; 2.436 ± 1.507) or pyruvate (0.726 ± 0.159; 0.664 ± 0.286). Neither the production of alanine (0.462 ± 0.133; 0.528 ± 0.016) and lactate (32.370 ± 4.407; 25.370 ± 3.058) suffered any significant variation.

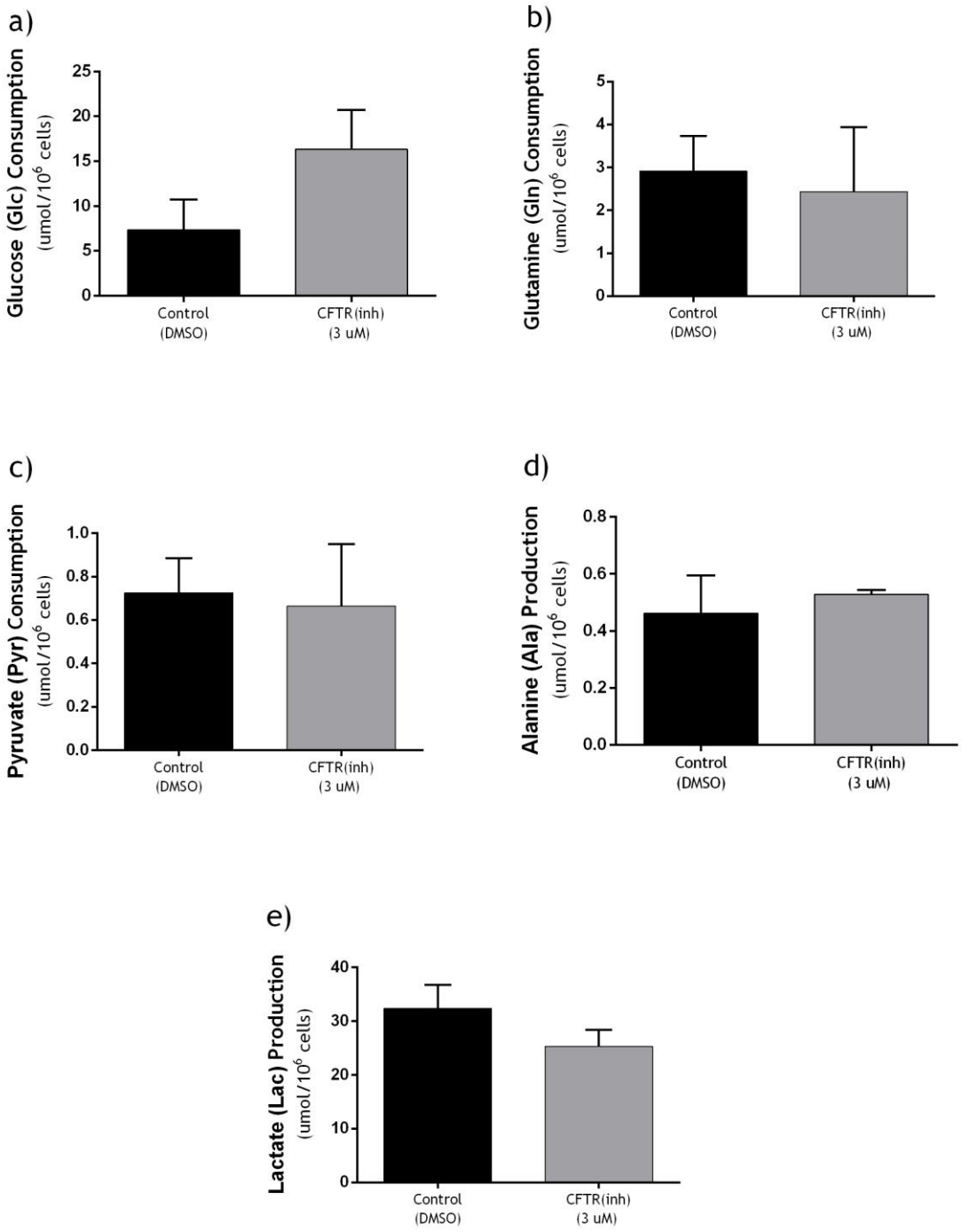

Figure 15: Effect of 24-hours treatment with $CFTR_{(inh)}$-172 (3 µM) on the metabolic profile related to human Sertoli cells. All the results are presented in µmol/10^6 cells. (a) Evaluation of glucose consumption. (b) Evaluation of glutamine consumption. (c) Evaluation of pyruvate consumption. (d) Evaluation of alanine production. (e) Evaluation of lactate production.

$CFTR_{(inh)}$-172 does not influence glutaminase protein expression in human Sertoli cells

The significant variation in glutamine consumption observed in the NMR assay of the Phloretin treated group led us to analyze glutaminase protein expression also in the $CFTR_{(inh)}$-172 group. The result is presented in fold variation to control, which was obtained through the measurement of total protein expression using a Ponceau Staining (Figure 16, panel a). An illustrative western blot is also available, regarding the expression of 73 KDa glutaminase bands (Figure 16, panel b). The obtained result did not show any significant variation in the expression of glutaminase, between control group (1.000 ± 0.3241) and treated group (1.256 ± 0.2514).

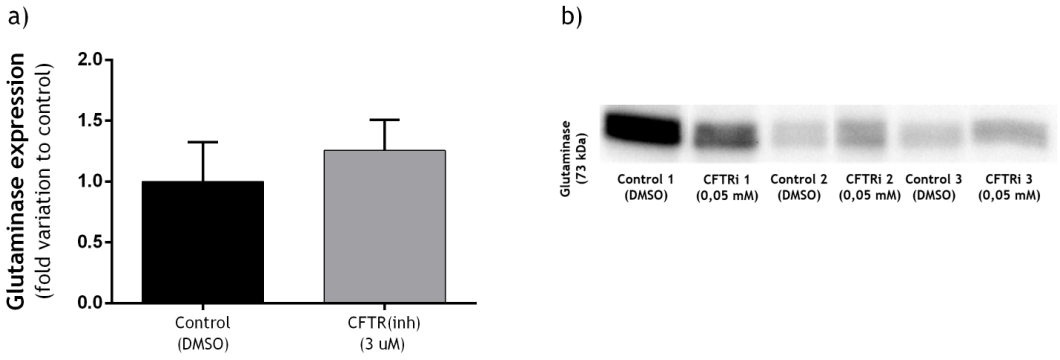

Figure 16: Effect of 24-hours treatment with $CFTR_{(inh)}$-172 (CFTRi, 3 µM) in human Sertoli cells glutaminase protein expression. (a) Evaluation of glutaminase expression. Result is expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition), in fold variation to control. (b) Illustrative western blot experiment, regarding three samples.

Discussion

Despite being a problem that affects millions of couples worldwide, the alarmingly decrease in male fertility over the last few decades have been somewhat overlooked. Such fact caused a lack of knowledge regarding this subject, making it hard to establish an accurate diagnosis for the observed anomalies. However, this trend seems to be changing, since there has never been so much talk about infertility and subfertility as nowadays. To understand male reproductive problems, it is important to realize that male reproductive tissues (and testes, in particular), are highly complex structures, being constituted by several specialized cells [4]. Indeed, testes present two main functions: the synthesis of steroid hormones and the production of male gametes [7]. These two functions present a common goal: the control of spermatogenesis. Spermatogenesis is a biological process that represents the maturation and development of immature germ cells into specialized spermatozoa. Spermatogenesis is supported by SCs, a somatic type of cells that are in close association to germ cells, allowing their differentiation [12].

