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Bicarbonate Transporters in Male Fertility: Identification and Functionality in Testicular Cells

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O conteúdo do presente trabalho é da exclusiva responsabilidade do autor:

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(Raquel Alexandra Lages Bernardino)

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Resumo

A formação de espermatozóides competentes é um processo complexo dependente do ambiente criado ao longo do trato reprodutor masculino. A regulação do conteúdo iónico dos fluidos luminais é essencial para a maturação dos espermatozóides. O bicarbonato é essencial não só para a homeostase iónica, como também tem um papel fundamental na manutenção do pH dos diversos fluidos ao longo do trato reprodutor masculino.

A *diabetes mellitus* (DM) representa uma das maiores ameaças à saúde na sociedade moderna, e afeta cada vez mais homens em idade reprodutiva. A DM é uma doença metabólica caracterizada por hiperglicémia, resultante de defeitos na secreção e/ou ação da insulina. Esta doença pode ser dividida, maioritariamente, em dois subtipos, tipo 1 e tipo 2 (T2DM). A T2DM é conhecida como a diabetes não-insulino-dependente, e inclui indivíduos com resistência à insulina, em que geralmente a secreção de insulina é insuficiente. Este tipo de diabetes pode ser prevenido se for detetado precocemente, no estado de pré-diabetes, que usualmente antecede o aparecimento desta doença.

Tem sido descrito que a DM afecta a regulação do pH intracelular (pHi) em células de mamíferos, principalmente devido à alteração significativa da atividade de alguns transportadores iónicos, particularmente de alguns mecanismos de transporte dependentes de bicarbonato. Pouco se sabe, no entanto, sobre os efeitos desta patologia nos mecanismos de transporte de membrana envolvidos na regulação do pH em células do trato reprodutor masculino, e ainda menos se sabe sobre os efeitos das diferentes fases envolvidas na progressão desta patologia, particularmente do pré-diabetes.

O primeiro objetivo deste trabalho foi analisar possíveis alterações nos níveis dos transportadores de bicarbonato mais relevantes da família Slc4 (trocador aniónico 2 -AE2; trocador Cl^-/HCO_3^- dependente de Na^+ - NDCBE; Na^+/HCO_3^- co-transportador eletrogénico 1 - NBCe1; Na^+/HCO_3^- co-transportador eletroneutro 1 - NBCn1) nos testículos e epidídimos de um modelo animal de pré-diabetes. Foram avaliados os níveis de expressão de proteína e mRNA por western blot e real-time PCR, respetivamente. Assim, foi possível confirmar a presença de todos estes transportadores de bicarbonato da família Slc4 em testículo e epidídimo. A nível testicular, embora não tenham sido detetadas alterações na expressão de proteína, os níveis de mRNA de NBCe1, NBCn1 e NDCBE encontraram-se significativamente aumentados em animais pré-diabéticos. Por outro lado, a nível epididimal, a condição pré-diabética causou um aumento significativo nos níveis proteicos de AE2 e uma diminuição significativa nos níveis de proteína de NDCBE. Estas variações poderão traduzir-se em alterações no fluxo transepitelial de HCO_3^- no epidídimo *in vivo*, que pode comprometer a sobrevivência dos

espermatozoides durante o seu armazenamento e maturação. Deste modo, os nossos resultados podem correlacionar-se com resultados descritos anteriormente, que demonstraram um aumento significativo na anormalidade morfológica em espermatozoides de ratos pré-diabéticos.

Por outro lado, vários estudos apresentam uma associação direta entre homens com altos níveis de 17ß-estradiol (E_2) e o aumento do risco de diabetes e, para além disso, o E_2 é responsável pela modulação da expressão de transportadores iónicos específicos no trato reprodutor masculino. Assim, o segundo objetivo do nosso trabalho foi determinar o efeito desta hormona esteróide sexual na expressão e funcionalidade dos transportadores de bicarbonato selecionados da família Slc4, em culturas de células de Sertoli (SCs). Os quatro transportadores estudados foram identificados e quantificados nas SCs (usando RT-PCR e real time PCR, respetivamente). Nas células tratadas com E_2 (100 nM) foi observado um aumento significativo nos níveis de expressão de mRNA de AE2, NBCn1 e NBCe1. Posteriormente, foi também avaliado o efeito do E_2 (100 nM) no transporte transcelular em SCs cultivadas em suportes semipermeáveis, usando a técnica de Voltage-Clamp. As SCs tratadas com E_2 apresentaram alterações significativas na variação da corrente de curto-circuito (ΔI_{sc}) induzida por Adenosine-5-triphosphate (ATP), bem como na recuperação dessa corrente de curto-circuito (I_{sc}) após estimulação com ATP. Estas alterações poderão ser promovidas pelo aumento da expressão de AE2 observados em células tratadas com E_2, visto que foi descrito que nestas células o I_{sc} envolve a secreção do Cl^- através da membrana apical por canais de Cl^- ativados pelo ATP, e a sua absorção através da membrana basolateral por mecanismos dependentes de HCO_3^-. Um aumento dos níveis de AE2 certamente será responsável por promover a variação de I_{sc} após a estimulação com ATP.

Assim, os nossos resultados mostram uma relação do pré-diabetes, assim como do aumento dos níveis de E_2, com a expressão/função dos transportadores de bicarbonato em epidídimo e SCs de rato, fornecendo novas evidências sobre os mecanismos pelos quais esta fase precoce da DM e algumas das suas características podem afetar a função reprodutivas masculina.

Palavras- Chave:

Transportadores de bicarbonato, fertilidade masculina, transportadores membranares, *diabetes mellitus*, estrogénios.

Resumo Alargado

O estabelecimento da fertilidade masculina envolve processos complexos que requerem a interação entre diferentes tecidos do trato reprodutor masculino. Nos mamíferos, os testículos são os elementos centrais do sistema reprodutor masculino, estando envolvidos na síntese de hormonas esteróides e na produção de gâmetas masculinos, os espermatozóides. A formação de espermatozóides competentes é um processo complexo dependente dos ambientes estabelecidos ao longo do trato reprodutor masculino. A regulação das propriedades iónicas do conteúdo dos diversos fluidos luminais é essencial para a maturação dos espermatozóides, bem como a regulação do seu pH. No interior dos túbulos seminíferos a espermatogénese é suportada pelas células de Sertoli (SCs), que promovem o suporte físico e nutricional das espermatogónias, espermatócitos, espermatídios e finalmente os espermatozóides, que são libertados no lúmen dos túbulos seminíferos. As SCs regulam entre outras coisas, a passagem de iões, água e metabolitos energéticos para o lúmen tubular. Depois de completa a espermatogénese, os espermatozóides são transportados até ao epidídimo, que com o seu microambiente luminal ajuda a transformar os espermatozóides imaturos e sem mobilidade em células competentes e capazes de fertilizar.

A normal ocorrência dos processos celulares necessita que o pH intracelular (pHi) e extracelular (pHo) sejam mantidos dentro de limites estreitos. O ajustamento do pH dos fluidos corporais é de extrema importância para uma função normal de todas as células e tecidos. Sabe-se que os transportadores de bicarbonato são de elevada importância para a regulação do pHi e pHo dos fluidos de muitos tecidos. Na realidade, na maioria dos tecidos, o bicarbonato é essencial não apenas para a manutenção do pH, mas tem também um papel fundamental na homeostase iónica e osmolaridade, e os tecidos reprodutivos masculinos não serão a exceção.

A *diabetes mellitus* (DM) representa uma das maiores ameaças à saúde na sociedade moderna. A sua incidência tem vindo a aumentar drasticamente e afeta cada vez mais pessoas jovens, ainda em idade reprodutiva. A DM é uma doença metabólica caracterizada por hiperglicémia, resultante de defeitos na secreção e/ou ação da insulina. Esta doença pode ser dividida em dois tipos, tipo 1 (T1DM) e tipo 2 (T2DM), ambos relacionados com várias complicações sistémicas. A T1DM geralmente desenvolve-se em idade jovem, e é causada pela destruição autoimune das células ß do pâncreas. Requer uma terapia de reposição diária de insulina. A T2DM é conhecida como a diabetes não-insulino-dependente, e ocorre quando a produção de insulina pelas células ß não é suficiente para manter os níveis fisiológicos no sangue. Este tipo de diabetes pode ser prevenido se for detetado precocemente, no estado de pré-diabetes, que usualmente antecede o aparecimento desta doença. A transição de um

estado de pré-diabetes para T2DM ocorre quando a capacidade secretora das células ß não é capaz de compensar a resistência à insulina.

Tem sido descrito que a DM altera a regulação do pHi em células de mamíferos, principalmente, devido à alteração significativa da actividade de alguns transportadores iónicos, nomeadamente de alguns mecanismos de transporte dependentes de bicarbonato. No entanto, pouco se sabe sobre os efeitos desta patologia nos mecanismos de transporte de membrana envolvidos na regulação do pH nas células do trato reprodutor masculino, e ainda menos se sabe sobre os efeitos das diferentes fases envolvidas na progressão desta patologia, particularmente o pré-diabetes.

O primeiro objetivo deste trabalho foi analisar possíveis alterações nos níveis dos transportadores de bicarbonato mais relevantes da família Slc4 (trocador aniónico 2 -AE2; trocador Cl^-/HCO_3^- dependente de Na^+ - NDCBE; Na^+/HCO_3^- co-transportador eletrogénico 1 - NBCe1; Na^+/HCO_3^- co-transportador eletroneutro 1 - NBCn1) nos testículos e epidídimos de um modelo animal de pré-diabetes. Foram avaliados os níveis de expressão de proteína e mRNA por western blot e real-time PCR, respetivamente. Assim, confirmamos a presença de todos estes transportadores de bicarbonato da família Slc4 em testículo e epidídimo. A nível testicular, embora não tenham sido detetadas alterações na expressão de proteína, os níveis de mRNA de NBCe1, NBCn1 e NDCBE encontravam-se significativamente aumentados em animais pré-diabéticos. Por outro lado, a nível epididimal, o pré-diabetes causa um aumento significativo nos níveis proteicos de AE2 e uma diminuição significativa nos níveis de proteína de NDCBE. Estas variações poderão traduzir-se em alterações no fluxo transepitelial de HCO_3^- no epidídimo *in vivo*, que podem comprometer a sobrevivência dos espermatozóides durante o seu armazenamento e maturação. Deste modo, os nossos resultados podem correlacionar-se com resultados descritos anteriormente, que demonstraram um aumento significativo na anormalidade morfológica em espermatozoides de ratos pré-diabéticos.

Por outro lado, vários estudos apresentam uma associação entre homens com elevados níveis de 17ß-estradiol (E_2) e o aumento do risco de diabetes e, para além disso, o E_2 é responsável pela modulação da expressão de transportadores iónicos específicos no trato reprodutor masculino. Assim, o segundo objetivo do nosso trabalho foi determinar o efeito desta hormona esteróide sexual na expressão e funcionalidade dos transportadores de bicarbonato selecionados da família Slc4, em culturas de SCs de rato. Os quatro transportadores estudados foram identificados e quantificados nas SCs (usando RT-PCR e real time PCR, respetivamente). Nas células tratadas com E_2 (100 nM) foi observado um aumento significativo nos níveis de expressão de mRNA de AE2, NBCn1 e NBCe1. Posteriormente, também foi avaliado o efeito do E_2 (100 nM) no transporte transcelular em SCs cultivadas em suportes semipermeáveis, usando a técnica de Voltagem-Controlada. As SCs tratadas com E_2 apresentaram alterações significativas na variação da corrente de curto-circuito induzidas por ATP (ΔI_{sc}), bem como na recuperação da corrente de curto-circuito (I_{sc}) depois da estimulação

com ATP. Estas alterações podem ser promovidas pelo aumento reportado nos níveis de AE2 observados em células tratadas com E_2, visto que nestas células o I_{sc} envolve a secreção do Cl^- através da membrana apical, através de canais de Cl^- ativados por ATP, e a sua absorção através da membrana basolateral por mecanismos dependentes de HCO_3^-. Um aumento dos níveis de AE2 será certamente responsável por promover a variação de I_{sc} após a estimulação com ATP.

Assim, os nossos resultados mostram uma relação do pré-diabetes, assim como do aumento dos níveis de E_2, com a expressão/função dos transportadores de bicarbonato em epidídimo e SCs de rato, fornecendo novas evidências sobre os mecanismos pelos quais esta fase precoce da DM e algumas das suas características podem afetar a função reprodutivas masculina.

Abstract

The formation of competent spermatozoa is a complex event that depends on the establishment of adequate environments throughout the male reproductive tract. The maintenance of a proper ionic content in the luminal *milieus* is crucial for spermatozoa maturation. Bicarbonate is not only essential to ionic homeostasis, as HCO_3^- concentration plays an essential role in the pH maintenance along the male reproductive tract.

Diabetes mellitus (DM) is one the most prominent public health threats in modern societies and its incidence is drastically increasing in men with reproductive age. This metabolic disease is characterized by hyperglycaemia that can result from defects in insulin secretion and/or insulin action. There are two types of DM, type-1 DM and type-2 DM (T2DM). T2DM is referred to as non-insulin-dependent diabetes, and encompasses individuals who have insulin resistance and usually have a relative insufficient insulin secretion. This type of diabetes can be prevented if detected early, in a status called pre-diabetes, which usually precedes the appearance of the disease.

It has been reported that DM alters pHi regulation in mammalian cells mainly by markedly altering the activity in some ion transporters, particularly some bicarbonate-dependent mechanisms. Little is known on the effects of this pathology on the membrane transport mechanisms involved in pH regulation on male reproductive tract cells and even fewer on the effects of the different stages involved in the progression of this pathology, particularly during pre-diabetes.

The first objective of this work was to analyse possible alterations on the levels of the most relevant bicarbonate transporters of the Slc4 family (anion exchanger 2 -AE2; Na^+-driven Cl^-/HCO_3^- exchanger - NDCBE; electrogenic Na^+/HCO_3^- co-transporter 1- NBCe1; electroneutral Na^+/HCO_3^- co-transporter 1 - NBCn1) in testis and epididymis of a pre-diabetic animal model. Protein and mRNA expression levels were evaluated by western blot and real-time PCR, respectively. We were able to confirm the presence of all the bicarbonate transporters of the Slc4 family studied both in testis and epididymis. At testicular level, although no alterations were detected in protein expression, the mRNA levels of NBCe1, NBCn1 and NDCBE were significantly increased in pre-diabetic animals. On the other hand, at epididymal level, pre-diabetes caused a significant increase on AE2 protein levels and a significant decrease of NDCBE protein levels. Hence, these alterations might translate into changes of the HCO_3^- transepithelial epididymal fluxes *in vivo*, which might represent a threat for sperm survival during storage in the epididymis. Our results might correlate with previous results that reported a significant increase in abnormal sperm morphology in pre-diabetic rats.

Furthermore, as several studies support an association of men with higher 17ß-estradiol (E_2) levels and the increased risk of diabetes and, moreover, E_2 is responsible for the modulation of the expression of specific ion transporters in the male reproductive tract, the second objective of our work was to determine the effect of this sex steroid hormone on the expression and functionality of selected bicarbonate transporters of the Slc4 family in cultured Sertoli cells (SCs). All the selected four transporters were identified and quantified in SCs (using RT-PCR and real time PCR, respectively). In cells treated with E_2 (100 nM) a significant increase in mRNA expression levels of AE2, NBCn1 and NBCe1 was observed. Subsequently, we also evaluated the effect of E_2 (100 nM) on transcellular transport in SCs, grown in semi-permeable supports, using the Voltage-Clamp technique. E_2-treated SCs presented a significant alteration on the shift of the short-circuit current (ΔI_{sc}) induced by ATP, as well as on short-circuit current (I_{sc}) recovery after stimulation. These alterations may be promoted by the increase of AE2 mRNA levels observed in E2-treated cells, as in these cells the I_{sc} involves the secretion of Cl^- through the apical membrane by an ATP-activated Cl^- conductance and its absorption via HCO_3^--dependent mechanisms through the basolateral membrane. An increase on AE2 levels will surely be responsible for a prompter effect of this transporter on I_{sc} variation following ATP activation.

Thus, our results show a relation of the pre-diabetes, as well as increased E_2 levels, with the expression/function of bicarbonate transporters in rat epididymis and SCs, providing new evidence on the mechanisms by which this prodromal stage of DM and its associated features can affect male reproductive function.

Keywords:

Bicarbonate transporters, male fertility, membrane transporters, *diabetes mellitus*, estrogens.

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Abbreviations

AE – Anion exchanger

AUCg - Area under the curve

ATP – Adenosine-5-triphosphate

BTB – Blood testes barrier

CA – Carbonic anhydrase

CFTR – Cystic fibrosis transmembrane

CLD – Congenital chloride diarrhea

CSF – Cerebrospinal fluid

DM – Diabetes mellitus

DMEM: Ham's F12 - Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12

DRA – Down – regulated in adenoma

E_2 - 17ß-Estradiol

EDTA – Ethylene diamine tetra acetic acid

EtOH- Etanol

ERα – Estrogen receptor α

ERß - Estrogen receptor ß

ErKO – ER knockout mouse

EST – Expressed sequence tags

FBS – Fetal bovine serum

FSH – Follicle – stimulating hormone

GPER30 – G-Protein coupled receptor 30

GnRH – Gonadotropin releasing hormone

HBSS - Hank's Balanced Salts Solution

HED – High energy diet

HPT – Hipothalamic-pituitary testis

I_{sc} – Short -circuit current

ITS supplement - Insulin-Transferrin-Sodium Selenite supplement

LH – Luteinizing hormone

M-MLV RT - Moloney Murine Leukemia Virus Reverse Transcriptase

mRNA - Messenger Ribonucleic Acid

NBCe – Electrogenic Na^+/HCO_3^- cotransporters

NBCn – Electroneutral Na^+/HCO_3^- cotransporters

NCBe - Electrogenic Na^+ - coupled HCO_3^- Transporters

NCBn - Electroneutral Na^+ - coupled HCO_3^- Transporters

NCBE - Na^+ - coupled HCO_3^- exchanger

NCBTs - Na^+ - coupled HCO_3^- Transporters

NDCBE - Na^+ - driven Cl^-/HCO_3^- exchanger

NHE3 – Na+/H+ exchanger 3

P450arom – Aromatase enzyme cytochrome P450

PAT-1 – Putative anion transporter 1

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDS- Pendred syndrome

pHi - intracellular pH

pHo – extracellular pH

qPCR -Real-time PCR

RIPA - Radio-Immunoprecipitation Assay

RNAt - total RNA

RTF – Rete testis fluid

sAC – Soluble adenylyl cyclase

Slc4 – Solute carrier 4

Slc26 – Solute carrier 26

SCs- Sertoli cells

STF- Seminiferous tubular fluid

T1DM – Type 1 Diabetes Mellitus

T2DM – Type 2 Diabetes Mellitus

TBS- Tris-buffered saline solution

WHO- World Health Organization

I. Introduction

1. General aspects

The establishment of male fertility is a complex process that requires concerted interactions between different tissues of the male reproductive tract and accessory glands, and between the different cell types that compose these organs. The male reproductive tract is composed of highly heterogeneous tissues, including testis, efferent ducts, epididymis and vas deferens (Figure 1) (R. Jones & Murdoch, 1996; Orgebin-Crist & Davies, 2003; Pastor-Soler, Piétrement, & Breton, 2005). The mammalian testis is a complex organ, divided in compartments, testicular lobules and each lobule encloses coiled seminiferous tubules (Shubhada, Glinz, & Lamb, 1993; W.H. Walker & Cheng, 2005), which contain the Sertoli cells (SCs) and the germinal cells in different development stages. These tubules are avascular and no nerves penetrate through their walls (B. Setchell, 1986). Besides, the lobules formed by seminiferous tubules, are separated by extensions of the tunica albuginea, that open on both ends into the rete testis (Figure 1) (Pastor-Soler et al., 2005; Saladin, 2003).

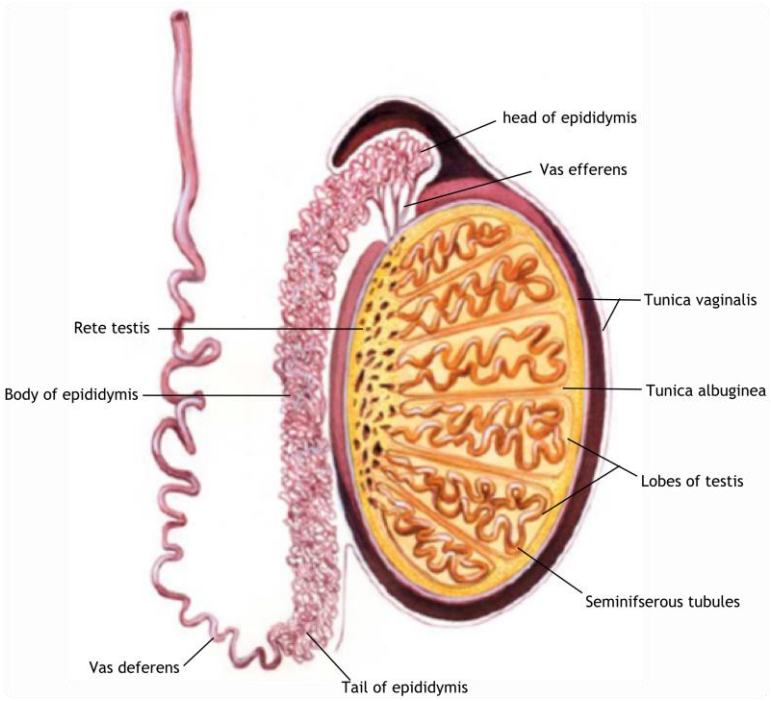

Figure 1: Schematic representation of the mammalian testes and epididymis. The testis is encapsulated by two layers: the tunica vaginalis that is the most outer tunic; and tunica albuginea that divides the testicles into lobules, filled by seminiferous tubules. The seminiferous tubules end in the rete testis, which converge to vas efferens that is connected to the epididymis. The epididymis is attached to the vas deferens and can be divided into three sections: head, body and tail. Adapted from Pastor-Soler et al. (2005).

