Universidade da Beira Interior

Faculdade de Ciências da Saúde

Tese de Mestrado em Ciências Biomédicas

Hormonal regulation of lactate production and NHE3 expression by Sertoli cells *ex vivo*: possible roles for sex steroids hormones in spermatogenesis?

Luís Pedro Ferreira Rato

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Regulação hormonal da produção de lactato e expressão do NHE3 pelas células de Sertoli *ex vivo*: possível papel das hormonas esteróides sexuais na espermatogénese?

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

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Resumo

As células de Sertoli desempenham um papel importante no estabelecimento de um ambiente luminal adequado nos tubulos seminíferos do tracto reprodutivo masculino. A secreção do fluído do tubulo seminífero, bem como a regulação do seu pH é essencial para a fertilidade masculina. As células de Sertoli expressam na sua membrana vários tipos de transportadores de iões que estão envolvidos no movimento de partículas básicas e ácidas através da membrana. Entre eles está o transportador de Na^+/H^+ 3 (NHE3) que pertence à família de transportadores de Na^+/H^+, uma das famílias mais relevantes de transportadores iónicos epiteliais, que cataliza o transporte de um Na^+ extracelular por um H^+ intracelular. Vários autores têm demonstrado que o NHE3 é importante para a fertilidade masculina e que a sua expressão parece ser regulada pelos esteróides sexuais. Por outro lado, as células germinativas são incapazes de usar a glucose para o seu metabolismo (as células de Sertoli metabolizam a glucose e a maioria é convertida a lactato, que é preferencialmente usado pelas células germinativas em desenvolvimento). Existe uma crescente consciencialização de que os androgénios e estrogénios têm papéis metabólicos gerais que vão para além dos processos reprodutivos. Assim, é importante perceber o papel dos esteróides sexuais na expressão do NHE3 em células de Sertoli, bem como, a sua modulação no metabolismo destas células. Deste modo, foram estabelecidas culturas primárias de células de Sertoli a partir de ratos com 20 dias de idade, em meio livre de soro com o suplemento de insulina, transferrina e selénio (meio ITS) e divididas por 7 grupos experimentais, sendo sujeitas a tratamento hormonal durante 50 horas. Os grupos experimentais foram: 17β-estradiol (E_2); dihidrotestosterona (DHT); ICI 182,720 (ICI); flutamida (Flut); ICI/ E_2; Flut/DHT e controlo. A concentração hormonal para todos os grupos experimentais foi 100nM, excepto para o grupo controlo que não foi tratado. A presença do NHE3 nas células de Sertoli foi confirmada por RT-PCR e por western blot. O NHE3 foi semi-quantificado por RT-PCR em todos os grupos experimentais, não tendo sido registadas diferenças significativas quando comparado com o controlo. Quanto à análise da secreção de metabolitos ou consumo pela cultura de células de Sertoli, foi feita uma recolha de 250 µL de meio de cultura às 5h, 15h, 25h, 35h e 50h após o início do tratamento hormonal, para análise do espectro de ressonância magnética. Os resultados obtidos mostraram que o consumo da glucose foi significativamente maior após 50 horas nas células tratadas com DHT quando comparadas com o grupo tratado com E_2 e com o controlo. Inesperadamente, as células tratadas com DHT produziram menos lactato que o grupo tratado com E_2 e

que o controlo. Isto pode ser devido a vários factores tais como a diminuição da produção de lactato, atraso no transporte de lactato para o meio extracelular ou mesmo a utilização do lactato como substrato nas células tratadas com DHT. No consumo de piruvato não se verificaram alterações significativas com o tratamento hormonal, no entanto a produção de alanina foi maior nas células tratadas com E_2. Em conclusão, este estudo demonstra que os esteróides sexuais não exercem efeitos significativos na expressão do NHE3 pelas células de Sertoli. É provável que a regulação do pH intracelular nas células de Sertoli e acidificação luminal dos tubulos não depende directamente da acção dos estrogénios e androgénios mediada pelos seus receptores. Por outro lado, conclui-se que a DHT aumenta o consumo de glucose nas células de Sertoli e o E_2 aumenta a produção de alanina. Deste modo, os esteróides sexuais parecem desempenhar um papel importante na modulação do metabolismo das células de Sertoli.