SCs are epithelial cells located in the seminiferous tubules showing a pivotal role in the support of nutritional and structural development of several germ cells, each. Besides, SCs are also responsible for the establishment and maintenance of the BTB, which presents anatomical, immunological and physiological components, essential for the development of a correct spermatogenesis [21, 22]. Finally, they also regulate the composition of the seminiferous tubular fluid. Indeed, a balanced environment, with proper ionic content, is a pivotal factor to germ cells development. Regarding this topic, SCs have the ability to regulate the mobile physiological buffer bicarbonate, which has been shown to be related with the control of fast changes in pH [32]. All SCs main functions are achieved through several transport proteins, among which CFTR and AQPs should be highlighted.

CFTR is a glycoprotein that acts as a plasma-membrane cAMP-regulated Cl^- channel, responsible for transepithelial Cl^-/HCO_3^- and water transport [41, 42]. CFTR malfunction has been associated with several diseases, such as CF and SD. Its importance in male reproductive system involves *vas deferens*, epididymis and seminiferous tubules [66]. In particular, CFTR expression in SCs is critical, being associated with several other ion channels, protein transporters, receptors and kinases. These interactions include AQPs.

AQPs are hydrophobic, integral membrane channel proteins that permeate water transport across biological membranes, enhancing the movement of solutes in epithelial cells [73]. This family of proteins includes thirteen members, from AQP0 to AQP12, which are divided into three subgroups: the classical AQPs, Aquaglyceroporins and unorthodox AQPs [82]. AQPs show important roles in male reproductive tract, mainly in the composition of the ductal luminal fluids and in the maturation, concentration and storage of spermatozoa in seminiferous vessels. AQPs cooperate with CFTR, an interaction that was already confirmed in the airway epithelial cells, in Xenopus oocytes and, more recently, in epididymal and SCs [94-96].

Furthermore, studies from our group had already highlighted the expression and role of the interaction involving AQP9 and CFTR in SCs.

AQP9 is an Aquaglyceroporin permeable to water, monocarboxylates, polyols, carbamides and pyrimidines [97]. It presents a high expression in brain, specifically in astrocytes. It also reveals a high expression along the male reproductive system, having already been identified in testes, efferent ducts and epididymis. In testes, it is particularly expressed in early germ cells, Leydig cells and SCs. In fact, previous studies proved that AQP9 interacts directly with CFTR in rat SCs, strongly suggesting that CFTR might act as a regulator of AQPs and water homeodynamics in these cells [68].

Starting with this background information, we aimed to define the physiological significance of the interaction between AQP9 and CFTR in human SCs and its implications for male fertility. Since there is a lack of information regarding CFTR-AQP9 interaction in human male reproductive system, to achieve our goal, we started by study AQP9 and CFTR functions independently, using primary cultures of human SCs.

Our results evidenced a high expression of AQP9 mRNA in human SCs. To the best of our knowledge, it was the first time that the mRNA of this water channel was identified in human SCs. To further disclose AQP9 presence, we tried to identify protein expression in these same cells. Using rat liver as positive control, we were able to get important results regarding this point. In fact, previous studies have already identified a two bands AQP9 protein expression: a most abundant and with lower molecular weight band, regarding the non-glycosylated form of the protein, and a less common with higher molecular weight band, regarding the glycosylated form of the protein [117]. It is also important to highlight that our results regarding AQP9 protein expression in human SCs evidenced a band pair with a molecular weight that is almost the double of the expected one. This may be explained by previous studies, showing that the usual tetramer conformation of AQPs is very difficult to break into the monomeric (~31.5 KDa) form. This problem arises from the fact that the 4 monomers constituting the tetramer are connected by very strong bonds. This is a controversial subject, since some studies refer the implication of hydrogen bonds [118, 119], while others implicate disulfide bonds [120, 121] in these connections. The presence of such strong bonds makes it hard to split the tetramer into its monomers and makes the denaturation process partially ineffective. With that said, it is easy to understand why the majority of the studies identified AQPs in an incomplete dimeric form, which is concomitant with the molecular weight (~63 KDa) obtained in our study [122, 123].