The interstitial space of the testis, that comprises all the spaces between the seminiferous tubules, contains all the blood and lymphatic vessels, which are essential for the

movement of hormones and nutrients into, and out of the testis (O'donnell, Robertson, Jones, & Simpson, 2001). In this interstitium we can also find the nerves, the Leydig cells which are the primary sites of steroidogenesis in the testis, and a significant population of macrophages (B. Setchell, 1986).

The testes perform two main functions: synthesis of steroid hormones and production of spermatozoa in a process called spermatogenesis (S. Carreau, Genissel, Bilinska, & Levallet, 1999; L. Rato, Socorro, Cavaco, & Oliveira, 2010; Saez, 1994). Indeed, a chain of complex local interactions involving the various testicular cells types, such as germ, Sertoli, peritubular and Leydig cells are responsible for the control of spermatogenesis (Shubhada et al., 1993; W.H. Walker & Cheng, 2005). Within the seminiferous tubules, the SCs reside on the basement membrane, under which are the lymphatic endothelium and the peritubular myoid cells (Dym & Fawcett, 1970). The SC plays a central role in the development of functional testis and, subsequently, in the manifestation of a male phenotype (Mruk & Cheng, 2004; R. M. Sharpe, McKinnell, Kivlin, & Fisher, 2003). Without the physical and metabolic support of the SCs, germ cell differentiation, meiosis and transformation into spermatozoa would not occur (R. Sharpe, 1994; R. M. Sharpe et al., 2003). It is well known that normal testicular development and maintenance of spermatogenesis are controlled by gonadotrophins and testosterone whose effects are modulated by locally produced factors. Moreover, estrogens are also pivotal to this process (S. Carreau et al., 1999; Saez, 1994).

Adjacent SCs form tight junctions with each other to form a basal and adluminal compartment. In these compartments, meiotic and post-meiotic steps of spermatogenesis proceed and occurs the formation of a fluid-filled lumen (Dym & Fawcett, 1970). As a result, the developing germ cells present in the adluminal compartment become effectively protected from direct access to plasma components and thus become dependent on the secretion of factors by the SC (L. Rato et al., 2012; R. Sharpe, 1994; R. M. Sharpe et al., 2003). The structural basis of the tubular barrier has been well characterized and has been reported to mainly reside in the specialized junctions between pairs of SCs (Dym, 1973; Dym & Fawcett, 1970). SCs also control the composition of the seminiferous tubular fluid (STF) and the physicochemical milieu where spermatogenesis occurs (L. Rato et al., 2010). These cells regulate, among other things, the passage of ions and the selective flow of water, steroids and carbohydrates into the tubular lumen (Abraham, 1991). After completion of spermatogenesis within the seminiferous tubules, spermatozoa are carried to the rete testis and from there across the efferent ductules to the epididymis. This movement is carried out by cilliary movement in the efferent ductules, for muscle contraction, and for fluid flow (Saladin, 2003). The efferent ductules concentrate the dilute testicular fluid and spermatozoa by reabsorbing approximately 90% of the fluid secreted by the testes (Clulow, Jones, Hansen, & Man, 1998; Newcombe, Clulow, Man, & Jones, 2000).

The functional aspects of the testes are very complex, its normal function depends on the vascular system delivering oxygen, nutrients and hormones into testicular interstitial fluid and removing waste and secretory products (Bergh & Damber, 1993). All these processes are dependent on numerous factors, which act in cascade, and any anatomical, physiological, hormonal or electrolytic abnormality can change reproductive parameters.

2. Spermatogenesis and hormonal regulation

Spermatogenesis is a process controlled by a network of endocrine and other regulatory factors (L. Rato et al., 2012; Verhoeven, Willems, Denolet, Swinnen, & De Gendt, 2010; W.H. Walker, 2011) in which immature germ cells undergo division, meiosis and differentiation to give rise to mature cells (M.G. Alves et al.,2013a; L. Rato et al., 2010). This process occurs in seminiferous tubules, the functional unities of the testis, through close association of germ cells with the epithelial somatic cells, the SCs (O'donnell et al., 2001; L. Rato et al., 2010; Shubhada et al., 1993; W.H. Walker & Cheng, 2005). The spermatogenesis can be divided into four different phases (Saladin, 2003) that include mitosis, meiosis, spermiogenesis and spermiation (Cheng, Wong, Yan, & Mruk, 2010). Spermatogonia are the immature germ cells in the testis, and include type A and type B spermatogonia, the last of which are committed to differentiation. The spermatogonia stay in basal compartment, in touch with the SCs. The spermatogonia migrate between SCs to the adluminal compartment, where undergo numerous mitotic cycles (L. D. Russell, Ettlin, Hikim, & Clegg, 1993; L. Rato et al., 2012; Saladin, 2003). After the last mitosis of type B spermatogonia, primary spermatocytes are formed (L. D. Russell et al., 1993). These cells replicate their DNA and thus initiating meiosis (O'donnell et al., 2001). They undergo the first meiotic division to yield secondary spermatocyte, that in turn undergo second meiotic division and to yield the haploid round spermatid (O'donnell et al., 2001). Spermiogenesis is the final stage of spermatogenesis, in which the maturation of spermatids into spermatozoa occurs. (L. D. Russell et al., 1993). Briefly, spermatids undergo morphological changes such as the establishment of the flagellum, the formation of the acrosome and the elongation of the nucleus (Figure 2) (M.G. Alves et al., 2013b). A great part of the cytoplasm is also eliminated and the chromatin is gradually condensed together with the changes of histones by transition proteins and then by protamines (Zini & Agarwal, 2011). When germ cell development is complete, the spermatozoa are released from the SCs into the tubule lumen (spermiation), and proceed through the rete testis, until they enter the epididymis via the efferent ducts. During passage through the epididymis, the spermatozoa undergo a series of biochemical changes to become motile and capable of fertilization (R. Jones & Murdoch, 1996; O'donnell et al., 2001; Orgebin-Crist & Davies, 2003).

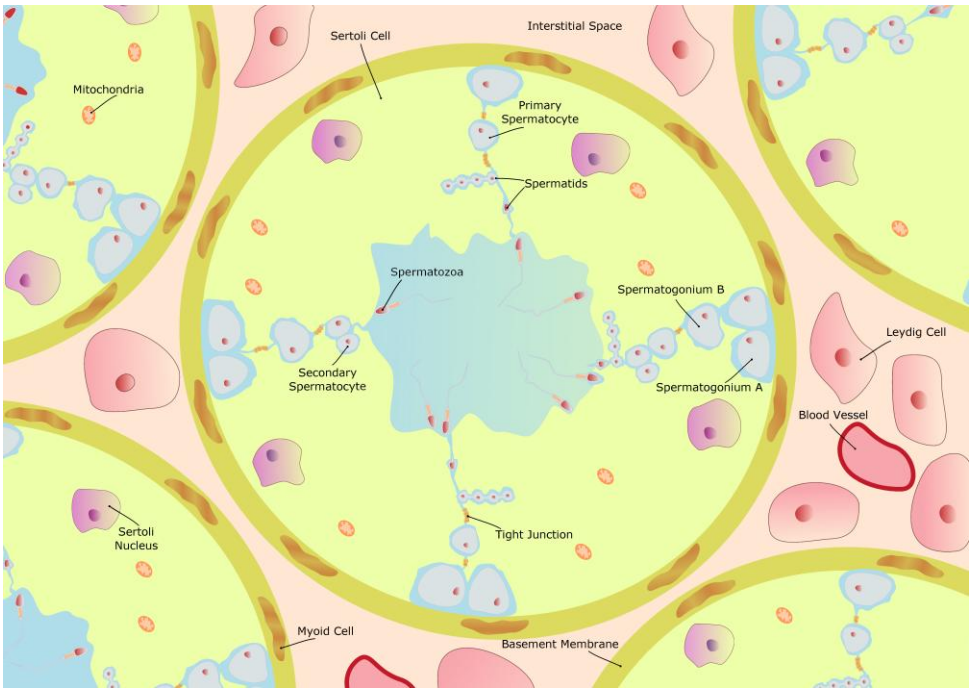

Figure 2: Schematic illustration of seminiferous tubule, spermatogenesis and cells in interstitial tissue outside the tubule. The Sertoli cells (SCs) reside on a basement membrane, under which are the lymphatic endothelium and the peritubular myoid cells. Two adjacent SCs abut a tight junction which limits intercellular transport and represents the blood testes barrier (BTB). Outside the BTB is the basal compartment, where spermatogonial renewal occurs, and inside the BTB is the adluminal compartment, where meiosis, spermiogenesis and spermiation occurs. Spermatogenesis is the process by which immature spermatogonium within the testis, divide and differentiate. Spermatogonium type A that divides mitotically in spermatogonium type B. After two meiosis, primary and secondary spermatocyte are formed. Then, takes place the spermiogenesis that produces spermatids, and the mature elongated spermatid that is subsequently released to the seminiferous epithelium (spermiation). Adapted from Alves et al.(2013**b**).

The endocrine glands of the male reproductive system includes the hypothalamus, the pituitary and the testes forming the hypothalamic-pituitary testis axis (HPT). Within this axis, neurons of the hypothalamus produce gonadotropin releasing hormone (GnRH). Pulsatile GnRH signals stimulate gonadotroph cells in the anterior pituitary to secret follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that then act on the testis to regulate the spermatogenic potential (W.H. Walker & Cheng, 2005). The testicles are involved in the production of the sex steroid hormones that exert a negative feedback on the hypothalamus and the pituitary to control the secretion of the gonodotropins, LH and FSH (L. Rato eta al.,2012; O'donnell et al., 2001). GnRH enters the hypothalamic-pituitary portal system and binds to receptors on the plasma membranes of pituitary cells, resulting in the synthesis and release of LH and FSH (Saladin, 2003). LH binds to receptors on the surface of Leydig cells in the testis and stimulates the production of testosterone, a steroid hormone that diffuses into the seminiferous tubules. Within the seminiferous tubules, only SC possesses receptors for testosterone and FSH and thus the major targets of the ultimate hormonal signals that

regulate spermatogenesis (Figure 3) (M. G. Alves et al., 2013c; Hoesl, Saad, Pöppel, & Altwein, 2005; Mruk & Cheng, 2004; W.H. Walker & Cheng, 2005). SCs also produce glycoprotein hormones, inhibin, activin, and follistatin, which regulate the secretion of FSH. Testosterone, 17β-estradiol (E_2), inhibin, activin, and follistatin are major testicular hormones that regulate the release of the gonadotropins LH and FSH. Generally, testosterone, E_2 and inhibin reduce the secretion of LH and FSH, whereas activin stimulates the secretion of FSH and follistatin inhibits FSH secretion (Saladin, 2003).

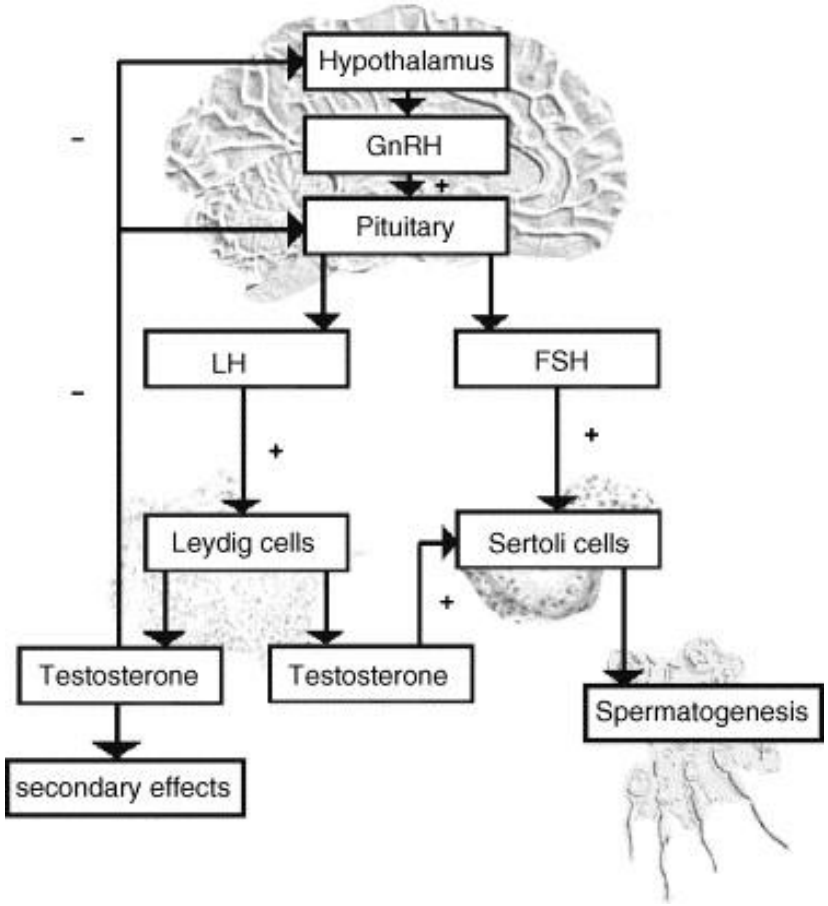

Figure 3: Hormonal regulation of male reproductive tract. The hypothalamus synthesizes the gonadotropin releasing hormone (GnRH), which will stimulate the pituitary to produce the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH bind membrane receptors on Leydig and Sertoli cells (SCs), respectively, and stimulate the testosterone production and spermatogenesis. The release of GnRH by the hypothalamus and LH by the pituitary is inhibited by increasing levels of testosterone. This androgen is responsible for male secondary sexual characteristics, and acts on SCs to stimulate spermatogenesis. Legend: + stimulating, - inhibition. Adapted from Hoesl et al. (2005).

Androgens are considered the sex male hormones, particularly testosterone, whereas estrogens are considered to be the sex female hormone, namely E_2. Nevertheless, androgens and estrogens are present in both sexes. Thus, sexual distinctions aren't qualitative

differences, but result from quantitative divergence in hormone concentrations and differential expressions of steroid hormone receptors (S. Carreau & Hess, 2010; P. Oliveira et al., 2011a). Estrogens are produced from testosterone by aromatase enzyme cytochrome P450 (P450arom), encoded by the *CYP19* gene (Boon, Chow, & Simpson, 2010; E. R. Simpson et al., 1994). This enzyme is involved in the irreversible conversion of androgens into estrogens and is present in the endoplasmic reticulum of many tissues (E. R. Simpson et al., 1994). In the mammalian testis it's well known that aromatase is mainly localized in Leydig cells (S. Carreau et al., 1999). In rodents, the source of testicular estrogens has been a considerable subject of interest. It has been reported that SCs are the major source of estrogens in immature animals but in adult rats only Leydig cells synthesize this hormone. This can be justified by low quantities of the aromatase transcripts in the adult SCs (S. Carreau et al., 2003). Moreover it has been reported the existence of P450arom transcripts in the epithelial cells of the rat epididymis (Wiszniewska, 2002). Expression of aromatase is stimulated by FSH and the maximum stimulatory effect of FSH in aromatase gene expression occurs in 20-days old rat SCs and seems to parallel SCs differentiation (S. Carreau, De Vienne, & Galeraud-Denis, 2008).

The role of E_2 in the development and physiology of male reproductive tract of mammals is still a matter of debate, even though there is a growing body of evidence suggesting that E_2 are playing a role via their specific receptors, estrogen receptor α (ERα) estrogen receptor β (ERβ) and G-protein coupled receptor-30 (GPER30) (M.G. Alves., 2013a; Fisher et al., 1997; R. Hess et al., 1997; Prossnitz et al., 2008). ERs are distributed all along the genital tract (S. Carreau et al., 2002; O'donnell et al., 2001; Scobie et al., 2002; Sirianni et al., 2008). Testicular estrogen should interact with ERs which in turn mediate the transcription of tissue specific genes (S Carreau et al., 2011). The biological significance of ER subtypes is unclear but may provide an explanation for the selective actions of estrogens in different target tissues.

The ERα are expressed in various cellular types of the male reproductive tract (Cavaco, Laurentino, Barros, Sousa, & Socorro, 2009; Fisher et al., 1997; R. A. Hess, Bunick, & Bahr, 1995), such as SCs (Lucas et al., 2008), Leydig cells, efferent ductules (Fisher et al., 1997; R. Hess et al., 1997),testis (Pelletier, Labrie, & Labrie, 2000), spermatocytes (Pentikäinen, Erkkilä, Suomalainen, Parvinen, & Dunkel, 2000), spermatids (Durkee, Mueller, & Zinaman, 1998) and spermatozoa (Durkee et al., 1998). The presence of ERβ has been described in reproductive tract tissues and was visualized in SCs (Pelletier et al., 2000), Leydig cells (Pelletier et al., 2000), spermatocytes (Saunders, Fisher, Sharpe, & Millar, 1998), spermatids (S Carreau et al., 2011), spermatogonia (Saunders et al., 1998), spermatozoa (Pentikäinen et al., 2000) and prostate (Weihua, Warner, & Gustafsson, 2002). GPER30 is able to mediate E_2 action, has been identified in a variety of human and rodent estrogen target tissues (Chagin & Sävendahl, 2007). Expression of GPER30 in several endocrine organs

including the testes and spermatogonia, highlight for a role of this receptor in controlling mouse spermatogonia cell proliferation in response to E_2 (Sirianni et al., 2008).

Animal models with ER knockout (ERKO) presented compromised spermatogenesis, steroidogenesis and male fertility (S. Carreau & Hess, 2010; Joseph, Shur, & Hess, 2011). Hess and collaborators (1997) described that αERKO animals have reduced fertility because of abnormal fluid reabsorption in the efferent ductules, which leads to germ cell destruction, and diluted sperm into epididymis, rather than concentrated, resulting in infertility, this model also revealed several abnormalities in the epididymis (R. A. Hess, 2000). Spermatogenesis, steroidogenesis and fertility are not affected in βERKO animals (Hewitt, Harrell, & Korach, 2005). Otto and collaborators (2009) described that GPER30 deficient mice are fertile.

Spermatogenesis in rodents is therefore at least partly under the E_2 control, particularly stem germ cell number and spermatid/sperm formation (S. Carreau & Hess, 2010; Li, Papadopoulos, Vidic, Dym, & Culty, 1997; Shetty, Krishnamurthy, Krishnamurthy, Bhatnagar, & Moudgal, 1997). E_2 are involved not only in some regulating steps of spermatogenesis but also through, for instance, the cadherin synthesis that mediates Sertoli-germ cell interactions (R. M. Sharpe, 1998). Furthermore, E_2 has an important role for sperm motility (S. Carreau & Hess, 2010), as reported in aromatase-deficient men, in which motility and number of spermatozoa are reduced (Carani, Fabbi, Zirilli, & Sgarbi, 2002). Some *in vivo* and *in vitro* studies revealed that E_2 can act as germ cell survival factor and that this effect is dose-dependent (Pentikäinen et al., 2000). For example, E_2 prevents apoptosis of germ cells within human seminiferous tubules *in vitro* even in the absence of gonadotropins (Pentikäinen et al., 2000). However, proapoptotic effects of E_2 on spermatogenesis have also been observed (Mishra & Shaha, 2005; S. Laurentino et al., 2011; V. Simões et al., 2012).

A better knowledge about the role of E_2 and its receptors in the regulation of the homeostasis and functions of male reproductive tract will be important for a deeper understanding of the control of male fertility.

2.1 Sertoli cells

Sertoli cells, the somatic constituents of the seminiferous epithelium, extend from the base to the apex of the epithelium, directly interacting with the developing germ cells (Mruk & Cheng, 2004). SCs are the first cells to recognizably differentiate in the indifferent foetal gonad, an event which enables seminiferous cord formation (Mackay, 2000). There appear to be fundamental differences between species as to SCs proliferative capacity. In rodents all proliferation occurs in foetal and neonatal life. In contrast, in humans, these periods are separated by a decade or more and in lower primates by periods of months (R. M. Sharpe et al., 2003). SCs are irregularly shaped, columnar cells that extend from the basal to

the adluminal compartment and occupy a volume of approximately 17–19% in the seminiferous epithelium of adult rats (L. D. Russell, Ren, Hikim, Schulze, & Hikim, 2005). They have an enormous surface area, which allows them to sustain a vast number of developing germ cells. This attribute, in itself, is crucial not only to spermatogenesis, but also to germ cell movement (Mruk & Cheng, 2004). SCs have several functions, including: (1) providing nourishment for the developing sperm cells; (2) destroying defective sperm cells; (3) secreting fluid that helps in the transport of sperm into the epididymis; (4) releasing of the hormone inhibin that helps regulate sperm production (Sikka & Wang, 2008; M.G. Alves., 2012). Hence, these somatic cells are known as the "nurse cells" for their role in providing structural and nutritional support for the developing germ cells, and creating an immunologically protected space for the germ cells (Griswold, 1998; Mäkelä et al., 2011; Meroni, Riera, Pellizzari, & Cigorraga, 2002).