Abstract

Sertoli cells cells play a key role on the establishment of an adequate luminal environment in the seminiferous tubules of the male reproductive tract. The secretion of the seminiferous tubular fluid (STF), as well as, the control of the pH of this fluid is crucial for male fertility. Sertoli cells express various types of ion membrane transporters that are directly involved on the movement basic and acidic particles across the membrane. Among them, is Na^+/H^+ exchanger (NHE3), which belongs to the Na^+/H^+ exchanger family, one of the most relevant epithelial ion transporter families, catalyzes the electroneutral transport of extracellular Na^+ for intracellular H^+. Several authors have provided confirmation that estrogens and androgens play an important role in male fertility, and regulate fluid transport on the male reproductive tract. On the other hand, as germ cells are unable to use glucose for their energy metabolism (Sertoli cells metabolize glucose and the majority of it is converted to lactate, which is preferentially used by developing germ cells). There is a growing awareness that androgens and estrogens have general metabolic roles that reach far beyond reproductive processes. Thus, is important to understand the role of the sex steroids in expression of NHE3 in Sertoli cells, as wells as, its modulation in metabolism of these "nurse" cells. For this purpose, primary Sertoli cell cultures were prepared from 20 days-old rats in serum-free medium supplemented with insulin, transferrin and selenium supplement (ITS medium) and divided in 7 experimental groups, being subjected to hormonal treatment during 50 hours. The groups were: E_2 (17β-estradiol); dihydrotestosterone (DHT); ICI 182,720 (ICI); flutamide (Flut); ICI/ E_2; Flut/DHT and control. The hormonal concentration was 100nM for all groups, except control group, which was not treated. The presence of NHE3 in Sertoli cells was confirmed by RT-PCR and western blot analysis. NHE3 was semi-quantified by RT-PCR for all experimental groups, there have been no significant differences when compared to control group. As for, analysis of the metabolites secretion or consumption by Sertoli cell culture, it was recovered 250 µL of the culture medium at 5h,15h, 25, 35h and 50h, after beginning treatment, for hydrogen nuclear magnetic resonance spectra analysis. The results obtained showed that glucose consumption was significantly higher in DHT-treated Sertoli cells after 50 hours than in control conditions or E_2-treated cells. Unexpectedly, DHT-treated cells produced less lactate than those treated with E_2 or in control conditions. This may be due to several factors such as to a decrease in cellular production of lactate, to a delay in lactate transport to extracellular medium or even to lactate utilization as substrate by DHT-treated cells. In pyruvate

consumption there were no significant changes with hormonal treatment, however alanine production was higher in E_2-treated cells. In summary, this study demonstrates that sex steroids do not exert significant effects in NHE3 expression by Sertoli cells. It is likely that control of intracellular pH of the Sertoli cells and luminal acidification in seminiferous tubules do not depend on directly of estrogen and androgen actions mediated by its receptors. In other hand, DHT increases glucose consumption in Sertoli cells and E_2 increase alanine production. Thus, sex steroids seem to play an important role in modulation of Sertoli cells metabolism.