To further analyze AQP9 relevance for human SCs viability, we selected three concentrations (0.05 mM, 0.01 mM and 0.005 mM) of an AQP9 inhibitor (Phloretin) and studied its cytotoxic effects through a MTT assay. The results showed that none of the inhibitor concentrations affected treated cells viability. This was an intriguing result since, given the manifest AQP9

expression in human SCs, it was expected that AQP9 activity would be essential to the sustenance of these same cells. In accordance with our results, a previous study regarding the use of Phloretin in the regulation of mouse spermatozoa water channels also verified the absence of differences in cellular viability between Phloretin-treated group and the control group [124]. To understand if there is any compensation process that protects SCs from extreme osmotic variations and, subsequently, from dying, we proceeded our study with the group of cells exposed to the higher concentration of the inhibitor: Phloretin (0.05 mM).

We also evaluated the possible impact of AQP9 inhibition on the differentiation status of SCs. Thus, we determined the variation of GATA4 and SOX9 mRNA expression between control group and Phloretin group. These two biomarkers have been previously shown to perform a very important function in promoting SCs differentiation in mammals, from fetal period until adulthood [115, 116]. In fact, studies regarding loss of function [125] or deletion [126] of *Sox9* induced male-to-female sex reversal. On the other hand, duplication [127] or ectopic expression of this gene in females [128] induced a female-to-male sex reversal. In either these cases, individuals showed to be infertile, highlighting the pivotal role of this gene expression to the correct differentiation of the male reproductive system. Surprisingly, our results revealed that the group of SCs exposed to Phloretin had a significant reduction of both biomarkers mRNA expression, when compared to control. This information is crucial and evidences that human SCs may suffer a reversal in the differentiation process when exposed to the selected concentration of the AQP9 inhibitor at these experimental conditions. Thus, this result points out the pivotal role of AQP9 presence in human SCs for the development of a full male reproductive function.

In order to evaluate if AQP9 protein expression was affected by Phloretin (0.05 mM), a western blot analysis was performed. Bands intensity of glycosylated, non-glycosylated and total AQP9 were evaluated and the results showed no significant differences between the control and the treated group. Although we performed a treatment with an AQP9 inhibitor, this result was expected, since the studies regarding Phloretin action showed that it affects AQP9 activity and not its expression [97]. With that said, the quantity of channels expressed in SCs was supposed to be roughly the same.

Proceeding with the study, we evaluated the metabolic profile of Phloretin-treated cells, as compared with non-treated SCs. Our results revealed that Phloretin (0.05 mM) was able to significantly reduce glutamine consumption in human SCs, although no other metabolite suffered any variation. This result is of major significance, given the pivotal role of glutamine in cells' metabolism. In fact, culture cells usually depend on glutamine as carbon, nitrogen, free energy and reducing equivalents precursors that are necessary to support cells growth and division [129]. The glutamine oxidation has been reported to support much of the required energy of SCs, being involved in anabolic pathways. Finally, glutamine is degraded in the Krebs Cycle to generate ATP [25]. With that said, it is easily understandable that the

significant reduction in glutamine consumption may cease SCs growing and differentiating and, consequently, may also be intimately associated with the reduction of GATA4 and SOX9 mRNA expression in these same cells.

Regarding glutamine variation, we further investigated if a glutaminolysis participant, glutaminase, had any variation in protein expression between the control group and the treated group. Glutaminase is responsible for the conversion of glutamine to glutamate, so the variation on glutamine consumption observed could be caused by a disturbance in glutaminase expression or activity. In our study, western blot experiment did not show any significant differences in glutaminase expression between the groups, which led us to assume that glutamine variation did not result from glutaminase. Further investigations should be performed to better understand the reduced glutamine consumption in the Phloretin treated group.

Concomitantly, an investigation was also performed in order to unveil the possible role of CFTR in maintaining SCs differentiation and functionality. We were able to identify CFTR mRNA expression in human SCs. However, the band did not match the molecular weight observed on the positive control (human testis). This is an interesting result, because after repeated attempts, we always obtained the same result. Since the primers used cover a portion of mRNA codifying region (N-terminus), we believe that we may be in the presence of a new CFTR isoform, specific for human SCs. Additional experiments are needed to consolidate this hypothesis.