Adjacent SCs form tight junctions with each other such that nothing larger than 1kDa can pass from the outside to the inside of the tubule (W. H. Walker, 2010; M.G. Alves.,2013a), regulating the movement of products, such as nutrients and wastes, both into and out of the seminiferous epithelium (Madara, 1998). The tight junctions between all SCs form the blood-testis barrier (BTB) that divides the seminiferous epithelium into basal and apical compartments. In the apical compartment occur spermiogenesis and spermiation, thus there are located the post-meiotic germ cells (B. Setchell, Scott, Voglmayr, & Waites, 1969), whereas outside of the barrier, are located germ cells at the beginning of meiosis (Mruk & Cheng, 2004). Once the germ cells move beyond BTB, they lose the access to serum constituents. These cells become high dependent upon SCs to supply nutrients and growth factors (Mruk & Cheng, 2004; W. H. Walker, 2010). The BTB controls the access of plasma substances to the seminiferous epithelium, maintaining different levels of substances between rete testis fluid and lymph or plasma. (B. Setchell et al., 1969).Thus, the differentiation of SCs and the formation of a competent BTB are essential to the establishment of normal spermatogenesis (Sikka & Wang, 2008).

3. The mammalian epididymis

The epididymis consists of a highly coiled single tubule of approximately a few meters long in the mammals. These tubular structure are adherent to the testis by the epididymo-testicular connective tissue and distally by both, the caudal connective tissue and the epididymal fat pad (T. T. Turner, 2008). The 3 main segments of the epididymis include the caput (head), corpus (body), and cauda (tail), although additional segments are recognized by the microenvironments (Serre & Robaire, 1998). Epididymal lumen diameter gradually increases from the caput to the cauda, with the large diameter tubules of the cauda reflecting the cauda's storage function (Foley, 2001). Human epididymis is approximately 43%

caput, 27% corpus, and 29% cauda (Figure 4) (Joseph et al., 2011; Robaire, Hinton, & Orgebin-Crist, 2006).

The epididymal epithelium consists of a pseudostratified columnar epithelium. The lining cells have a large brush border and are designed for secretive and resorptive functions, and many cells have prominent stereocilia. Five cell types have been described in epididymal epithelium: principal, narrow, clear, basal, and halo cells (Robaire & Hermo, 1988), all of which differing in their relative abundance depending on the epididymal region and species studies. The epididymal epithelial cells show cell-cell tight junctions composed of a number of cell adhesion molecules (Cyr et al., 2007), which impose a blood-epididymal barrier similar in effect to the BTB (Howards, Jessee, & Johnson, 1976), that is, the blood-epididymal barrier provides a specialized, immune-privileged microenvironment in which sperm remain isolated from other body compartments (Hinton & Keeper, 1985).

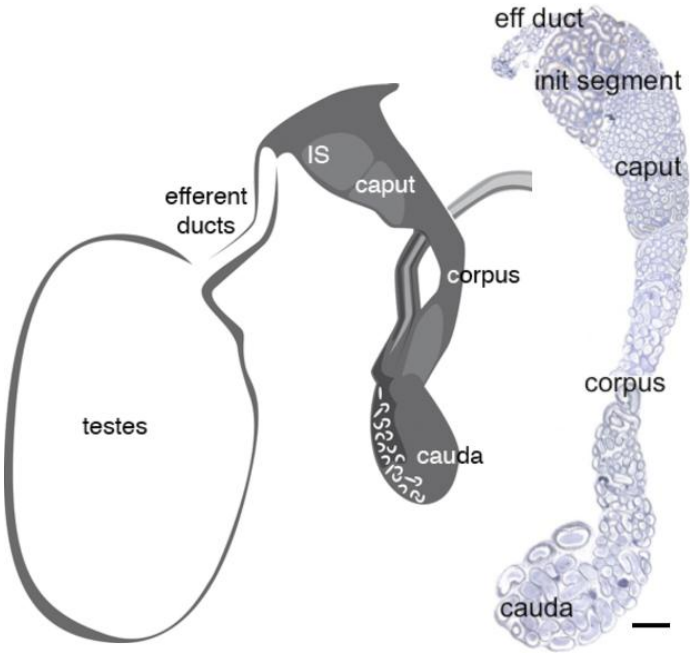

Figure 4: Schematic and histological representation of the male reproductive tract and excurrent ducts. Schematic (left) showing relative orientation of the efferent ducts and the proximal (IS and caput) and distal segments (corpus and cauda) of the epididymis. The cauda connects to the ejaculatory duct or vas deferens. Sagittal section (right) of the efferent ducts and epididymis depicting the convoluted nature of the duct as well as its complex and changing epithelium. eff duct, efferent ductules; IS and init segment, initial segment. Bar =1.5 mm. Adapted from Joseph et al. (2011).

Epididymal function is dependent on circulating sex steroid hormones and testicular factors in the luminal fluid. The luminal microenvironment of the epididymis is important for sperm maturation. Indeed, the establishment of the epididymal-blood barrier allows the epididymis to regulate and modify the luminal fluid contents, which is crucial in sperm maturation. Both epididymal secretion and reabsorption of luminal fluids establish and modify

the epididymal microenvironment (Foley, 2001; Serre & Robaire, 1998). The epididymis performs several crucial functions for male fertility, namely in the transport, concentration and storage of spermatozoa. However, the main function is to provide a luminal environment that transforms spermatozoa info fully mature cells (Robaire et al., 2006).

Sperm are moved through the epididymis in part by hydrostatic pressures originated from fluids secreted in the seminiferous tubules (B. Setchell, 1974), and by peristaltic-like contractions of the tubules (Hinton & Setchell, 1978). The contractions of the tunica albuginea of the testis also potentially play a role in the generation of positive fluid pressure in the head of the epididymis (Banks et al., 2006). Peristaltic contractions of the peritubular myoid cells surrounding the epididymis originate positive hydrostatic pressure, which causes fluid movement of the caput to distal duct (Foley, 2001). Net transport rates are estimated to be rapid in the efferent ducts and proximal epididymis, where fluid is nonviscous and water is being rapidly absorbed from the lumen. However, the transport rates decrease in the more distal tubule where the lumen content becomes more viscous (T. Turner, Gleavy, & Harris, 1990). In the epididymal lumen fluid, the concentration of ions, small organic molecules, and specific proteins secreted by the epithelium is likely important for sperm maturation or for the regulation of downstream activities of the epididymal epithelium. (Foley, 2001).

The increase in sperm concentrations between the efferent ducts and cauda epididymis is caused by fluid reabsorption subsequent to electrolyte transport (Wong, Gong, Leung, & Cheuk, 2001). Ion transporters like the cystic fibrosis transmembrane (CTFR) and the sodium membrane transporters cause osmotic shifts that draw water movements from the epididymal lumen, through aquaporin channels (Da Silva, Piétrement, Brown, & Breton, 2006). This reabsorption of water results in a gradual increase in intraluminal sperm concentrations and, ultimately, to a dense sperm pack filling the lumen of the cauda epididymis (Foley, 2001). Approximately 55%–65% of the total epididymal sperm in humans are stored in the cauda epididymis (Amann, 1981). Electrolytes and small organic molecules change in characteristic patterns along the epididymis (T. Turner, 2002). The exposure of sperm to this ever-changing microenvironment is necessary for its full maturation (Robaire et al., 2006). In fact, the epididymal microenvironment continuously changes as the sperm move from the proximal to the distal epididymis. At any point along the duct, the luminal environment is the result of net secretory and absorptive processes of the epithelium, which continuously changes along this duct (Robaire et al., 2006). These changes include net of H_2O, Na^+, Cl^- and HCO_3^- reabsorption, K^+ secretion and luminal acidification (T. Turner, 2002). Electrolyte and water transport in the epididymis is an important function of the epididymis because it affects the concentration of all major constituents in the epididymis. Furthermore, fluid transport has an immediate effect on sperm because spermatozoa are bathed in a milieu created by the epithelium. (Wong et al., 2001). In general, the epididymal secretions function

to protect, stabilize, or modify the sperm surface, with the end product being spermatozoa that are viable, motile, and capable to fertilize an egg (Robaire et al., 2006).

4. Diabetes mellitus and male fertility

Diabetes Mellitus (DM) is one of the most prominent public health threats in modern societies and its prevalence is drastically increasing. The World Health Organization (WHO) reported that in 2000 there were 177 million people with DM worldwide, but this number is likely to increase. In fact, the WHO estimates that there will be 300 million people living with the disease in 2025 (WHO, 2002).

DM is a chronic, metabolic disease characterized by hyperglycaemia that can result from defects in insulin secretion and/or insulin action (Association, 2012; M.G. Alves et al.,2013d). It causes several systemic complications and co-morbidities such as renal failure or hypertension (Kumar, Nugent, Kalakunja, & Pirtle, 2003; M.G. Alves et al., 2013d), besides a severe alteration in carbohydrate, lipid and protein metabolism (Association, 2012). The DM is a pathological process that affects the whole body system. Skeletal muscle, fat, and liver are considered as the insulin-sensitive tissues. Alterations of the functional status of these tissues may result in insulin resistance of the body (Ai et al., 2005). The vast majority of the diagnosed DM cases are classified as Type 1 Diabetes Mellitus (T1DM) or Type 2 Diabetes Mellitus (T2DM). T1DM is responsible for only 5-10% of those with DM and generally develops at young age with the great majority of the patients being diagnosed before the age of 30 (Agbaje et al., 2007). It is caused by autoimmune destruction of pancreatic β-cells, requiring daily insulin replacement therapy, in addition to diet and physical activity. In this form of diabetes, the rate of β-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults) (Association, 2012; Berdanier, 2001). Untreated T1DM is characterized by hyperglycaemia, hypoinsulinemia, ketonuria, and hyperlipidaemia, resulting from a general metabolic failure (Emilien, Maloteaux, & Ponchon, 1999). On the other hand, T2DM, which is responsible for 90-95% of the diagnosed DM patients, is referred to as non-insulin-dependent diabetes or adult onset diabetes, and encompasses individuals who have insulin resistance and usually have relative insufficient insulin secretion. Most patients with T2DM are obese, and obesity itself causes some degree of insulin resistance (Association, 2012).

The complexity of DM diagnostic, especially in obese patients, led the investigators to establish an intermediate state, often called as pre-diabetic state. This intermediate state is characterized by resistance to insulin-mediated glucose disposal and compensatory hyperinsulinemia (M. Alves et al., 2013b; Reed et al., 2000). Pre-diabetes associates with the metabolic syndrome, representing a group of abnormalities, including overweight (visceral abdominal fat distribution), dyslipidaemia, hypertension, and impaired glucose metabolism,

with insulin resistance as the postulated underlying pathogenic mechanism (Kasturi, Tannir, & Brannigan, 2008). Pre-diabetic patients have significant metabolic disorders that increase the risk for T2DM development (Engelgau, Narayan, & Herman, 2000) and associated complications. In addition to mild glycaemia, pre-diabetic individuals also present impaired glucose tolerance and insulin secretion as well as relative insulin insensitivity (M.G. Alves et al., 2013b; Bock et al., 2006; Engelgau et al., 2000). The transition from pre-diabetes to T2DM occurs when the secretory capacity of the pancreatic β-cell is no longer able to compensate for the insulin resistance. However, hyperglycaemia in patients with T2DM is not associated with absolute hypoinsulinemia. Usually, day-long circulating insulin concentrations in patients with T2DM are comparable in absolute terms to the values in non-diabetics patients (Reed et al., 2000).

Infertility is a major health problem in both, developed and developing world, with up to one in six couples requiring specialist investigation or treatments in order to conceive (Bener, Al-Ansari, Zirie, & Al-Hamaq, 2009). It is defined as the state in which a couple wanting a child cannot conceive after 1 year of unprotected intercourse. Male factor infertility accounts for up to half of all cases of infertility and affects one man in 20, in the general population (Tremellen, 2008). Male infertility can occur either as an isolated disorder or within the framework of a known complex disorder or syndrome. The number of causes of male infertility is broad, including gene mutations, aneuploidies, infectious diseases, ejaculatory duct occlusion, varicocele, radiation, chemotherapy, erectile dysfunction, anatomopathologic abnormalities, aging and drugs. However, nearly 50% of infertile men are classified as idiopathic (Ollero et al., 2001).

Relating the incidence of DM with fertility rates of modern societies, it shows that they are closely linked and increasing incidence of DM is often related with increasing infertility cases (M.G. Alves et al., 2013d; Lutz, 2006). This is partly due to the increasing incidence of DM in man with reproductive age. Sexual disorders, such as erectile dysfunction (Sexton & Jarow, 1997) or retrograde ejaculation (Fedele, 2005), are known to occur in diabetic individuals. It is also well known that DM alters the HPT axis, which is responsible for some of the problems related to DM, such as impotence (Ballester et al., 2004). Diabetes are also reported to significantly decreased seminiferous tubule diameter and increased testicular blood vessel numbers (Anderson & Thliveris, 1986). The endocrine control of spermatogenesis, is in fact severely altered in DM (Ballester et al., 2004), and sperm quality of diabetic men can be compromised. This may be a direct consequence of the unique characteristics of glucose metabolism that testicular cells present (M.G. Alves et al., 2013b). In testes, glucose metabolism is also a pivotal event. Moreover, spermatogenesis maintenance *in vivo* depends upon glucose metabolism (Zysk, Bushway, Whistler, & Carlton, 1975), although there are low levels of this sugar in tubular fluid (Robinson & Fritz, 1981). SC is responsible for the conversion of glucose, a non metabolized substrate by developing germ cells, in lactate which

is the preferential substrate for those cells (M.G. Alves et al., 2013b). Indeed, impairment of glucose metabolism is often related with increased fatty acid metabolism. Some studies reported that DM caused an increased endogenous oxygen uptake and reduced lactate production by testicular cells (Sharaf, Kheir El Din, Hamdy, & Hafeiz, 1978). However, the molecular mechanisms of testicular glucose metabolism in diabetic conditions are far from being disclosed.

5. pH Regulation

Intracellular processes functioning occur only over a narrow pH range, and adjustment of body fluids pH is a process of paramount importance for the normal functions of the cells and tissues. Under a variety of physiological conditions, pH may change transiently, producing an acute alteration in the cell function as a component of a signal transduction process (J. M. Jones, Lorton, & Bavister, 1995). Given the large number of cellular processes that are sensitive to $[H^+]$, the study of pH, has become an emerging area of crucial interest for understanding the regulation of cellular function. Changes in intracellular pH (pHi) affect the ionization state of all weak acids and weak bases, a bewildering array of cellular molecules that includes all peptides and proteins, and thus may potentially affect a wide array of biological processes (Boron, 2004).

CO_2 is the major end product of carbohydrate and lipid metabolism, and the CO_2/HCO_3^- buffer pair constitutes one of the most important pH buffers in nearly all body compartments. Carbonic anhydrase (CA), an enzyme that catalyses the reversible hydration of CO_2, is present in most body and subcellular compartments and serves to accelerate the normally slow equilibration between CO_2 and HCO_3^- (Figure 5) (S. L. Alper, Chernova, & Stewart, 2002; Boron, 2004).

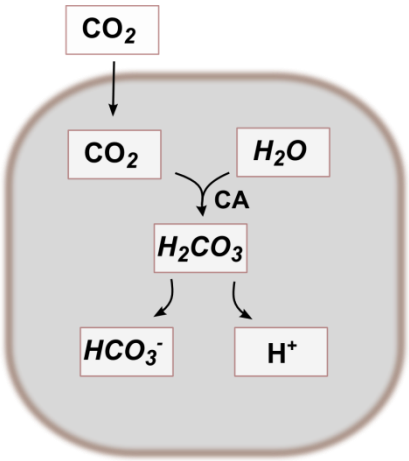

Figure 5: Schematic representation of the bicarbonate synthesis. The bicarbonate buffering system is an important buffer system in acid-base homeostasis. Carbon dioxide (CO_2) cross the plasma membrane combines with water (H_2O) to form carbonic acid (H_2CO_3). The carbonic anhydrase (CA) is the enzyme

that catalyses this reversible hydration of CO_2. H_2CO_3 can dissociate into H^+ and HCO_3^-, to maintain the ionic balance.

Cells possess in their plasmatic membrane a wide range of ion transporters that participate in pH regulation. HCO_3^- is the mobile physiological pH buffer that protects cells from fast and local changes in pHi (Casey, Grinstein, & Orlowski, 2009). The membrane proteins which mediate the transport of HCO_3^- play an important role in maintaining both intracellular and extracellular pH (pH_o) within narrow limits (Boron, 2004). Acid-base transporters are divided as to functionality into two groups: (1) acid-extruders (Na^+-H^+ exchangers, Na^+-driven HCO_3^-/Cl^- transporters, Na^+/HCO_3^- co-transporters, and V-ATPases) which move H^+ out of the cell and/or move bases such as HCO_3^- and are utilized to increase pH_i when acidosis occurs, and (2) acid-loaders (Na^+-independent HCO_3^-/Cl^- transporters and Na^+/HCO_3^- co-transporters), responsible for moving acids into the cell and/or bases out of the cell and utilized to decrease pH_i when alkalosis occurs (Boron, 2004). The ability to function either as an acid loader or as an acid extruder also depends on the ionic gradients established through the membrane (P. Oliveira, Sousa, Barros, Moura, & Rebelo da Costa, 2009).

As said, almost all bicarbonate transporters are important for the regulation of pH_i, but some specific HCO_3^- transporters also play key roles in the regulation of cellular volume, as well as on the transport of acid/base equivalents across epithelia. For instance, electrogenic Na^+/HCO_3^- co-transporters (NBCe) play key roles in bicarbonate reabsorption by the renal proximal tubule (Boron, Chen, & Parker, 2009; Maunsbach et al., 2000) and bicarbonate secretion by the pancreatic duct (Abuladze et al., 1998); electroneutral Na^+/HCO_3^- co-transporters (NBCn) regulate pH_i in vascular smooth muscle (I. Choi, Aalkjaer, Boulpaep, & Boron, 2000; Damkier, Nielsen, & Praetorius, 2006) and in axons in the brain (M. D. Parker, Musa-Aziz, et al., 2008; C. Z. Wang, Yano, Nagashima, & Seino, 2000).

As it happens in other tissues, the control of HCO_3^- concentration is essential to maintain pH along the male reproductive tract. In mouse, the luminal HCO_3^- concentration reaches its highest value in the lumen seminiferous tubules, the lowest in the caput epididymis, and then slightly rises in the cauda epididymis and vas deferens (Levine & Marsh, 1971). In the acidic fluid of the cauda epididymis, the matured spermatozoa are rendered in quiescence with little motility and no ability to fulfill fertilization (M. Chang, 1951). Unlike this epididymal fluid which is acidic and contains very low concentration of HCO_3^- (Levine & Marsh, 1971), the semen from mammals is rich in HCO_3^- and has an alkaline pH, which varies in a broad range from 7.2 to 8.4 in human (Owen & Katz, 2005).

The control of the STF pH is crucial for male fertility and regulation of pHi of SCs, the somatic component of the BTB, should also play a major role in this process (Mruk & Cheng, 2004; Tuck, Setchell, Waites, & Young, 1970). pH_i is kept mainly through the net balance between production and elimination of protons and by intracellular buffers (Roos & Boron, 1981).The composition of the STF is influenced by net movements of water, K^+ secretion, Na^+,

Cl^- and HCO_3^- reabsorption, and luminal acidification (Au & Wong, 1980; Levine & Marsh, 1971; T. Turner, 1984). Substantial differences in the ionic composition of STF have been reported, especially in the concentration of K^+ but also in that of Na^+, Cl^- and HCO_3^- (L. Rato et al., 2010). There are important differences in composition of rete testis fluid (RTF) and STF, although both fluids contain appreciably more K^+ and less Na^+ than blood plasma or testicular lymph from a lymphatic vessel in the spermatic cord. RTF and STF also contain considerably more of some organic compounds, such as inositol and some amino acids, and much less of others, such as glucose, protein and particularly immunoglobulins than blood plasma or lymph (B. Setchell, 1986). The SCs are responsible for water transport from the interstitial space to the lumen (B. Setchell et al., 1969), they also control the seminiferous fluid pH and ionic composition (P. Oliveira et al., 2009; P. F. Oliveira, Sousa, Barros, Moura, & da Costa, 2009; L. Rato et al., 2010). In these cells, distinct types of transport proteins have been identified, such as membrane pumps (Na^+/K^+-ATPase and Ca^{2+} -ATPase)(Byers & Graham, 1990; Feng, Hershlag, Han, & Zheng, 2006), various H^+/HCO_3^- membrane transporters (NDCBEs, NBCes and Na^+/H^+ exchangers)(Boron, 2001; P. Oliveira et al., 2009; P. F. Oliveira et al., 2009), ion channels (voltage-dependent Cl^- channels activated by acidic extracellular pH, CFTR Cl^- channels, K^+ channels and L^- T^- and N-type Ca^{2+} channels)(Auzanneau, Thoreau, Kitzis, & Becq, 2003; Boockfor, Morris, DeSimone, Hunt, & Walsh, 1998; Loss et al., 2004; Taranta, Morena, Barbacci, & D'Agostino, 1997; Von Ledebur, Almeida, Loss, & Wassermann, 2002), ion co-transporters (Na^+-K^+-$2Cl^-$ co-transporter and Na^+/Ca^{2+} exchanger)(Grasso, Joseph, & Reichert, 1991; A. J. Pace et al., 2000) and water channels (AQPs 0 and 8)(Badran & Hermo, 2002; Hermo, Krzeczunowicz, & Ruz, 2004; Tani et al., 2001). Although the involvement of such transporters in the establishment of the STF is not yet completely disclosed, it is certain that they have a key role in the cellular mechanisms responsible for determining ion composition, osmolarity and pH of the fluid (L. Rato et al., 2010).

The semen provides the necessary HCO_3^- for sperm to obtain initial motility at the time of ejaculation (Huggins, Scott, & Heinen, 1942; Owen & Katz, 2005). The generation of competent sperm is a complex multistep process that initiates in the seminiferous epithelium. One critical feature is the secretion of STF, which is known to be maintained slightly acidic (C. R. Caflisch & Dubose, 1990; Levine & Marsh, 1971). The capacitation of sperm occurs after mixing with the prostatic and seminal vesicle fluids and is triggered by an influx of HCO_3^-, which is abundant in these fluids. Low pHi suppresses sperm metabolism and motility (Carr & Acott, 1989), whereas HCO_3^- is known to be an activator of sperm adenylyl cyclase (sAC) (Y. Chen et al., 2000; Sinclair et al., 2000), and consequently increases cAMP production. The luminal fluid in which spermatozoa reside undergoes significant modifications as it moves from the proximal to the distal regions of the epididymis. The values of pH and HCO_3^- decrease along the epididymis (C. R. Caflisch & Dubose, 1990; R. Jones & Murdoch, 1996; Rodriguez-Martinez, Ekstedt, & Einarsson, 1990) and achieve the lowest value in the cauda.

The acidic pH is crucial for the maintenance of spermatozoa in a quiescent state during their maturation and epididymal storage (Newcombe et al., 2000). As measured in rats *in vivo*, the intraluminal pH in seminiferous tubules is 7.0–7.3, and progressively acidifies to 6.5 along the epididymis. Several authors report a trend to increase acidification of the luminal fluid along the epididymis (C. Caflisch, 1993; Levine & Kelly, 1978). Narrow and apical cells in epididymis, appear to be responsible for H^+ secretion and HCO_3^- reabsorption (Pushkin, Clark, Kwon, Nielsen, & Kurtz, 2000). The data indicate that, in the rat, the efferent ducts are the region of highest luminal pH and HCO_3^- concentration, and the major region for the processing of HCO_3^- within the extratesticular ducts. By reabsorbing more than 95% of the testicular HCO_3^- output, the efferent ducts contribute substantially to the establishment of the HCO_3^- /pH status of the epididymal fluids, and thus to the establishment of a luminal environment suitable for maintaining epididymal fluid (Newcombe et al., 2000). Failure to maintain the pH homeostasis in the male reproductive tract may impair the production and/or maturation of spermatozoa, and therefore cause infertility or subfertility (Liu, Wang, & Chen, 2012).

6. Bicarbonate transporters in the male reproductive tract

Cells possess in their plasmatic membrane a wide range of ion transporters that participate in pHi regulation, among which are the basic and acidic particles membrane transporters (Boron, 2004). Membrane bicarbonate transporters are divided into two main families of transporters, Slc4 (solute carrier 4) and Slc26 (solute carrier 26) (Liu et al., 2012; Romero, 2005).

The Slc4 gene family consists of ten human genes (Romero, Fulton, & Boron, 2004). These genes encode membrane proteins facilitating the exchange of Cl^-, HCO_3^-, Na^+ and borate across the plasma membrane of mammalian cells and thus contribute to regulation of pHi, cell volume, and secondarily to membrane potential (S. L. Alper, 2005; Lohi et al., 2000). The Slc26 gene family includes anion transporters structurally different from the Slc4 family (Everett & Green, 1999). This family also consists of 10 genes, encoding proteins which use several different substrates, namely Cl-, OH^-, I^-, SO_4, HCO_3^-, $HCOO^-$, and C_2O_4 (A. Sindic, M. H. Chang, D. B. Mount, & M. F. Romero, 2007). The physiological role of each Slc26 anion exchangers depends on both of substrate specificity as tissue expression (M. H. Chang et al., 2009).

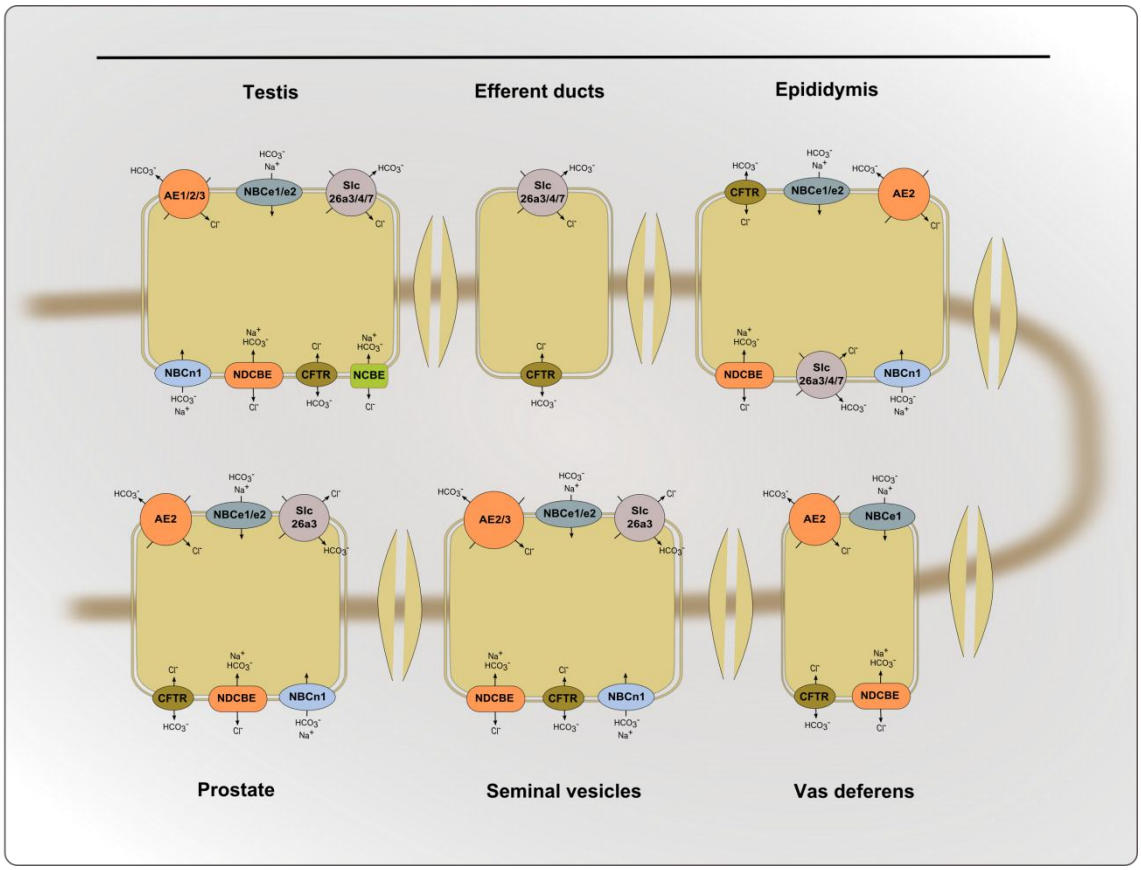

Figure 6: Schematic representation of the distribution of bicarbonate transporters in male reproductive system. In the testis are described twelve HCO_3^- transporters, named AE1/2/3, NBCe1 and NBCe2, CFTR, Slc26a3/4/7, NCBE and NDCBE. CFTR is quite distributed in male reproductive tract. Besides the testis, it is also described in efferent ducts, epididymis, vas deferens, prostate and seminal vesicle. Slc26a3 is also expressed in efferent ducts, epididymis, prostate and seminal vesicles. In epididymis is described the expression of AE2, NBCe1/2, NBCn1, NDCBE, Slc26a4 and Slc26a7. The Slc26a7 is also present in efferent ducts. In prostate and seminal vesicles have been also detected, AE2, NBCe1/2, NBCn1, NDCBE. In addition to these transporters located in the seminal vesicle, another anion exchanger, the AE3, is also found. Finally, AE2, NBCe1 and NDCBE are present in vas deferens.

6.1 Slc4 family

Membrane transporters of the Slc4 family have in common the characteristic of transporting basic particles, namely HCO_3^- or CO_3^{2-}. They diverge in the ability to mediate the concurrent transport of Na^+ and/or Cl^- (S. L. Alper, 2009; Pushkin & Kurtz, 2006) (Figure 7). The Slc4 family represents the major subfamily of HCO_3^- transporters, which includes two subgroups: the Na^+-independent, and the Na^+-dependent HCO_3^- transporters (S. L. Alper, 2005; Romero et al., 2004).

6.1.1 Na^+-independent bicarbonate transporters

The three first members of the Slc4 family are anion exchangers AE1 (Slc4a1), AE2 (Slc4a2), AE3 (Slc4a3) belonging to the Na^+-independent transporters subgroup. They mediate electroneutral Cl^--HCO_3^- exchange (S. L. Alper, 2009) (Figure 7). The AE1 polypeptide is expressed in greatest abundance in erythrocytes and in intercalated cells of renal collecting duct (S. L. Alper, 2005; Romero et al., 2004).

The AE1 transporter is highly expressed in erythrocytes and intercalated cells of renal collecting duct (S. L. Alper, 2005; Romero et al., 2004). It has been proposed that this transporter acts as a key player on numerous physiological functions in a variety of cell types, including pHi and cell volume regulation (Garcia-Romeu, Borgese, Guizouarn, Fievet, & Motais, 1996; Kopito et al., 1989). AE1 also plays a key role in delivering cellular metabolism-derived HCO_3^- to the lung (Pushkin & Kurtz, 2006) and, in the kidney, it participates in the transepithelial Cl^- and acid/base transport, being expressed on the basolateral membrane of collecting duct cells (S. Alper, Natale, Gluck, Lodish, & Brown, 1989; Pushkin & Kurtz, 2006; Verlander, Madsen, Low, Allen, & Tisher, 1988).

AE2 is also a Na^+-independent transporter of the Slc4 family, but unlike the other AEs, this anion exchanger is widely distributed, and thus is termed "house-keeping" AE. Its expression has been described in an ample variety of mammalian tissues, including choroid plexus (S. Alper, Stuart-Tilley, Simmons, Brown, & Drenckhahn, 1994), stomach (A. Stuart-Tilley et al., 1994), intestine (S. L. Alper et al., 1999), kidney (S. L. Alper, Stuart-Tilley, Biemesderfer, Shmukler, & Brown, 1997; A. K. Stuart-Tilley, Shmukler, Brown, & Alper, 1998), cochlea (Mhatre, Charachon, Alper, & Lalwani, 1998), salivary glands (Vazquez et al., 1995), and liver (Garcia, Montuenga, Medina, & Prieto, 1998; Martínez-Ansó, Castillo, Diez, Medina, & Prieto, 2005). AE2 presence is of extreme importance for pHi regulation and its functioning is activated by exposure to alkaline media and inhibited by exposure to acidic media (Humphreys, Jiang, Chernova, & Alper, 1995; Zhang, Chernova, Stuart-Tilley, Jiang, & Alper, 1996).

Like AE1 and AE2, AE3 mediates Na^+-independent anion exchange, although it is not as highly abundant as AE2. AE3 expression was primarily described in cells of excitable tissues, such as the nervous system (Kobayashi, Morgans, Casey, & Kopito, 1994) and the cardiac muscle tissue (Linn, Askew, Menon, & Shull, 1995; Yannoukakos et al., 1994). Similarly to AE2, in the cardiac tissue, AE3 function is activated by alkaline pH (Stewart, Chernova, Kunes, & Alper, 2001).

Along the male reproductive tract, the presence of AE1 has only been detected in testicular interstitial cells (or Leydig cells), although a weak imunnoreactivity was described in these cells (Uhlen et al., 2010). Contrastingly, the expression of AE2 has been widely

identified in the majority of the cells of the male reproductive tract, as could be expect, as it has been discussed, this transporter is widely expressed and termed "house-keeping" AE. In the testes, AE2 mRNA expression was described in the various somatic cells (Sertoli, peritubular and Leydig cells) and in developing germ cells and "mature" sperm cells (Holappa et al., 1999). It has also been suggested that the expression of AE2 mRNA was variable to some extent according to the epithelial cell cycle stage, with the strongest expression being observed at stages VII–XIV (except for stage X), which are associated with the major structural and morphological changes in developing germ cells (Holappa et al., 1999). According to the authors of that study, the expression patterns suggest that AE2 regulates HCO_3^- transport in late developing germ cells and mature sperm cells, playing a key role on the spermatogenic event. Indeed, studies on knockout mice have shown that AE2 plays essential roles in spermatogenesis (Liu et al., 2012). In mouse, disruption of Slc4a2 gene (encoding AE2) slightly reduced the number of spermatogonia and spermatocytes, substantially reduced the number of spermatids, and totally abolished the formation of mature spermatozoa (Medina et al., 2003). Expression of AE2 was also reported in the epithelial cells lining the lumen of the epididymis and of other excurrent ducts. According to Medina et al. (2003), AE2 is exclusively localized in the basolateral membrane of cells in the male reproductive excurrent ducts, being highly expressed along the proximal regions of the ducts, including the initial segment, the intermediate zone, as well as caput epididymis and in relatively low abundance in the distal regions, including the cauda epididymis and vas deferens (Jensen, Stuart-Tilley, et al., 1999) (Table 1). Additionally, a weak AE2 expression has also been detected in the epithelial cells of the prostate and the seminal vesicles (Uhlen et al., 2010) (Figure 6).

Contrastingly to the wide expression of AE2, the presence of AE3 has not been detected in most of the male reproductive tract tissues. So far, its expression has only been detected in few testicular cells, particularly in developing germ cells (Johnston et al., 2008) and in seminal vesicle glandular cells (Uhlen et al., 2010) (Figure 6).

6.1.2 Na^+-dependent bicarbonate transporters

The Na^+-dependent members include five Na^+-coupled HCO_3^- transporters, also termed NCBTs (Boron et al., 2009), and one Na^+-coupled borate transporter (Slc4a11) (M. D. Parker, Ourmozdi, & Tanner, 2001). Due to their transport stoichiometry, the Na^+-coupled HCO_3^- transporters are divided into two sub-groups: one electrogenic (NCBe) and the other electroneutral (NCBn) (L.-M. Chen, Liu, & Boron, 2011).

The electrogenic members include the co-transporters NBCe1 (Slc4a4) and NBCe2 (Slc4a5), whereas the electroneutral members include the Na^+-HCO_3^- co-transporter NBCn1 (Slc4a7), the Na^+-driven Cl^--HCO_3^--exchanger NDCBE (Slc4a8) and the Na^+-coupled HCO_3^--exchanger NCBE (Slc4a10) (L.-M. Chen et al., 2011) (Figure 7). Slc4a9 stoichiometry and function remain a matter of debate and need further investigation. This transporter was

initially described as an anion exchanger (AE4), mediating the Na^+-independent Cl^-/HCO_3^- exchange (M. D. Parker et al., 2001; Tsuganezawa et al., 2001), however in a more recent study it has been described as an electroneutral sodium bicarbonate co-transporter (NBCn) without Cl^-/HCO_3^- exchange activity (M. Parker, Boron, & Tanner, 2002) (Figure 7).

The two electrogenic Na^+/HCO_3^- co-transporters (NBCe1 and NBCe2) mediate the movement of one Na^+ and two or three HCO_3^- in same direction (Romero et al., 2004). The direction and stoichiometry of the transport depends on the tissues in which they are expressed (Figure 7). For example, the NBCe1 present in the pancreatic duct cells uptakes the ions into the cytosol, whereas in the renal proximal tubules it extrudes these ions (Damkier, Nielsen, & Praetorius, 2007). NBCe1 (Slc4a4) was firstly described as a Na^+/HCO_3^- co-transporter by Boron and Boulpaep (1983) in cells of the renal proximal tubule of the salamander. Thereafter, the expression of this transporter has been reported in several tissues, namely in brain (Majumdar et al., 2008), kidney (Zhu et al., 2010), eye (Bok et al., 2001; Usui et al., 2001), pancreas (Abuladze et al., 1998; Satoh et al., 2003) and colon (Abuladze et al., 1998). Although in the kidney NBCe1 operates with an Na^+:HCO_3^- stoichiometry of 1:3 (Zhu et al., 2010), mediating the net HCO_3^- efflux across the basolateral membrane of proximal tubule epithelia, in the majority of the other cell types, it operates with a 1:2 stoichiometry and mediates net HCO_3^- influx (Majumdar et al., 2008).

NBCe2 (Slc4a5 or NCB4) was the second of two electrogenic NCBTs to be described and seems to be primarily a luminal transport protein, in contrast with the basolateral electrogenic NBCe1 (Damkier et al., 2007). Although these two transporters exhibit very similar transport properties, NBCe2 seems to have distinctive role in transepithelial bicarbonate transport (Damkier et al., 2007). In the kidney, NBCe2 is expressed on the apical membrane of uroepithelial cells lining the renal pelvis, where it seems to play a key role in protecting the renal parenchyma from alterations in urine pH (Abuladze et al., 2004), while in the skeletal muscle it seems to regulate the pH of sarcolemmal vesicles (Kristensen, Kristensen, & Juel, 2004). This electrogenic bicarbonate transporter is predominantly detected in the liver (Abuladze et al., 2004), kidney (Abuladze et al., 2004; Damkier et al., 2007; Sassani et al., 2002), brain (Bouzinova et al., 2005), heart (Pushkin, Abuladze, et al., 2000), pancreas and muscle (Sassani et al., 2002), testes and spleen (Pushkin, Abuladze, et al., 2000).

NBCn1 is an electroneutral Na^+-HCO_3^- co-transporter that moves HCO_3^- across the cell membranes (Pushkin & Kurtz, 2006; Romero et al., 2004). This transporter plays an important role in transepithelial acid–base movement in various epithelial tissues (Praetorius et al., 2004), operating mostly as an acid-extruder (Damkier et al., 2007). Additionally, it has also been described that the NBCn1 co-transporter contributes to pHi regulation in many tissues, namely, neurons (Cooper et al., 2009), arterial myocytes (Boedtkjer, Praetorius, Füchtbauer, & Aalkjaer, 2008), vascular smooth muscle cells (Pushkin, Abuladze, et al., 2000).

The NDCBE (Na^+-driven Cl^-/HCO_3^- exchanger) subfamily is constituted by at least four protein variants (NDCBE A–D) that seem to be the major pHi regulators in many cells (Boron, 2001; Boron et al., 2009; M. D. Parker, Bouyer, Daly, & Boron, 2008). The NDCBE is a pHi regulator that transports extracellular Na^+ and HCO_3^- into cells in exchange for intracellular Cl^- and H^+, playing an important role in cellular alkalinisation (J. M. Russell & BORON, 1976). Its expression has been described in the brain (L. M. Chen, Kelly, Parker, et al., 2008; Schwiening & Boron, 1994), vascular endothelial cells (Sun, Vaughan-Jones, & Kambayashi, 1999), sperm (Zeng, Oberdorf, & Florman, 1996), kidney (Ishibashi, Rector, & Berry, 1993) and pancreatic beta-cells (C. S. Pace, Tarvin, & Smith, 1983) (Figure 7).

Finally, the NCBE (Na^+-coupled bicarbonate exchanger) is encoded by the Slc4a10 gene and various NCBE transcripts have been detected in the peripheral nervous system (L. M. Chen, Kelly, Rojas, et al., 2008) and in some acid-secreting epithelia, including the of the stomach, the duodenal epithelium and kidney (Giffard, Lee, Ouyang, Murphy, & Monyer, 2003; C. Z. Wang et al., 2000). This transporter utilizes the transmembrane gradient of Na^+ to mediate the cellular uptake of HCO_3^- (or CO_3^{2-}) and mediates acid extrusion. It has been reported that this transporter participates on pHi regulation in neurons (Schwiening & Boron, 1994) and it is involved in the regulation of cerebrospinal fluid (CSF) composition, through transepithelial transport of Na^+, HCO_3^- (Brown, Davies, Speake, & Millar, 2004). Disruption of NCBE gene is known to cause impaired pHi regulation in hippocampal neurons, small brain ventricles, reduced neuronal excitability and increased seizure thresholds (Jacobs et al., 2008).

The expression of all these members of the NCBT family has been identified throughout the male reproductive tract (Liu, Xu, Wang, Wang, & Chen, 2011) (Figure 6). Analogous to what happens in other epithelia, in cells of the male reproductive tract these transporters must have a central role in the control of pHi in cells, as well as in the transport of acid-base equivalents (and/or salt) across the various epithelia, and be responsible by the pH maintenance of the lumen of the tubules/ducts (C. R. Caflisch & Dubose, 1990).

Along the male reproductive tract, NBCe1 expression has also been detected in all major regions, including the testis, epididymis, vas deferens (Liu et al., 2011), as well as prostate (Nishimura & Naito, 2005) and seminal vesicles (Uhlen et al., 2010). Jensen et al. (Jensen, Schmitt, et al., 1999) showed that NBCe1 is expressed in the basolateral membrane of principal and apical/narrow cells of rat epididymis (Figure 6). It has been proposed that NBCe1 is localized in the basolateral membrane of those cells and that, by facilitating HCO_3^- extrusion into interstitial space, it might contribute to luminal HCO_3^- uptake by this epithelium (Pastor-Soler et al., 2005).