To further evaluate mRNA expression results, we tried to identify CFTR protein in human SCs. Curiously, western blot experiments did not allow us this identification, even knowing that we tried to perform the identification with two distinct antibodies. This was an intriguing result, since a previous study was able to identify the expression of CFTR in these same cells, by immunocytochemical analysis [69]. In fact, the two antibodies used in our study were already successfully used in CFTR identification in rat SCs [68]. After further investigation, we figured out that the immunocytochemical study, the only one that was capable to identify CFTR presence in human SCs so far, was performed using an antibody that binds to the C-Terminus of the protein. On the other hand, the antibodies used in our study bind to the N-terminus of CFTR. This result, in accordance to mRNA expression results, made us consider an interesting hypothesis: CFTR expressed in human SCs may present a different nucleotide/amino acid sequence fragment in its N-terminus, which suggests a specific CFTR isoform expressed in these cells.

Then, we evaluated if human SCs viability was affected by treating them with a CFTR inhibitor ($CFTR_{(inh)}$-172), using different concentrations (3 µM, 300 nM and 30 nM). The MTT assay did not evidence any significant differences between the treated groups and the control one. In fact, a previous study already proved that $CFTR_{(inh)}$-172, until a 5 µM concentration,

fully and selectively inhibit CFTR conductance in murine kidney tissues, without affecting cells viability [130]. With that said, we proceeded for the remaining experimental activities with the highest concentration of the inhibitor: $CFTR_{(inh)}$-172 (3 μM).

Following our investigation, GATA4 and SOX9 mRNA expressions were evaluated after CFTR inhibitor treatment and the results were similar to those obtained concerning Phloretin treatment. A significant decrease was observed regarding both biomarkers. This led us to suggest that $CFTR_{(inh)}$-172 provokes SCs to an undifferentiated stage, as previously discussed. We can speculate two possible options: one, in which this decrease in GATA4 and SOX9 mRNA expression is caused by the direct influence of $CFTR_{(inh)}$-172 in the CFTR protein; or a second hypothesis, in which this result is caused by a disruption of the possible interaction between CFTR and AQP9. In fact, it was already proved that rat AQP9 contains a putative PDZ binding motif, in its C-terminus, which binds CFTR C-terminus, through the mediation of ERM Binding Protein 50 (EBP50) [131]. Knowing that the C-Terminus is a conserved catalytic portion of CFTR protein, we can suggest that the inhibition of CFTR may, consequently, inhibit AQP9, leading to a similar outcome of the one obtained with the Phloretin treated group.

In order to evaluate if AQP9 protein expression was affected by $CFTR_{(inh)}$-172 (3 μM), a western blot analysis was performed. Bands intensity of glycosylated, non-glycosylated and total AQP9 did not show any significant differences between the control group and the treated group. This result indicates that inhibiting CFTR does not affect AQP9 protein expression. This is an understandable result, since $CFTR_{(inh)}$-172 was shown to affect the CFTR gating mechanism and not CFTR or AQP9 protein expression [132].

Further analysis of the metabolic profile did not present any significant result, regarding the metabolites aforementioned. This result may be justified by knowing that cells were able to maintain their viability in the treated group. Although no significant results were obtained regarding glutamine consumption, we further evaluated, in similarity to Phloretin group, if glutaminase protein expression had suffered any variation. Our results did not evidence any significant variation in this protein, which is in accordance with the result obtained regarding glutamine expression in the $CFTR_{(inh)}$-172 treated group.