Furthermore, it has been demonstrated that NBCe1 is regulated via estrogen signaling in the epididymal epithelia (Joseph et al., 2010). In ERα null mice (which are infertile) the

expression of NBCe1 is substantially decreased in the proximal portion of the epididymis and the pH of the epididymal luminal milieu of those mice was significantly higher than that of the control normal mice, resulting also in an increase in pHi and a decrease in sperm motility. The authors of that study suggested that NBCe1 (together with CA and Na^+-H^+ exchanger 3) plays critical role in male reproduction by regulating luminal pH of epididymal tract (Joseph et al., 2010). NBCe1 expression has also been detected in primary epithelial cell cultures from porcine *vas deferens*, where it has been suggested to play a role in the regulation of luminal pH (Carlin et al., 2006; Liu et al., 2011) (Figure 6).

The expression of NCBe2 has also been reported throughout the whole male reproductive tract (Chan, Ko, Zhao, Fu, & Wong, 1996; Liu et al., 2011; Uhlen et al., 2010) (Figure 6). NCBe2 mRNA and protein expression has been described in the testis, epidydimis, prostate and seminal vesicles (Liu et al., 2011; Uhlen et al., 2010). Additionally, using cultured rat epididymal epithelia, Chan et al. (1996) showed an electrogenic Na^+-dependent HCO_3^- secretion activity that was present at the apical membrane of the cells. In agreement with what happens in other epithelia, the authors suggest that the NBCe2 expressed in epididymis might be involved in HCO_3^- secretion (Chan et al., 1996).

The presence of electroneutral NBCn1, usually located in the basolateral membrane of epithelial cells (Boron et al., 2009), has been also reported throughout the male reproductive tract (Chan et al., 1996; Uhlen et al., 2010), although in the testis, prostate and seminal vesicles, solely NCBn1 mRNA expression has been described, based on microarray and expressed sequence tags (EST) studies (Uhlen et al., 2010). Divergent to what is described in other tissues, in rat epididymis, the presence of NBCn1 has been clearly identified (Figure 6). However, it has been localized at the apical membrane of specialized epididymal cells (narrow cells and clear cells) (Pushkin, Clark, et al., 2000).

Expression of NDCBE has been reported at very high levels in the testis (Boron, 2001; Grichtchenko et al., 2001) and it has also been described in SCs (P. Oliveira et al., 2009; P. F. Oliveira et al., 2009). As said, it was shown that this exchanger is a key pHi regulator in the neural cells, where it is also abundantly expressed (Schwiening & Boron, 1994). Likewise, it has been suggested that NDCBE might play an important role in maintaining SCs pHi homeostasis (P. Oliveira et al., 2009; P. F. Oliveira et al., 2009). Furthermore, a strong presence of NDCBE has also been detected in the epididymis, vas deferens prostate and seminal vesicles (Liu et al., 2011; Uhlen et al., 2010) (Figure 6).

On the other hand, along the male reproductive tract, NCBE expression was only described in testis as weakly present (C. Z. Wang et al., 2000), and particularly in Leydig cells (Uhlen et al., 2010) (Figure 6). No data regarding the function of this transporter in the cells of the male reproductive tract is available.

6.2 Slc26 family

The Slc26 family consists of ten members that show diverse transport stoichiometries and specificity, which predominantly mediate Na^+-independent anion transport (Pushkin & Kurtz, 2006). The Slc26 family members are membrane proteins capable of transporting several anions (Cl^-, HCO_3^-, OH^-, SO_4^{2-}, and oxalate) with different specificities (Kujala et al., 2007; A. Sindic, M.-H. Chang, D. B. Mount, & M. F. Romero, 2007). Among the Slc26 family members, those that are permeable to HCO_3^- include Slc26a3, Slc26a4, Slc26a6, Slc26a7 and Slc26a9 (Mount & Romero, 2004).

The Slc26a3 transporter is an electrogenic Na^+-independent $Cl^-/HCO_3^-/OH^-$ exchanger (Shcheynikov et al., 2008) (Figure 7) initially identified as a candidate tumor suppressor, as it was reported that its expression was down-regulated in adenomas (Schweinfest, Henderson, Suster, Kondoh, & Papas, 1993), hence its original name: DRA (down-regulated in adenomas). This transporter is highly expressed in colonic mucosa (Jacob et al., 2002; Schweinfest et al., 1993) and also in the sweat gland (Haila et al., 2000). Studies with knockout models of this transporter, suggested that it is an electroneutral transporter found in the apical membranes of epithelial cells participating in bicarbonate secretion and chloride reabsorption (Schultheis et al., 1998).

Slc26a4 is a Cl^-/HCO_3^- exchanger capable of transporting HCO_3^- also I^- (Shcheynikov et al., 2008) (Figure 7). This transporter has been identified in cells of the kidney (Inatomi et al., 2004; Wall, 2005), thyroid (Pryor et al., 2005), lung (Izuhara et al., 2009) and inner ear (Bizhanova & Kopp, 2010). Loss of Slc26a4 function defects (Dossena et al., 2009) result in autosomal-recessive Pendred syndrome (PDS), characterized by deafness and goiter (B. Choi et al., 2011; Maciaszczyk & Lewinski, 2008). Consequently, this transporter is also named Pendrin. Pendrin has been implicated in base secretion in the inner ear (Wangemann et al., 2007) and in the renal tubule, participating in the control of vascular volume and arterial pH (Kim et al., 2005).

Slc26a6, also known as PAT-1 (putative anion transporter-1), is capable of transporting all of the substrates described for this family (Jiang, Grichtchenko, Boron, & Aronson, 2002; Xie, Welch, Mercado, Romero, & Mount, 2002). When transporting HCO_3^-, it has been reported that this transporter can function as a coupled electrogenic Cl^--HCO_3^- exchanger with a stoichiometry of $1Cl^-:2HCO_3^-$ (S. B. H. Ko et al., 2002; Shcheynikov et al., 2006; Xie et al., 2002), or as an electroneutral transporter with a stoichiometry of $1Cl^-:1HCO_3^-$ (J. E. Simpson et al., 2007) (Figure 7). It has been identified in various tissues, such as the pancreas (S. B. H. Ko et al., 2004; Steward, Ishiguro, & Case, 2005), salivary glands (S. B. H. Ko et al., 2004), intestine (Soleimani, 2008; Z. Wang, Petrovic, Mann, & Soleimani, 2002), kidney (Z. Wang et al., 2005), heart (Shcheynikov et al., 2006) and stomach (Soleimani, 2008). Slc26a6 has also been associated with the activity of other bicarbonate transporters,

particularly CFTR. Indeed, deletion of Slc26a6 in the pancreatic duct results in deregulation of CFTR evidencing the essential role of Slc26a6 in pancreatic HCO_3^- secretion (Y. Wang et al., 2006).

Slc26a7 is also a HCO_3^- transporter whose functional properties are still a matter of debate. Initially, studies revealed that Slc26a7 functions as a Cl^-/HCO_3^- exchanger (Petrovic et al., 2004), but more recent reports revealed that this transporter is a highly selective Cl^- channel with minimal HCO_3^- permeability (Amlal, Xu, Barone, Zahedi, & Soleimani, 2012). This pHi-regulated Cl^- channel may serve as a sensor of pHi in cells that secrete acid or base equivalents with uncoupled Cl^- transport (Amlal et al., 2012) (Figure 7). Its expression has been identified in the kidney, stomach and nasal epithelium (Lohi et al., 2002). Slc26a7 has been localized to the basolateral membrane of the gastric cells, the renal medullary collecting ducts (Xu et al., 2006) and the apical membrane of cells of the proximal tubules (Vincourt, Jullien, Kossida, Amalric, & Girard, 2002).

Finally, Slc26a9 mediates Cl^-/HCO_3^- exchange, as well as Cl^- independent HCO_3^- transport (Figure 7), by mechanisms not yet described in any other protein of this family of transporters (M. H. Chang et al., 2009). Slc26a9 was initially identified in gastric epithelia (Xu et al., 2005), but its expression has also been found in other tissues, such as the lung and stomach (M. H. Chang et al., 2009), airway (Lohi et al., 2002), gastric surface epithelial cells (Xu et al., 2005), and with lower expression levels in the kidney (M. H. Chang et al., 2009). As functional and pharmacological studies on the Slc26a9 transporter are still scarce, the controversy about its properties and whether the protein is a exchanger or a channel remains (Anagnostopoulou et al., 2012; M. H. Chang et al., 2009). Nevertheless, recent studies points towards this transporter functioning as a Cl^- channel with minimal HCO_3^- conductance, regulated by CFTR (Anagnostopoulou et al., 2012).

The presence of the bicarbonate transporting members of this Slc26 family along the male reproductive tract has not been as thoroughly studied as happens with Slc4 family members. Indeed, most studies discuss only information concerning the expression of five Slc26 members in the male reproductive tract. Excluding Slc26a3, no systematic studies on the functional properties of these proteins in the male reproductive tract were performed (Hihnala et al., 2006). The Slc26a3 transporter (DRA) is highly expressed in testis (Lacroix et al., 2001), epididymis, prostate (Uhlen et al., 2010) and efferent ducts (Hihnala et al., 2006), and in the male seminal vesicle (Haila et al., 2000). Mutations in DRA gene are associated with congenital chloride diarrhea (CLD), a autosomal recessive disease with a defect in the intestinal Cl^--HCO_3^- exchange (Hihnala et al., 2006; Wedenoja et al., 2011). CLD is associated with male subfertility, especially tendency to form spermatoceles and oligoasthenozoospermia, which is suggested to be caused by the disruption of Slc26a3 at multiple sites of the male reproductive tract (Hihnala et al., 2006). In males suffering from

CLD, high concentration of Cl^- and a low pH in the seminal plasma resultant from the defective Slc26a3 functioning has been reported (Höglund et al., 2006).

The expression of Slc26a4 and Slc26a7 has also been identified in cells of the testis (Uhlen et al., 2010), epididymis and efferent ducts (Blomqvist, Vidarsson, Söder, & Enerbäck, 2006; Kujala et al., 2007) (Figure 6). Although functional studies concerning the role of some of these anion exchangers in the tissues of the male reproductive tract are not yet available, it has been suggested that Slc26a4 is an important transporter for sperm maturation (Liu et al., 2012).

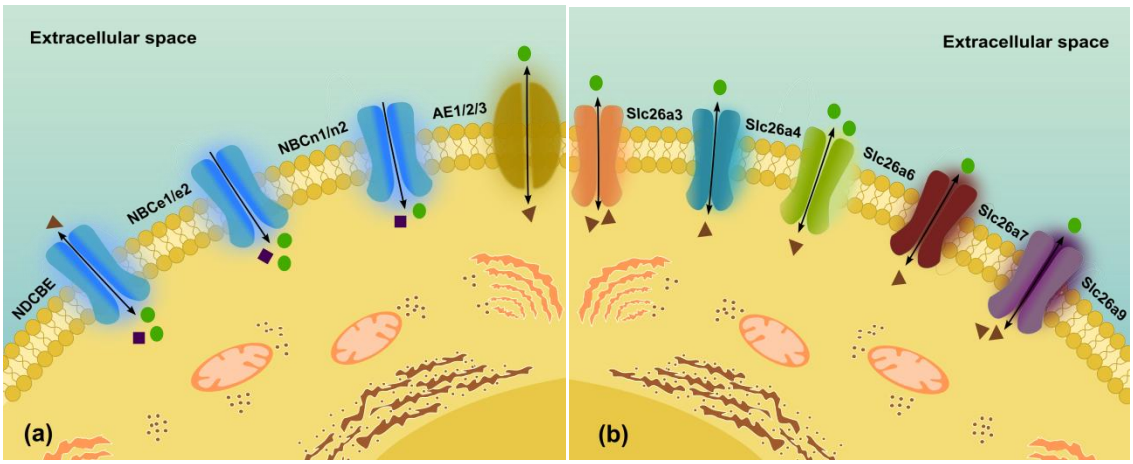

Figure 7: Bicarbonate transporters. (a) Protein transporters of bicarbonate of family slc4 in plasmatic membrane. The Na+ -driven Cl^-/HCO_3^- exchanger NDCBE mediating the electroneutral exchange of Cl^- for Na^+ and HCO_3^-. NBCe1/e2 are an electrogenic transporter, operate with an apparent $Na^+:HCO_3^-$ stoichiometry of 1:2. NBCn1/n2 is an electroneutral Na^+-dependent transporter, transports sodium and bicarbonate in the same direction. AE1/2/3 are anion exchangers, Na^+ -independent Cl^-/HCO_3^- exchangers, mediating 1:1 electroneutral exchange of Cl^- for HCO_3^-. **(b)** Protein transporters of bicarbonate of family slc26 in plasmatic membrane. Slc26a3 is an electrogenic Na^+-independent Cl^-/HCO_3^- exchanger, with stoichiometry 2:1. Slc26a4 may be considered an electroneutral Cl^-/HCO_3^- exchanger. Slc26a6 is a electrogenic Cl^-/HCO_3^- exchanger with a stoichiometry of $1Cl^-/2HCO_3^-$. Slc26a7 is also a HCO_3^- transporter, functions as a Cl^-/HCO_3^- exchanger. Slc26a9 is a protein nCl^-/HCO_3^- exchanger. Symbols: ● - HCO_3^-; ▲- Cl^-; ■- Na+.

7. Diabetes, estrogens and pH regulation

All functions of the body require the maintenance of an adequate pH_i, which is kept strictly within a narrow range at relatively alkaline values (Feuvray, 1997; J. M. Jones et al., 1995). The regulation of pH_i largely depends upon the activity of plasma membrane carriers that mediate the transport of acid/base equivalents (Madshus, 1988). Instability in pH_i or in the processes regulating pH_i are expected to occur in diabetic individuals as a result of either altered cellular metabolism and/or cellular and subcellular membrane changes (Feuvray, 1997).

It has been reported that DM does not change steady-state pH_i but significantly alters pH_i regulation in cells of the cardiac muscle mainly by decreasing markedly the activity in some ion transporters, such as Na^+/H^+ exchanger (Feuvray, 1997). In those cells, several cellular changes associated with DM occur that might account for the depressed activity of Na^+/H^+ exchange (Dudeja, Foster, & Brasitus, 1987). For instance, alteration of the cellular membrane composition, causes a modification in the microenvironment of the exchanger that may induce a shift in its affinity for extracellular and intracellular H^+ and Na^+ (Pierce, Ramjiawan, Dhalla, & Ferrari, 1990). It has also been reported that in the ischemic diabetic rat heart, the recovery of pH_i occurs more rapidly in diabetic hearts receiving HEPES buffered solution than in those receiving bicarbonate buffered solution suggesting that the bicarbonate dependent mechanism of pH regulation may be depressed in diabetes (Khandoudi, Bernard, Cozzone, & Feuvray, 1995).

Additionally, DM and high energy diets (HED) are responsible for obesity and consequently increased adipose tissue (Cohen, 2001). In men, some 20% of the total E_2 is secreted by the testes, while the main source of E_2 is peripheral conversion of testosterone by aromatase, enzyme expressed in adipose tissue (Cohen, 2001; Wake et al., 2007). It has been suggested that the decrease in free testosterone concentration in these patients may be the result of an excessive aromatase dependent conversion of testosterone into E_2 (Cohen, 1999; Hofstra et al., 2008; Vikan, Schirmer, Njølstad, & Svartberg, 2010). In fact, it has been reported that E_2 concentrations are elevated in obese men as compared with lean men and that E_2 concentrations correlate positively with body mass index (BMI) (Hofstra et al., 2008; Muller, den Tonkelaar, Thijssen, Grobbee, & van der Schouw, 2003; Schneider, Kirschner, Berkowitz, & Ertel, 1979). Many studies support an association of men with higher E_2 levels and the increased risk of impaired fasting glucose and diabetes (Colangelo et al., 2009; Oh, Barrett-Connor, Wedick, & Wingard, 2002; Vikan et al., 2010).

It has also been described that E_2 is responsible for regulation of ion transporters expression in the male reproductive tract. Zhou et al. (2001) reported that ERα is responsible for regulation of the expression of the Na^+/H^+ exchanger 3 (NHE3) in efferent ductules and, thus, it influences Na^+ reabsorption and passive water transport. NHE3 is one essential protein involved in fluid transport in epithelial cells (Leung, Tse, Chew, & Wong, 2001). Mice αERKO and antiestrogen-treated mice presented loss or decreased expression of this protein, which can explain the fluid accumulation observed by the authors in efferent ductules and testes, that resulted in infertility (Zhou et al., 2001). More recently, Martins et al. (2012) identified the presence of NHE3 in SCs and described that E_2 is able to modulate its expression in cultured rat SCs. These findings indicate an effect of estrogens in other transporters expression and functionality, which is important for controlling the composition of luminal fluids in male reproductive tract.

Hence, several cellular changes associated with diabetes may account for the alteration of specific membrane ion transporters. It is expected that DM alters the cellular homeostasis, due to certain abnormalities in carbohydrate, fat, electrolyte and protein metabolism which ultimately lead to several acute and chronic complication (largely due to an increase in oxidative stress and production of reactive oxygen species) (M.G. Alves et al., 2013d) or due to an imbalance in the hormonal equilibrium, particularly in the sex steroid hormone levels.

Thus, it is of major relevance to deepen the knowledge on the effects of DM and pre-diabetes on the expression and functionality of specific membrane ion transporters, particularly in HCO_3-dependent membrane processes, in the male reproductive tract. This will elucidate whether or not the progression to pre-diabetes and/or more severe forms of diabetes is associated with the (dys)function of these membrane processes.

II. Aim of project

The bicarbonate is an essential element for maintenance of intracellular and extracellular pH in all cells and tissues. Herein we will focus on identification and on the role of bicarbonate transporters throughout the male reproductive tract.

The first objective of this study was examine the effect of pre-diabetes on bicarbonate transporters the Slc4 family (AE2, NDCBE, NBCe1 and NBCn1), in rat testes and epididymis. To accomplish that, mRNA and protein levels of those transporters were analysed. A second goal of this study was to study the effect of elevated concentrations of E_2 in the selected bicarbonate transporters. For that, mRNA expression and transcellular transport in SCs were evaluated.

III. Material and methods

1. Chemicals

Hank's Balanced Salts Solution (HBSS), Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM: Ham's F12), Ethylene Diamine Tetra Acetic acid (EDTA), Soybean Trypsin Inhibitor, DNAse, Collagenase type I, 17β-estradiol (E_2), Bovine Serum Albumin (BSA), Trypsin-EDTA, Insulin-Transferrin-Sodium Selenite supplement (ITS supplement), Adenosine-5'-triphosphate (ATP) and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS) was obtained from Biochrom AG (Germany). NZY M-MuLV Reverse Transcriptase (M-MLV RT), random hexamer primers, dNTPs and NZTaq 2x Green Master Mix, agarose and Greensafe were obtained from NZYTech (Lisboa, Portugal). Primers were obtained from STABVIDA (Oeiras, Portugal). Maxima SYBR Green/Fluorescein qPCR Master Mix was obtained from Thermo Scientific (California, USA). Tween 20 was obtained Applichem (Darmstadt, Germany). Dried milk was obtained Regilait (Saint-Martin-Belle-Roche, France). Antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

2. *In vitro* studies

2.1 Primary cultures of rat Sertoli cells

Male Wistar rats (22-day-old) were sacrificed by cervical dislocation, the testes were immediately excised in aseptic conditions and washed two times in a conical tube in 20mL of ice cold HBSS (potassium chloride 0,4 g/L, potassium phosphate monobasic anhydrous 0,06 g/L, sodium chloride 8 g/L, sodium phosphate dibasic 0,045 g/L, D-Glucose 1 g/L, Sodium bicarbonate 0,35 g/L) containing 10000 U/mL of penicillin, 10 mg/mL streptomycin and 25 µg/ml amphotericin B (pH 7.4). After removal of the adherent epididymis and vas deferens, the testes were decapsulated in HBSS, and the loosen tissue was washed three times in HBSS. SCs were isolated by a method described by Oliveira and collaborators (2011b) with slight modifications. Briefly, to remove contaminating peritubular cells, the tissue from decapsulated testes was washed in a conical tube and dispersed in glycine solution (HBSS plus 1 M glycine, 2 mM EDTA, 0,002% (w/v) Soybean Trypsin Inhibitor; pH 7.2). To further remove residual peritubular cells, the tubules were placed and dispersed in a Petri dish containing in glycine solution containing 0,5 mg/ml DNAse I during 10 minutes at room temperature. To uncoil the tubules and further release the interstitial tissue/cells, the dispersed tubules were forced through a large-pore Pasteur pipette. The tubular pellet was then digested for 15-20 minutes (at room temperature) in a HBSS plus 0,225 mg/ml collagenase type I and 0,05 mg/mL DNase I. After digestion, the disaggregated seminiferous tubules were washed three times in HBSS by centrifuging the cell suspension 3 minutes at 300.*g*. The SC suspension was collected and resuspended in Sertoli culture medium which consisted of a 1:1 mixture of DMEM: Ham F12, supplemented with 15 mM HEPES, 50 U/ml penicillin and 50 mg/mL streptomycin sulfate, 0,5 mg/mL fungizone, 50 µg/mL gentamicin and 10% heat inactivated

FBS. In order to disaggregate large SC clusters, the cellular suspension was forced though a 20G needle. For cell culture, the concentration of the clusters on the cellular suspension obtained was adjusted to 5000 clusters/ml, plated on 25 cm^2 culture flasks (Cell+; Sarstedt), and incubated at 33ºC in an atmosphere of 5% CO_2: 95% O_2. The cultures were left undisturbed until day 2, considering the day of plating day 0 of culture.

2.2 Hormonal treatment of rat Sertoli cells

When SCs cultures presented a 90-95% confluence, culture medium was replaced by serum and phenol-red free medium supplemented with insulin, DMEM: F12 supplemented with ITS, pH 7.4. In order to evaluate the effects of hormones on this work SCs were treated with 100 nM of E_2 in 0,025% ethanol (EtOH). The E_2 concentration was chosen based on intratesticular interstitial fluid concentrations of those hormones, are reaching values up to 200 nM. We used in this study a medium value this concentration.

Control group was treated with 0,025% EtOH. Treatments were performed during 50 hours in an atmosphere of 5% CO2, 95% O2 at 33ºC.

3. *In vivo* studies

3.1 Establishment of the pre-diabetes animal model

Twelve 2-month-old male Wistar rats were used in present study. The animals were housed in our accredited animal colony (Health Sciences Research Center, University of Beira Interior) and maintained with food and water ad libitum in a constant room temperature (20 ± 2°C) on a 12 hour cycle of artificial lighting. Rats were randomly divided in control and high-energy-diet (HED) groups. The control group animals were fed with a standard chow diet (4RF21 certificate, Mucedola, Italy) and the HED group received an additional high-energy emulsion as described elsewhere (L. Rato et al., 2013). Briefly, in the first 5 treatment days, animals were given progressively 1–5 mL of emulsion consisting of 20g lard oil, 1 g thyreostat, 5 g cholesterol, 1 g sodium glutamate, 10 g sucrose, 20 mL Tween 80, 30 mL propylene glycol prepared in a final volume of 100 mL by adding distilled water. Thereafter, they were administrated daily with 5mL of the emulsion until they reach 1 month of treatment. Water, food consumption and the animal's weight were monitored every 2 days in both experimental groups, during all the treatment. After the treatment, animals were killed by cervical dislocation. Blood was collected by cardiac puncture to non-heparinized tubes. Testes were removed, weighed and processed for testicular interstitial fluid collection, according to (Porter, Shetty, & Meistrich, 2006) or stored at -80°C. All animal experiments were performed according to the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and the European directives for the care and handling of laboratory animals (Directive 86/609/EEC).

4. RNA extraction

Extraction of total RNA (RNAt) was performed using the E.Z.N.A. Total RNA Kit (Omega bio-tek, Norcross USA) as indicated by the manufacturer. RNA from epididymis and testis was isolated using 15 mg of tissue. RNA from SCs was isolated after detaching cells from the culture flasks using a trypsin-EDTA solution. To eliminate residual trypsin, detached cells were washed with 3 mL of phosphate buffered saline (PBS), by centrifugation at 3000.*g* during 5 minutes. RNA concentration and absorbance ratios (A260/A280) were determined by spectrophotometry (NanophotometerTM, Implen, Germany).

5. RT-PCR

The RNAt obtained for each sample was reversely transcribed in a mixture containing 0,5 mM of each dNTP, 250 ng of random hexamer primers, 1 µg of RNAt and sterile H_2O up a volume of 13,50 µl. The mixture was initially incubated 5 minutes at 65ºC. Then, 200 U of M-MLV RT and 2 µl of Reaction Buffer were added and incubated sequentially at 25ºC for 10 minutes, 37ºC for 50 minutes and 70ºC for 15 minutes. The resulting complementary deoxyribonucleic acid (cDNA) was used with exon-exon spanning primer sets designed to amplify AE2, NDCBE, NBCe1 and NBCn1 cDNA fragments (Table 1). Polymerase chain reactions (PCR) were carried out using 1 µL of cDNA in 12,5 µL of final volume of a mixture containing 6,25 µL of NZYTaq Green Master Mix 2x, 1 µM of each primers and sterile H_2O. Primer sequences, optimal annealing temperature, the number of cycles required for exponential amplification phase of fragments and fragment sizes are indicated in Table 1. Kidney mRNA was used as positive control and cDNA-free sample was used as negative control. At the end of the experiments, samples were run in 1% agarose gel electrophoresis with 25 µL of Greensafe in 100 mL, during 30 minutes at 120V. The agarose gel was visualised using software Molecular Imager FX Pro Plus MultiImager (Biorad, Hercules, USA) coupled to an image acquisition system (Vilber Lourmat, Marne-la-Vallée, France). The size of the expected products was compared to a DNA ladder (NZYDNA Ladder VI, Nzytech).

6. Real time – PCR

Real-time PCR (qPCR) was performed to analyze AE2, NDCBE, NBCe1 and NBCn1 mRNA expression. Specific primers were designed for the amplification of the target and housekeeping transcripts (Table 1). qPCR was carried out in an iQ5 system (Bio-Rad, Hercules, USA) and efficiency of the amplification was determined for all primer sets using serial dilutions of cDNA (1:3, 1:15 and 1:75). qPCR conditions and reagents concentrations were previously optimized and specificity of the amplicons was determined by melting curves. qPCR amplifications used 1 µg of synthesized cDNA in a 20 µL reaction containing: 10 µL

Maxima SYBR Green/Fluorescein qPCR Master Mix and 0,3 μM of sense and antisense primers for each gene. Amplification conditions comprised a initial denaturation step of 5 minutes at 95ºC, followed by 35 runs of a 3 steps cycle: (1) a denaturation step of 10 s at 95ºC, (2) an annealing step of 30 s with a specific temperature for each set of primers (Table 1) and (3) an extension step of 10 s at 72ºC. β-2-microglobulin transcript levels were used to normalize the mRNA expression levels of AE2, NDCBE, NBCe1 and NBCn1. Fold variation of the expression levels was calculated following the mathematical model proposed by Pfaffl using the formula: $2^{-\Delta\Delta Ct}$ (2001).

Table 1: Genes, oligonucleotide sequence and respective conditions for PCR amplification of AE2, NDCBE, NBCn1, NBCe1 and ß2-Microglobulin.

Gene	Sequence (5'-3')	AT (ºC)	Amplicon (bp)	Cycles
AE2 NM_017048.2	Sense: ATGCCAAAGGGTCTACACAG Antisense: GCTCCTGGTTTTTGTCCAAC	53	138	35
NDCBE NM_199497.2	Sense: GAGACCTACCCCATCCACAT Antisense: TATGAACTCCCCGTGCATCT	53	189	35
NBCn1 NM_058211.2	Sense: GATGAAATGGCCAAAACTGCC Antisense: ATTGTCACACTCACAGGCTT	53	107	35
NBCe1 NM_053424	Sense: GCCTGGAGAACAACCAAAGT Antisense: CATACAGAACAGGCATGGGG	53	131	35
ß-2-Micoglobulin NM_012512.2	Sense: ATGAGTATGCCTGCCGTGTG Antisense: CAAACCTCCATGATGCTGCTTAC	58	92	30

Abbreviation: AT- Annealing temperature.

7. Total protein extraction

Tissue samples (50 mg) were homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer (1x PBS, 1% NP-40, 0,5% sodium deoxycholate, 0,1% SDS, 1mM phenylmethylsulfonyl fluoride (PMSF)) supplemented with 1% protease inhibitor cocktail, aprotinin and 100mM sodium orthovanadate with the aid of a Tissue Ruptor homogenizer (Qiagen, Hilden, Germany). The lysed tissue was allowed to stand 15 minutes on ice and the suspension was centrifuged at 14000.*g* for 20 minutes at 4ºC. The resulting pellet was discarded. The total protein concentration was determined using the Bradford assay as previously described (Bradford, 1976).

8. Western blot

Western Blot procedure was performed as previously described by Alves and collaborators (2011). Briefly, proteins samples (50 µg) were denaturated for 30 minutes at 37ºC.The proteins samples were fractionated on a 12% SDS-PAGE at 30 mA/gel for 90 minutes. After electrophoresis, proteins were electrotransferred to a PVDF membrane at 750 mA for 75 minutes. The membranes were blocked in a Tris-buffered saline solution (TBS) with 0,05% Tween 20 containing 5% skimmed dried milk for 90 minutes. The membranes were then incubated at 4ºC overnight with goat anti-Slc4a4 (1:250, Santa Cruz Biotechnology Heidelberg, Germany, Sc-162214), or goat anti-AE2 (1:250, Santa Cruz Biotechnology Heidelberg, Germany, Sc-46710), or goat anti-Slc4a8 (1:500, Santa Cruz Biotechnology Heidelberg, Germany, Sc-169346), or rabbit anti-Slc4a7 (1:250, Santa Cruz Biotechnology Heidelberg, Germany, Sc-99633). Mouse anti-actin was used as protein loading control (1:5000, Sigma, Roedermark, Germany, A-5441). The immune-reactive proteins were detected separately with donkey anti-goat IgG-AP (1:5000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 2020), or goat anti-rabbit IgG-AP (1:5000, Santa Cruz Biotechnology Heidelberg, Germany, Sc-2004), or goat anti-mouse IgG-AP (1:5000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 2005). Membranes were reacted with ECF detection system (GE, Healthcare, Weßling, Germany) and read with the BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK). The densities from each band were obtained using the Quantity One Software (Bio-Rad, Hemel Hempstead, UK), according to standard methods.

10. Voltage Clamp

SCs obtained from primary cultures of male Wistar rats (22-day-old) seminiferous tubules, as described above, were seeded in Snapwell cell culture inserts with a polyester membrane of 0.4 µm pore size (Corning, Tewksbury, USA) until reaching confluence. Confluence was assessed by measurin transepithelial resistance as described previously (Skalli, Avallet, Vigier, & Saez, 1992). After culture confluence, the cells were treated with E_2 100nM or 0,025% EtOH during 24 hours. Afterwards, 1.13 cm^2 Snapwell inserts were mounted in *Ussing* type chambers (Vertical diffusion chamber system, Navicyte, HARVARD APPARATUS, Massachusetts, USA). Sertoli Control Solution ((in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.8; KH_2PO_4, 1.8; $NaHCO_3$, 25; $C_6H_{12}O_6$, 14; Hepes, 25; pH=7.4) was added to each side of the preparation and the current was left to stabilize before the experiments took place.

The preparation was kept short-circuited throughout the experiments by means of an electronic voltage-clamp system (Model VCC MCx, Physiologic Instruments, San Diego, CA) and the values of the instantaneous currents were acquired at a sampling rate of 1 min^{-1}. The epithelial conductance was automatically estimated from the current pulse produced by displacing the transepithelial electrical potential difference by 1 mV during 15 s, every

minute. Data was acquired using a personal computer and software ACQUIRE & ANALYZE (Acquire & analyze, Physiologic Instruments, Version 2.3).

After a steady state period of at least 10 minutes, ATP (1mM or 2mM) was added to the apical side of the preparation and the short-circuit current (I_{sc}) was followed for a period up to 2 hours.

I_{sc} is plotted as the average (± s.e.m.) of the fractional values calculated by dividing each value by the initial I_{sc} value (acquired at time zero). Spontaneous Vt values were calculated as the average of the 10 initial readings or the average of the last 10 readings of each experiment. In this preparation the I/V curve is linear at least between -100 and +100 mV.

11. Statistical Analysis

The statistical significance of the samples variation among the experimental groups was assessed by two-way ANOVA, followed by Bonferroni post-test. T-test student was also performed in some samples. In some conditions, the control sample is represented by 1, and results are presented by relative variation in comparison with control group. All experimental data are shown as mean ± SEM (n=5 for each condition). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). $P<0.05$ was considered significant.

IV. Results

1. Bicarbonate transporters in testis and epididymis of HED-treated animals

The possible alterations in mRNA expression and protein levels of bicarbonate transporters, namely AE2, NBCe1, NBCn1 and NDCBE in testes and epididymis of HED-treated rats, were analysed by qPCR and western blot, and compared with control group.

1.1 Pre-diabetic rodent model characterization

High-energy diet fed rats developed mild hyperglycemia, glucose intolerance and hypoinsulinemia. Pre-diabetes rodent model was developed as previously described by our team (L. Rato et al., 2013). At the end of HED treatment, animals presented mild hyperglycemia (116±3 mg/dL), as oposed to the animals of the control group (101±4 mg/dL). Moreover, HED rats had significantly increased (by ~57%) area under the curve (AUCg) values, after receiving an intraperitoneal injection with 6 mL glucose 30% (w/v) per kg of body weight, compared to control group, evidencing that HED rats developed significant glucose intolerance. These results suggested an insulin dysfunction status, so we measured fasting blood insulin levels and, as expected, insulin levels of HED rats were significantly decreased (by ~61%) when compared to control group. These characteristics, particularly glucose intolerance and mild-hyperglycaemia, indicated that HED animals developed a pre-diabetic state.

1.2 HED increases protein levels of AE2 in the epididymis

The possible effect of HED on mRNA transcript levels of AE2 was evaluated by a qPCR. The mRNA expression of AE2 in testis was not significantly different when compared with the control group (0.92 ± 0.15) (Figure 8 Panel B). The western blot analysis was used in order to determinate the protein expression levels, which confirmed no effect of HED on AE2 protein levels as compared to the control group (1.03 ± 0.17) (Figure 8 Panel A and Panel B).

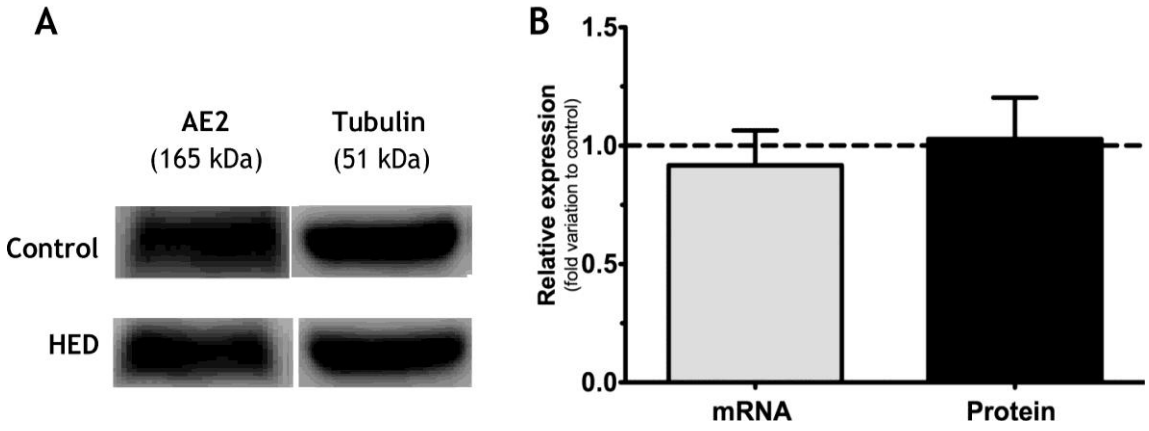

Figure 8: Effect of High Energy Diet (HED) on membrane transporter anion exchanger 2 (AE2) mRNA and protein levels in rat testis. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in testis tissue of HED rats when compared with testis from control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

In epididymis of HED treated animal, the mRNA expression of AE2 was not significantly different from that of control group (0.64 ± 0.08) (Figure 9 Panel B). However when we evaluated the protein expression levels of this transporter, the results showed that there was a significant increase of AE2 protein expression in the epididymis of HED-treated animals relative to the control (1.77 ± 0.50) (Figure 9 Panel A and 9 Panel B).

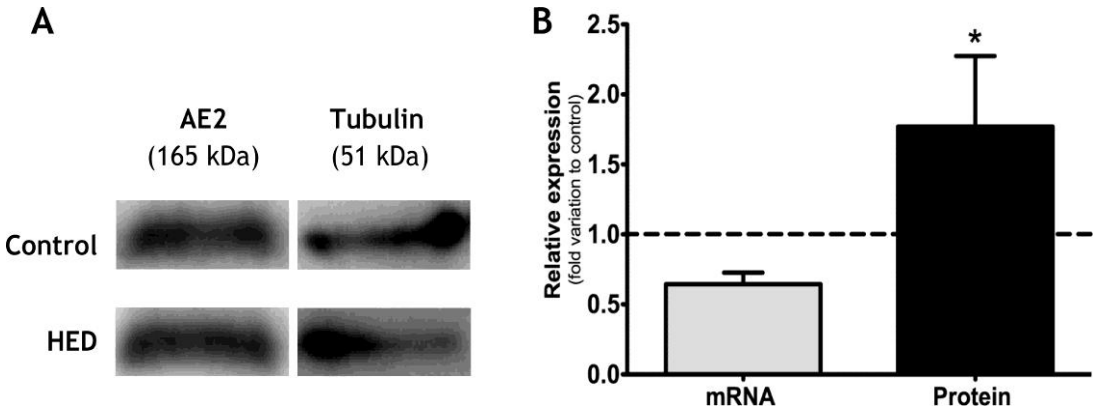

Figure 9: Effect of High Energy Diet (HED) on membrane transporter anion exchanger 2 (AE2) in mRNA and protein levels in rat epididymis. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in epididymis tissue of HED rats when compared with epididymis from control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

1.3 HED increases mRNA levels of NBCe1 in the testis

The protein levels of the NBCe1 were not significantly different in HED-treated animals testes when compared with the control group (0.81 ± 0.19) (Figure 10 Panel A and Panel B). On the other hand, the mRNA expression levels of NBCe1 in the testis of HED-treated rats presented a significant increase of 2.94 ± 0.21 fold relatively to the control (Figure 10 Panel B).

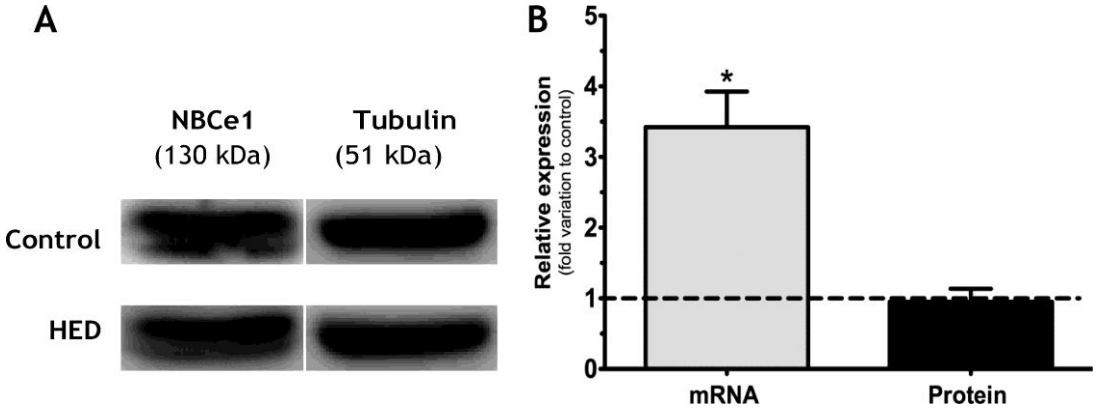

Figure 10: Effect of High Energy Diet (HED) on electrogenic Na^+/HCO_3^- co-transporters (NBCe1) in mRNA and protein levels in rat testis. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in testis tissue of HED rats when compared with testis from control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

In epididymis of HED-treated rats the mRNA expression of NBCe1 is decreased (0.71 ± 0.15 fold variation) when compared with control group, but this alteration was not statistically significant (Figure 11 Panel B). Likewise, the protein levels of this transporter were not significantly altered (0.95 ± 0.13 fold variation) in the epididymis of rats from the HED group (Figure 11 Panel A and Panel B).

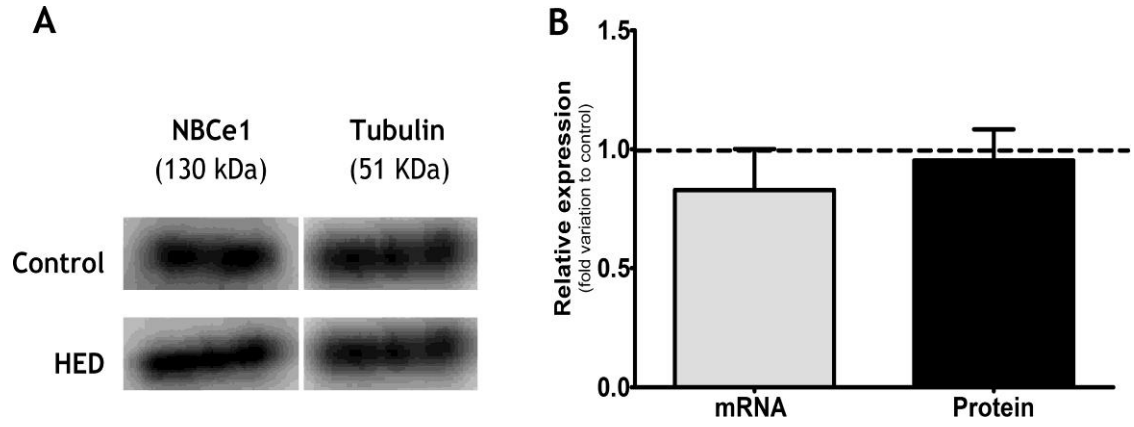

Figure 11: Effect of High Energy Diet (HED) on electrogenic Na^+/HCO_3^- co-transporters (NBCe1) in mRNA and protein levels in rat epididymis. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in epididymis tissue of HED rats when compared with epididymis from control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

1.4 HED increases mRNA levels of NBCn1 in the testis

The mRNA expression of the electroneutral transporter, NBCn1, was significantly increased in testis of HED-treated rats (4.22 ± 0.17 fold increased to control) (Figure 12 Panel B). This mRNA increase in testis HED was not followed by an increase in the protein expression levels of these transporters (0.811 ± 0.190 fold variation relative to control) (Figure 12 Panel A and Panel B).