In summary, this study examined the role of AQP9 and CFTR in primary cultures of human SCs. To the best of our knowledge, we are one of the few groups that have explored the important function and cooperation between AQP9 and CFTR in the development of SCs, spermatogenesis and, lastly, male fertility. With the results obtained in this project, we expected to answer some crucial questions regarding AQP9-CFTR interaction in human reproductive system. Previous studies focused on AQPs interaction with CFTR already proved that these proteins are key members in a healthy organism. Specifically, investigation regarding AQP9-CFTR interaction in rat SCs and epididymal cells evidenced a consistent role of these proteins in male reproductive system [68, 95]. Although observations in primary

cultures may not exactly represent an *in vivo* organism, the results presented in our study are of great significance and represent a huge step to elucidate the complex role of SCs (and, consequently, of the BTB) in developing a testicular homeostatic environment. We believe that further studies regarding AQP9 and CFTR in humans may be the key to the development of new therapeutic technics to fight some of the most serious problems threatening human species. This study is just the beginning of the strap, but we believe that it will attract the attention to a global scale problem: male infertility.

Conclusions

Male fertility is becoming a trending topic, given the terrifyingly raise on infertility cases. Although the majority of these situations have an unknown etiology, it is understandable that the source of male healthy reproduction is intimately associated with a correct development and function of the testes. Testes are two major organs that support the development of spermatogenesis, which represents the growth of immature germ cells into spermatozoa by close association with the somatic SCs. In fact, SCs are responsible for protecting and supporting germ cells and establishing the functional unit of the BTB. They also reveal a pivotal role, controlling the balance of the seminiferous tubular fluid composition. To regulate the seminiferous tubules content, SCs features several interactions with different partners, including CFTR and AQPs channels.

The glycoprotein CFTR acts as a plasma-membrane Cl^-/HCO_3^- channel, being responsible for transepithelial salt and water transport. CFTR mutations originate severe reproductive problems, like CBAVD. In addition to its main role, CFTR also interacts with several other membrane protein channels, in which AQPs are included. AQPs represent a family of thirteen water channels that permeate water and other metabolites across biological membranes. All AQP isoforms, apart from AQP12, were proved to be expressed in the testes. Among them, AQP9 must be pointed out, being one of the most abundant isoforms in male reproductive system and, particularly, in SCs. Recent studies pointed out the pivotal interaction between CFTR and AQP9.

In this study, we aimed to take the first step regarding CFTR and AQP9 function (and possible cooperation) in human SCs. This investigation followed previous discoveries achieved by our group, regarding AQP9-CFTR interactome presence in rat SCs. The results presented here are quite interesting. We were able to identify, for the first time, AQP9 mRNA and protein expression in human SCs. Using an AQP9 inhibitor at a specific concentration (Phloretin, 0.05 mM), we observed a highly decrease in GATA4 and SOX9 mRNA expression which, associated with a decrease in glutamine consumption by treated cells, represents an undifferentiation process developed by SCs. This process could culminate in infertility in an *in vivo* mammalian. No changes were observed in the protein expression of AQP9, neither of glutaminase. In other hand, we were also able to identify CFTR mRNA expression. This result, in association with the one obtained by the trials developed to identify CFTR protein, truly indicates that human SCs may present a CFTR isoform different from the ones previously described. Using the $CFTR_{(inh)}$-172 as a CFTR inhibitor for further investigation and after setting the concentration of 3 μM, we obtained a curious result. GATA4 and SOX9 mRNA expression had, in similarity with Phloretin treated group, a highly decrease in their expression. This result may be a consequence of the direct inhibition of CFTR or the indirect inhibition of AQP9 by the association of AQP9 with CFTR. No metabolic variation was observed, neither in AQP9 nor in glutaminase protein expression.

In conclusion, this is a pioneer study which raises several questions that need to be attended. Further investigation should unveil the cause of reduced glutamine consumption in Phloretin treated group; the cause of GATA4 and SOX9 decreased expression in Phloretin and $CFTR_{(inh)}$-172 treated groups; and the nucleotide and protein sequence of CFTR in human SCs. Concomitantly, an investigation on SCs histology after Phloretin and $CFTR_{(inh)}$-172 treatment should be performed, as well as a co-immunoprecipitation assay, in order to confirm the possible interaction between AQP9 and CFTR proteins.

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