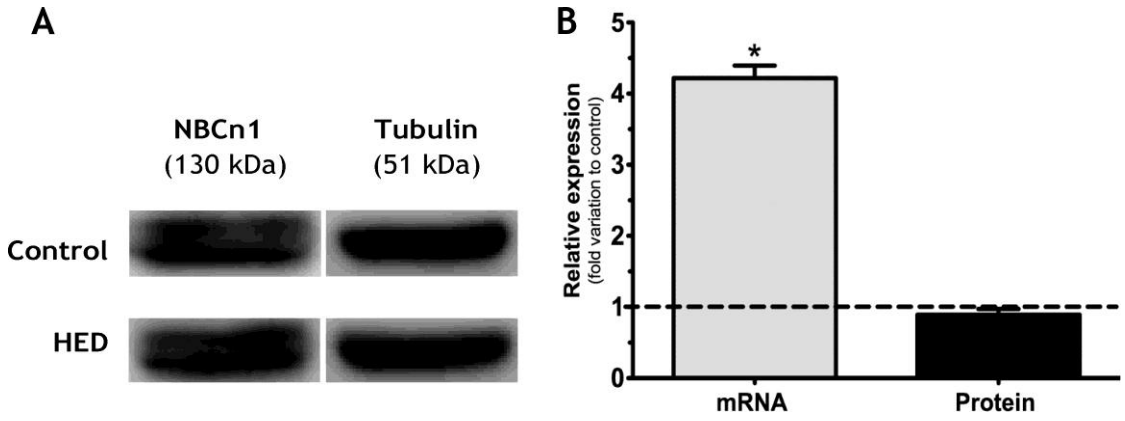

Figure 12: Effect of High Energy Diet (HED) on eletroneutral Na^+/HCO_3^- cotransporters (NBCn1) in mRNA and protein levels in rat testis.Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in testis tissue of HED rats when compared with testis from control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

The protein levels and mRNA expression of NBCn1 in epididymis were not significantly different in HED-treated animals when compared with the control group (Figure 13B). However, both mRNA and protein levels (Figure 13 Panel A and Panel B) from the epididymis of HED-treated animals presented a non-significant increase to 1.606 ± 0.265 and 1.817 ± 0.270 fold variation relative to control group, respectively.

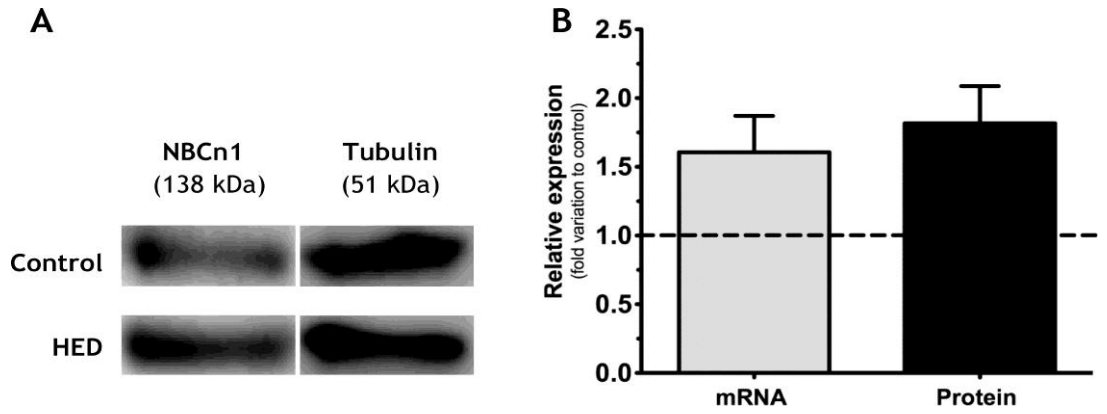

Figure 13: Effect of High Energy Diet (HED) on eletroneutral Na^+/HCO_3^- cotransporters (NBCn1) in mRNA and protein levels in rat epididymis. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in epididymis tissue of HED rats when compared with epididymis from the control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

1.5 HED decreases protein levels of NDCBE in the epididymis

Analysis of mRNA NDCBE level the testis of HED-treated rats showed a significant increase of 2.00 ± 0.31 fold relatively to animal from the control group (Figure 14 Panel B). When we evaluated the protein levels of this bicarbonate transporter, we observed no significant alteration in HED-treated rats when compared to control group (Figure 14 Panel A and Panel B).

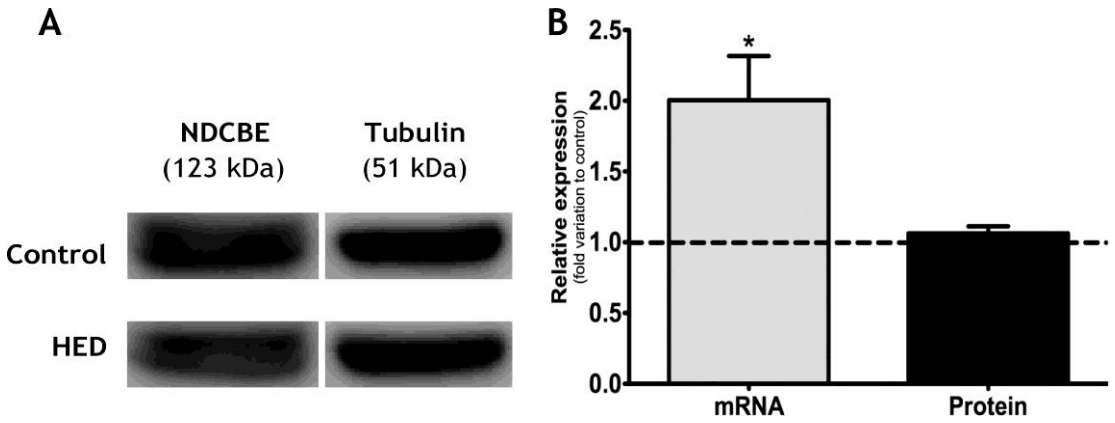

Figure 14: Effect of High Energy Diet (HED) on Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE) in mRNA and protein levels in rat testis. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in testis tissue of HED rats when compared with testis from control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

HED-treated animals did not present a significant alteration on NDCBE mRNA transcript levels (1.16 ± 0.15 fold variation to the control) in epididymis (Figure 15 Panel B). Nevertheless, protein expression levels were significantly decreased in epididymis of HED-treated rats (0.85 ± 0.04 fold decreased to control) (Figure 15 Panel A and Panel B).

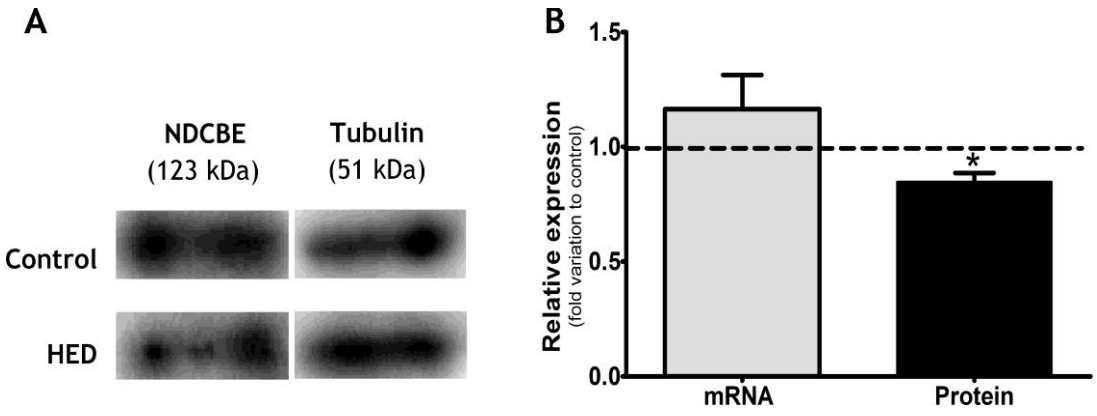

Figure 15: Effect of High Energy Diet (HED) on Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE) in mRNA and protein levels in rat epididymis. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA and protein levels found in epididymis tissue of HED rats when compared with epididymis from control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

2. 17β-Estradiol effects in bicarbonate transporters of Sertoli cells

To analyse the possible effect of E_2 on the expression of bicarbonate transporters, SCs were cultured for 50 hours in media containing 100 nM of E_2 or not (control group). The mRNA expression of the bicarbonate transporters in SCs was then determined.

Furthermore transcellular transport on cultured SCs was also analysed.

2.1 Relative expression of bicarbonate transporters in cultured Sertoli cells

We were able to confirm the transcripts presence of all the analysed bicarbonate transporters (AE2, NDCBE, NBCn1 and NBCe1) in rat cultured SCs (Figure 16 Panel A). The identification of these transporters in SCs was detected by a RT-PCR, using kidney lysate mRNA as positive control (Figure 16 Panel B).The mRNA abundance of AE2, NDCBE, NBCn1 and

NBCe1 was evaluated using the qPCR technique. AE2 mRNA is the most abundant in rat SCs (0.037 ± 0.006 arbitrary units), compared with NBCe1 (0.006 ± 0.002 arbitrary units), NBCn1 (0.010 ± 0.002 arbitrary units) and NDCBE (0.001 ± 0.001 arbitrary units) (Figure 16 Panel B).

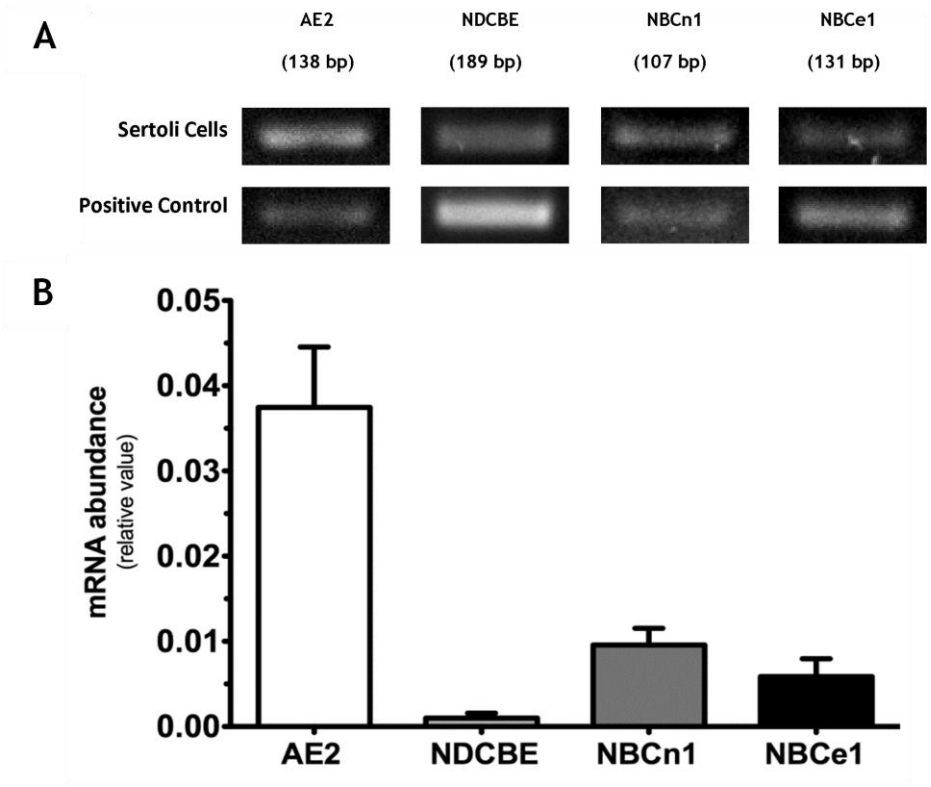

Figure 16: Identification and quantification of the mRNA levels of anion exchanger 2 (AE2), Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE), electroneutral Na^+/HCO_3^- co-transporters (NBCn1) and electrogenic Na^+/HCO_3^- co-transporters (NBCe1) in rat Sertoli cells. Panel A shows data indicating the mRNA abundance of these transporters in Sertoli cells. Panel B shows representative semi-quantitative RT-PCR. Results are expressed as mean ± SEM (n=5).

2.2 Elevated concentration of E_2 alter mRNA expression of AE2, NBCn1 and NBCe1 in rat Sertoli cells

The effect of E_2 (100nM) on mRNA transcript levels of AE2, NDCBE, NBCn1 and NBCe1 was evaluated by qPCR. The mRNA expression of AE2, NBCn1 and NBCe1 in E_2-treated cells was significantly increased when compared with the control group (2.30 ± 0.74, 1.38 ± 0.27 and 1.51 ± 0.20 fold increase, respectively). On the other hand the mRNA levels of NDCBE were decreased when compared with control group, but this difference was not significant (0.75 ± 0.04 fold decrease) (Figure 17).

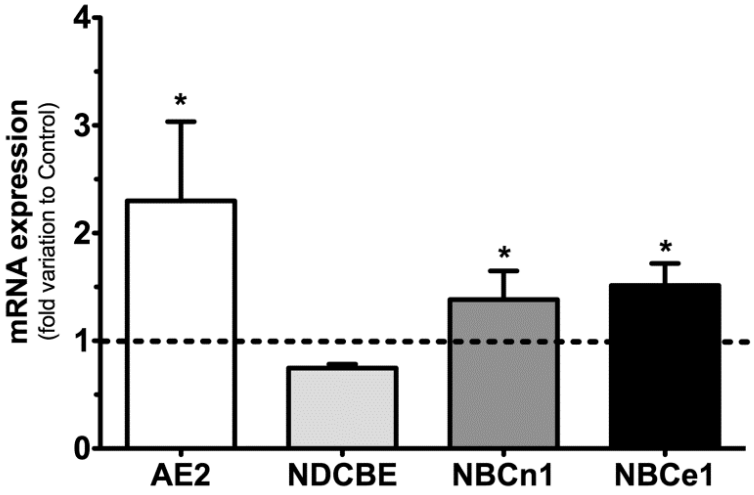

Figure 17: Effect of 100 nM 17ß-estradiol (E_2) on anion exchanger 2 (AE2), Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE), electroneutral Na^+/HCO^{3-} co-transporters (NBCn1) and electrogenic Na^+/HCO_3^- co-transporters (NBCe1) relative expression of mRNA in rat Sertoli cells, when compared with the control condition (dashed line). Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$).

2.3 Alterations in transcellular transport in E_2-treated rat Sertoli cells

The effect of E_2 (100 nM) in transcellular transport by SCs was evaluated using the Voltage-Clamp technique. SCs were seeded in Snapwell cell culture inserts (Corning, Tewksbury, USA) until reaching confluence. Afterwards, cells were treated with 100 nM of E_2 or with an equal volume of 0,025% EtOH (control group) during 50 hours.

Several secretory epithelial cells, including the rat SCs have shown to possess functional purinoceptors, which affect transepithelial transport. Ko et al. (1996) described that culture SCs transport electrolytes electrogenically when stimulated with ATP, a phenomenon that was inhibited by the removal of HCO_3^- or Cl^- from the bathing solutions, and by apical application of diphenylamine-2-carboxylate (DPC) or 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS), suggesting that the observed current could be attributed to HCO_3^--dependent Cl^- secretion (W. Ko, Chan, Chew, & Wong, 1998).

We performed a similar protocol using two different ATP concentrations, 1 mM and 2 mM. The representative curve for addition of ATP is represented in Figure 18 Panel A, when addition of ATP the I_{sc} increase followed by an I_{sc} recovery, which normally does not reach the initial value. The effect of ATP is shown in Figure 18. ATP (1 mM or 2 mM) added to the apical side caused a biphasic response. In the control conditions, I_{sc} rose rapidly to a peak level of 1.27 ± 0.05 arbitrary units (ATP 1 mM) or 1.40 ± 0.07 arbitrary units (ATP 2 mM). It then fell

to a stable plateau at about 1.22 ± 0.01 arbitrary units (ATP 1 mM) or 1.25 ± 0.08 arbitrary units (ATP 2 mM).

The peak level and the I_{sc} recovery to a sustained plateau current after ATP addition were reduced by incubation with E_2 (100 nM) (Figure 18 Panel B-E). The variation of I_{sc} (ΔI_{sc}) in SCs treated with E_2, after being stimulated with 1 mM ATP was significantly decreased as compared with SCs from the control group (0.05 ± 0.03 and 0.26 ± 0.05 arbitrary units, respectively) (Figure 18 Panel B). I_{sc} recovery in these cells (0.04 ± 0.01 arbitrary units) was also decreased, although not significantly, when compared with the control group (0.05 ± 0.01 arbitrary units) (Figure 18 Panel C). When cells were stimulated with an ATP concentration of 2 mM it originated similar results. The ΔI_{sc} in E_2-treated cells was 0.17 ± 0.04 arbitrary units, a value lower than in SCs from the control group (0.40 ± 0.07 arbitrary units) (Figure 18 Panel D). I_{sc} recovery in these E_2-treated cells stimulated with ATP 2 mM was much smaller (0.02 ± 0.01 arbitrary units) as compared with cells from the control group (0.15 ± 0.08) (Figure 18 Panel E).

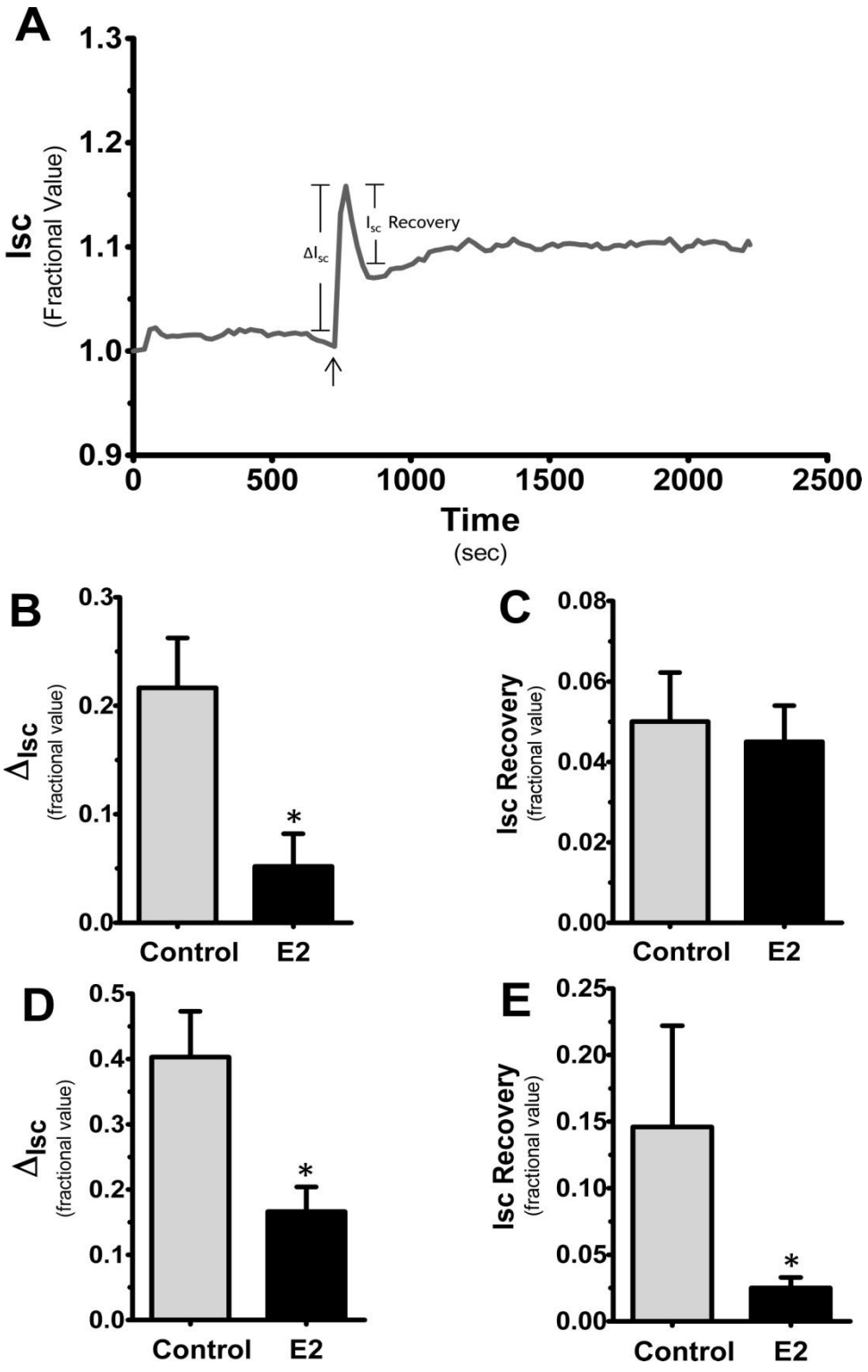

Figure 18: Short-circuit current (Isc) response to the addition of ATP to the apical bathing solution. Panel A shows the representative curve of I_{sc} when ATP was added to cultured Sertoli cells. Panel B shows fractional values of the variation of I_{sc} (ΔI_{sc}) after addition of 1 mM ATP to the apical bathing solution in SCs from the control group and E_2-treated (100 nM) cells. Panel C represent the I_{sc} recovery after being added 1 mM ATP. Panel D shows fractional value of ΔI_{sc} after addition of 1 mM ATP to the apical bathing solution in SCs from the control group and E_2-treated (100 nM) cells. Results are expressed as means ± SEM (n=5). * Indicates significantly different relatively to control ($p< 0.05$). Arrow represents the addition of ATP (1 mM or 2 mM).

V. Discussion

The formation of competent spermatozoa is a complex event that depends of the establishment of adequate environments in the seminiferous epithelium and also in the excurrent ducts. For instance, the maintenance of adequate luminal *milieu* in the epididymis is crucial and small ionic or pH perturbations can arrest spermatozoa maturation (L. Rato et al., 2012). Bicarbonate is not only essential to ionic homeostasis as HCO_3^- concentration plays an essential role in the pH maintenance along the male reproductive tract. Moreover, spermatozoa capacitation is an HCO_3^--dependent activation process that ends in the female reproductive tract and enables spermatozoon to fertilize the eggs (T. Turner, 2002; L. Rato et al., 2012). In this process, HCO_3^- is needed for activation of soluble adenylyl cyclase controlling several phosphorylation events. Nevertheless, the mechanisms through which the bicarbonate transport occurs and how they control the male reproductive health remain largely unknown. It is clear that, as it happens in other tissues, the several membrane proteins already identified in the male reproductive tract that transport HCO_3^- have at least one major function: maintain both pH_i and pH_o within narrow limits allowing not only a successful spermatogenesis but also spermatozoa capacitation and egg fertilization (Boron, 2004; Casey, Grinstein, & Orlowski, 2009; Robaire et al., 2006).

The presence of distinct HCO_3^- transport systems, with different expression levels in distinct testicular cells and spermatozoa, is clear evidence that HCO_3^- is a key player in the maintenance of male reproductive health. Moreover, the role of HCO_3^- throughout the male reproductive tract is essential for determining the ionic fluids composition, osmolariy and pH (Robaire et al., 2006; T. Turner, 2002). Any alteration in these processes may end-up in male subfertility and/or infertility.

In our work, we chose to analyze the possible alterations in the presence and function of four of the most widely distributed bicarbonate transporters of the Slc4 family, namely AE2 (Scl4a2), NBCe1 (Scl4a4), NBCn1 (Slc4a7) and NDCBE (Slc4a8). These membrane transporters of the Slc4 family have in common the characteristic of transporting basic particles, specifically HCO_3^-, but they differ in the ability to mediate the concurrent transport of Na^+ and/or Cl^- (Figure 19).

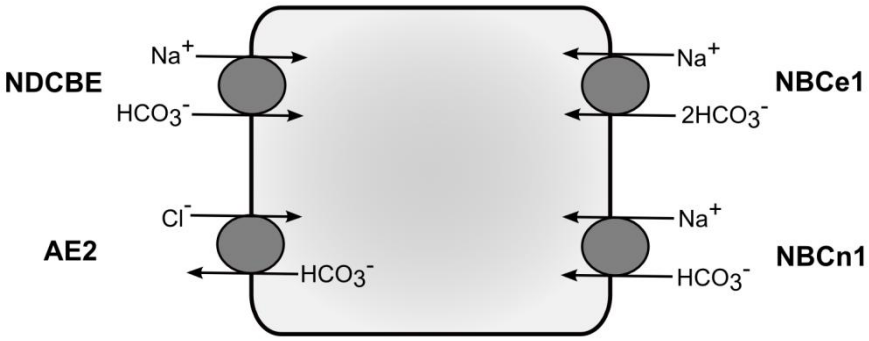

Figure 19: Diagram of a simplified cell with the bicarbonate membrane transport systems considered. AE2 – Anion exchanger 2 (Slc4a2); NBCe1 - electrogenic Na^+-dependent HCO_3^- co-transporter (Scl4a4), NBCn1 - electroneutral Na^+-dependent HCO_3^- co-transporter (Slc4a7); NDCBE - Na^+-driven Cl^--HCO_3^- exchanger (Slc4a8).

1. Bicarbonate transporters in testis and epididymis of HED-treated animals

Testes are responsible for two major tasks: testosterone production and formation of haploid germ cells. The two-compartment configuration of the testis (the seminiferous tubules and the intertubular areas), filled with the characteristic fluids (STF and testicular lymph or interstitial fluid) (Jegou et al. 1982), is of great relevance for its functioning. For instance, it has been shown that the composition of the fluid within the seminiferous tubules is very stable due to the existence of the BTB. The BTB regulates the passage of various endogenous and administered substances (Koskimies and Kormano 1973), supporting the fundamental relevance of the intraluminal fluid composition (A. J. Pace et al., 2000; D. Fisher, 2002; B.P. Setchell, 1890). It provides the milieu for developing spermatozoa and the vehicle to transport them from the testis. Furthermore, the luminal milieu is markedly distinct from the interstitial fluid and plasma and these differences are critical to the normal occurrence of spermatogenesis (A. J. Pace et al., 2000; D. Fisher, 2002).

A study by Caflisch & DuBose (1990) showed that the rat STF contains a very low concentration of HCO_3^-. These results led to the assumption that active HCO_3^- secretion is not an important factor in the formation of the STF. Nevertheless, testicular cells and particular seminiferous tubule sustentacular cells (SCs) must possess in their membranes transport mechanisms in order to be able to exert a strict regulation of the HCO_3^- concentration, although very few is known about these processes.

It has been reported that DM does not change steady-state pH_i but significantly alters pH_i regulation in mammalian cells mainly by markedly decreasing the activity in some ion

transporters (Feuvray, 1997). It has also been suggested that some bicarbonate-dependent mechanisms of pH regulation may be depressed in cells of DM individuals (Khandoudi et al., 1995), but little is known on the effects of the DM on the membrane transport mechanisms involved in pH regulation on male reproductive tract cells and even fewer of the different stages involved in the progression of this pathology, particularly pre-diabetes.

Hence, we induced a pre-diabetic state in male rats following the ingestion of a HED, as described previously by our team (L Rato et al., 2013), and evaluated the effect of this pathological state on the expression of the most relevant bicarbonate transporters of the Slc4 family, namely AE2 (Scl4a2), NBCe1 (Scl4a4), NBCn1 (Slc4a7) and NDCBE (Slc4a8), previously described in the cells of the male reproductive tract and specifically in testicular and epididymal cells.

At the testicular level, we were able to confirm the presence of all the bicarbonate transporters of the Slc4 family studied, both at mRNA and protein level. When we evaluated the alterations caused by pre-diabetes on the expression levels of these HCO_3^-, we were able to perceive that, although no alterations were detected in protein expression, the mRNA levels of NBCe1, NBCn1 and NDCBE were significantly increased.

Even though, as observed in our work, the protein levels do not always reflect the changes on mRNA transcript levels and, hence, the changes in mRNA level are not a direct measure of the variation on the protein quantity or functioning, they can be a clear indication of the effectiveness of the regulation exerted on the studied protein. The diminished mRNA levels could be explained by differential rates of synthesis or degradation or both. mRNA half-lives can increase or decrease in response to a variety of stimuli including hormones and growth factors (Hollams, Giles, Thomson, & Leedman, 2002). Thus, the possibility exists that, pre-diabetes modulation of the analyzed mRNA quantities is exerted at a transcriptional and/or post-transcriptional level, and/or that the modulation of protein quantities is regulated by other mechanisms or on a different timeframe.

On the other hand, at epididymal level we were able to determine that pre-diabetes is capable to alter the protein expression of several of the bicarbonate transporters examined. As previously referred, epididymal function is important to regulate and modify the luminal fluid content, which is crucial for male fertility, namely for the maturation, transport, concentration and storage of spermatozoa. Both epididymal secretion and reabsorption of luminal fluids establish and modify the epididymal microenvironment (Foley, 2001; Serre & Robaire, 1998), in order to provide an adequate environment to transform spermatozoa info fully mature cells (Robaire et al., 2006). The fluid milieu in the epididymis is acidic containing very low concentration of HCO_3^- (Okamura, Tajima, & Sugita, 1987; Rodriguez-Martinez et al., 1990) and this points towards a major role of bicarbonate membrane transport mechanisms on the establishment of luminal fluid pH and, hence, in epididymis function.

As it happened at testicular level, our results confirmed the expression of all the bicarbonate transporters of the Slc4 family studied, both at mRNA and at protein level. The evaluation of the alterations caused by pre-diabetes on the expression levels of these HCO_3^- transporters showed a significant increase on AE2 and a significant decreased of NDCBE protein levels. Expression of AE2 has been described in the basolateral membrane of epithelial cells lining the lumen of all the regions of the epididymis (Medina et al., 2003), and he is expressed in relatively low abundance in the cauda epididymis (Jensen, Stuart-Tilley, et al., 1999). The presence of AE2 in the basolateral membrane of epithelial cells of the proximal parts of the epididymis correlates with the low luminal concentration of bicarbonate attained in these segments, and thus it has been suggested that the basolateral AE2 might contribute, in parallel with other bicarbonate transporters, to the net bicarbonate reabsorption. Also, we were able to detect a decrease of NDCBE protein levels. This transporter has been described as a pH_i regulator that transports extracellular Na^+ and HCO_3^- in exchange for intracellular Cl^- and/or H^+, playing an important role in cellular alkalinisation (J. M. Russell & BORON, 1976) and in bicarbonate reabsorption.

As said, the establishment of a low HCO_3^- concentration in the lumen of the epididymis contributes to maintaining an optimum environment for proper sperm storage and viability. Alteration of the HCO_3^- transepithelial epididymal fluxes *in vivo* might, therefore, represent a real threat for sperm survival during storage in the epididymis and this might correlate with the results described by Rato et al. (2013) that reported a significant increase in abnormal sperm morphology in pre-diabetic rats.

2. Bicarbonate transporters in Sertoli cells: regulation by 17β-Estradiol

As previously mentioned, several studies support an association of men with higher E_2 levels and the increased risk of impaired fasting glucose and diabetes (Colangelo et al., 2009; Oh et al., 2002; Vikan et al., 2010). Furthermore, it has been described that E_2 is responsible for regulation of ion transporters expression in the male reproductive tract (Zhou et al., 2001). Hence, using SCs cultures incubated in the presence or absence of E_2 (100 nM) we were able to evaluate the effect of this estrogenic hormone on the mRNA levels of the most relevant bicarbonate transporters of the Slc4 family, namely AE2 (Scl4a2), NBCe1 (Scl4a4), NBCn1 (Slc4a7) and NDCBE (Slc4a8). The sex steroid hormone concentration was chosen based on published data which reported that intratesticular interstitial fluid concentrations of those hormones are notably higher than those of circulating plasma, reaching values up to 200 nanomolar (R. Hess, 2000; B. P. Setchell, 2004). Although observations *in vitro* may not exactly represent an *in vivo* situation, the results obtained in the present study are a step

further to identify key mechanisms by which E_2 can regulate SCs physiology and consequently spermatogenesis, with a direct influence in the reproductive capacity of individuals.

Thus, using primary cultures of rat SCs we were able to confirm the presence of the two HCO_3^- transporters already described in these cells, AE2 and NDCBE. Additionally, we were also able to identify for the first time the expression of the transcripts of NBCe1 and NBCn1 in these cells. Furthermore, using the qPCR technique we were able to quantitatively evaluate the expression of these four HCO_3^- transporters and, as could be expected, AE2 was the most abundant transcript present in cultured SCs. AE2 expression is widely distributed, and for this reason this transporter is termed a "house-keeping" AE. As referred, in the male reproductive tract, its expression has been reported in a variety of tissues and cells, particularly in the various somatic cells of the testis and in developing germ cells and spermatozoa (Holappa et al., 1999), where it has been suggested that it plays a key role on the spermatogenic event (Holappa et al., 1999).

The levels of NDCBE were also evaluated and it was possible to show that it is expressed at much lower levels than AE2 in SCs. Although its expression has been reported at high levels in the testis (Boron, 2001; Grichtchenko et al., 2001), its presence in SCs has been solely assessed by electrophysiological techniques (P. Oliveira et al., 2009; P. F. Oliveira et al., 2009) and a study of its expression levels has never been performed.

On the other hand, the expression of NCBe1 and NCBn1 has never been reported in SCs and, to the best of our knowledge, our results are the first to clearly demonstrate it in cultured rat SCs. Although their function has not been evaluated yet in these cells, it is expectable that, as it happens in the majority of the other cell types where NBCe1 serves primarily as a basolateral electrogenic transport protein (Damkier et al., 2007; Majumdar et al., 2008), this transporter may operate with a 1:2 stoichiometry and mediate net HCO_3^- influx (Figure 19). Similarly, no data has been published concerning NBCn1 function in SCs, which is an electroneutral Na^+-HCO_3^- co-transporter that moves HCO_3^- across the cell membranes (Figure 19). As it happens in other cell types (Damkier et al., 2007; Praetorius et al., 2004), this transporter might be crucial for transepithelial acid–base movement in the seminiferous tubules, mostly operating as an acid-extruder in SCs.

When we evaluated the effect of E_2 on the transcript levels of these four members of the Slc4 family of bicarbonate transporters we could observe that AE2, NBCe1 and NBCn1 transcript levels were significantly increased in cells treated with E_2 (100 nM) during 50 hours. In fact, these results are in agreement with those obtained by Zhou et al. (2001) that reported the effect of an anti-estrogenic treatment on the regulation of the NHE3 expression in efferent ductules. Those authors described that antiestrogen-treated mice presented decreased NHE3 expression, which would explain the fluid accumulation observed in efferent ductules and testes of those animals, resulting in infertility (Zhou et al., 2001).

Nevertheless and although, as previously said, changes in mRNA levels can be a clear indication of the effectiveness of the regulation exerted on the studied protein, they cannot be used as a direct measure of the variation on the protein quantity or functioning. Hence, it is of great relevance to conduct studies in order to better understand the effect of estrogen treatment on the SC membrane transport mechanisms. Keeping this in mind, we lay hand to the voltage-clamp technique in order to study the effect of that hormone on transcellular transport in SCs cultured in semi-permeable Snapwell inserts. Using a similar protocol to the one used by Ko et al. (1996), which described that culture SCs when stimulated with ATP present an increase on the electrogenic transport of electrolytes (I_{sc}), we were able to perceive that E_2 treatment causes a perturbation on the effect of ATP these cells.

This purinergic effect was attributed to an increased HCO_3^--dependent Cl^- secretion towards the apical compartment (W. H. Ko et al., 1996) and SCs treated with E_2 presented a lower magnitude on this I_{sc} activation by ATP (both by 1 or 2 mM), followed by a lower I_{sc} recovery. It has been postulated by Ko et al. (1996) that this electrogenic transport of electrolytes involves the secretion of Cl^- through the apical membrane by an ATP-activated Cl^- conductance and its absorption via HCO_3^--dependent mechanisms through the basolateral membrane (Figure 20). So far only four HCO_3^- transporters have been identified in cultured SCs, from which only one (AE2) involves the transport of Cl^-. If, as predicted, all these HCO_3^- transporters are present in the basal portion of SC membrane, AE2 will be responsible by the electroneutral absorption of Cl^- from the basolateral compartment, which is connected directly the outtake of HCO_3^-. The remaining HCO_3^- transporters (NBCe1, NBCn1 and NDCBE) should be responsible for the recycling of HCO_3^- to the interior of the cell (Figure 20).

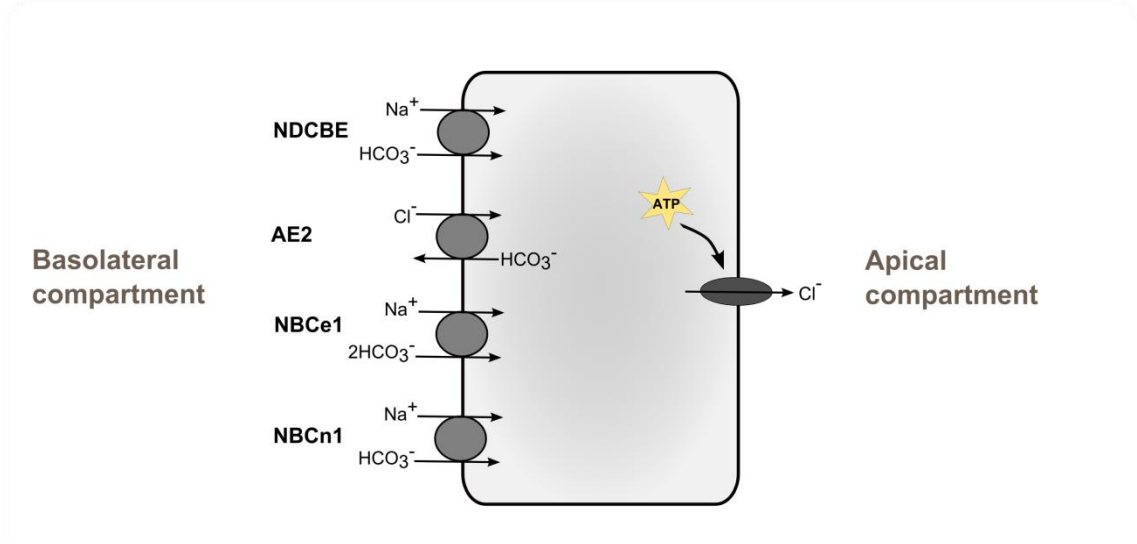

Figure 20: Diagram of a simplified polarized Sertoli cell with the chloride channel and bicarbonate membrane transport systems considered. AE2 – Anion exchanger 2 (Slc4a2); NBCe1 - electrogenic Na^+-dependent HCO_3^- co-transporter (Scl4a4), NBCn1 - electroneutral Na^+-dependent HCO_3^- co-transporter (Slc4a7); NDCBE - Na+-driven Cl^--HCO_3^- exchanger (Slc4a8).

Indeed, a higher expression of AE2 is consistent with the observed effect of ATP on the I_{sc} generated by SCs. If I_{sc} activation is due to ATP-activated Cl^- channels, which remove Cl^- from inside the cell, the resultant decrease of intracellular Cl^- will subsequently activate AE2 in order to replenish the cell with HCO_3^-. AE2 activation will be responsible for the decrease in intracellular HCO_3^- that in turn will counteract the increase on the transport of electrogenic electrolytes (Cl^-) to the apical compartment, and could be accountable for the I_{sc} recovery. Hence, if the observed effect of E_2 on the mRNA levels of the HCO_3^- transporters is converted into a change in protein levels, an increase of AE2 levels will surely be responsible for a prompter effect of this transporter on I_{sc} variation following ATP activation, which can translate into a faster I_{sc} recovery or even a smaller ΔI_{sc}, as detected in our experimental work.

VI. Conclusion

In conclusion, we were able to confirm the presence of the four selected bicarbonate transporters (AE2 (Scl4a2), NBCe1 (Scl4a4), NBCn1 (Slc4a7) and NDCBE (Slc4a8)) in testis and epididymis of 3 months old Wistar rats. Using the HED-fed rodent model, we detected alterations on the expression of these transporters at testicular and epididymal level, induced by the established pre-diabetic state. In fact, at testicular level, we could observe a significant increase on the mRNA levels of NBCe1, NBCn1 and NDCBE, although no differences were observed at protein level. On the other hand, at epididymal level we were able to determine that pre-diabetes is capable to alter the protein expression of some of the bicarbonate transporters studied, causing a significant increase on AE2 and a significant decreased of NDCBE protein levels. These results suggest an alteration in HCO_3^- homeostasis in the lumen of the epididymis, which may affect the establishment of an environment for proper sperm storage and viability and, hence, male reproductive potential.

Furthermore, using primary cultures of rat SCs we confirmed the presence of the four HCO_3^- transporters, AE2, NBCe1, NBCn1 and NDCBE. Additionally, we also reported that AE2 was the most abundant transcript present in cultured SCs. When we evaluated the effect of E_2 on the transcript levels of these four members of the Slc4 family of bicarbonate transporters, we could observe that AE2, NBCe1 and NBCn1 transcript levels were significantly increased in cells treated with E_2 (100 nM). Using the voltage-clamp technique, we studied the effect of E_2 on transcellular transport in SCs and we concluded that E_2 treatment causes a perturbation on the ATP-induced increase of the electrogenic transport of electrolytes (I_{sc}). This I_{sc} perturbation may be due to the alterations caused by E_2 on the expression levels of AE2, NBCe1 and NBCn1.

In face of the results obtained in our work, it is imperative to further disclose the molecular mechanisms involved in HCO_3^- transport and regulation, to identify and counteract possible alterations associated with pathological conditions that compromise the male reproductive potential, particularly DM and pre-diabetes. Indeed, further studies focused not only in membrane transport systems identification and expression but also in their functioning will be needed to enlighten the importance and the molecular basis of HCO_3^- membrane transport in the male reproductive tract.

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VIII. Annex I

List of publications resultant from the work developed during the M.Sc. in Biomedical Sciences:

Bernardino RL, Jesus TT, Socorro S, Alves MG, Oliveira PF. (2013) Bicarbonate transport in the male reproductive tract: involvement of membrane transporters. (Submitted)

Bernardino RL, Martins AD, Jesus TT, Sá R, Sousa M, Socorro S, Alves MG, Oliveira PF. Regulation by estrogens of bicarbonate secretion mechanisms in Sertoli cells. XXXVII IUPS Congress, 21-26 July 2013, Birmingham, England.

Alves MG*, Jesus TT*, Bernardino RL, Martins AD, Sá R, Sousa M, Oliveira PF. Water transport in seminiferous tubule: Aquaporins identification in Sertoli cells. XXXVII IUPS Congress, 21-26 July 2013, Birmingham, England (*both authors contributed equally).