Abbreviations and Symbols

AMH – anti-Müllerian Hormone

AR – androgen receptor

ATP – adenosine triphosphate

BSA – bovine serum albumin

BTB – blood-testis barrier

Ca^{2+} - calcium

cDNA – complementary DNA

Cl^{-} - chloride

DAB – 3,3' diaminobenzidine hydrochloride

D_2O – heavy water

DMEM – Dubelco's modified eagle's medium

DNA- deoxyribonucleic acid

DHT – dihydrotestosterone

E_2 – 17β-estradiol

ED – efferent ductules

EDTA – etilene diamine tetra acetic acid

ERα – estrogen receptor α

ERKOα – estrogen receptor knockout α

EtOH – ethanol

FBS – fetal bovine serum

Flu – flutamide

FSH – follicle-stimulating hormone

GnRH – gonadotropin-releasing hormone

GLUT – glucose transporter

GLUT 1 – glucose transporter 1

GLUT 3 – glucose transporter 3

GLUT 8 – glucose transporter 8

HBSS – Hank balance salt solution

HCO^{-}_3 – hydrogencarbonate

HPT – hypothalamic-pituitary testis axis

ICI – ICI 182,720

ITS – insulin, transferrin and sodium selenite supplement

K^{+} - potassium

LDH A – lactate dehydrogenase A

LH – luteinizing hormone

mA – milliAmpere

mM – millimolar

mRNA – messenger ribonucleic acid

MCT – monocarboxylate transporter

MCT1 – monocarboxylate transporter 1

MCT4 – monocarboxylate transporter 4

Mg^{2+} - magnesium

nM – nanomolar

NMR – nuclear magnetic ressonance

Na^{+} - sodium

NaOH – Sodium hydroxide

NaN_3 – sodium azide

NBCe – Na^{+}/ HCO^{-}_{3} co-transporters

NBCE – Na^{+}-driven HCO^{-}_{3}/Cl^{-} exchanger

NHE – Na^{+}/H+ exchangers

NHE3 – Na^{+}/H+ exchanger isoform 3

PBS – phosphate buffered saline

pH_i – intracellular pH

PKA – protein kinase A

RNA – ribonucleic acid

RNAt- total RNA

RT-PCR – reverse transcriptase polymerase chain reaction

SDS – sodium docedyl sulfate

STF – seminiferous tubular fluid

T – testosterone

TBS – Tris-buffered saline

WT – wild type

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I. Introduction

1. Testis physiology

1.1. Testis: anatomy and histology

The testes of mammals are paired organs that essentially perform two functions, production of spermatozoa and synthesis of steroids (Lee and Cheng, 2004, Hermo et al., 2009). The testis is composed of numerous seminiferous tubules and the interstitial space, all encased by a connective tissue capsule called the tunica albuginea (O'Donnell et al., 2001) and an outer two-layered tunic derived from an outpocketing of the peritoneum, called tunica vaginalis. Each plum-sized human testis is approximately 4 cm long and 2.5 cm in width (Marieb and Hoehn, 2007). Septa extending from the tunica albuginea divide the testis in 250 to 300 wedge-shaped lobules, each containing one to four tightly coiled seminiferous tubules (Marieb and Hoehn, 2007), the functional unities of the testis (Cheng et al., 2010). Each testis of a laboratory rat contains several convoluted seminiferous tubules, with an external diameter of about 250 µm and a total length of about 20 m per testis and a surface area of 340 cm^2 (Wing and Christensen, 1982). Surrounding each seminiferous tubule are smooth muscle-like myoid cells (figure 1b). These cells may help to squeeze sperm and testicular fluids through the tubules and out of the testes by contracting rhythmically (Marieb and Hoehn, 2007). Moreover the testicular capsule in several species has been shown to contract spontaneously in vitro (Setchell and Breed, 2006). Contractions of the capsule may be important in expelling the sperm from the testis into the epididymis, but the flow of fluid from the rete testis was not affected by removal of the capsule (Free et al., 1980). Seminiferous tubules are enveloped by a tunica propria or, limiting membrane, comprised of contractile myoid cells interposed between connective tissue layers of collagen and elastic fibers, with myoid cells moving immature spermatozoa toward the rete testis (Clermont, 1958, Dym and Fawcett, 1970). In both rodents and humans, the limiting membrane also contains cells of the immune system (Hermo and Clermont, 1976, Hermo et al., 1977). At the terminal part of seminiferous tubules they connect into the rete testis, termed the transitional distal seminiferous segment (Kerr et al., 2006).The tall elongated modified Sertoli cells orient themselves in a downstream direction, and their apices converge on one another distally in the direction of the rete testis (Kerr et al., 2006). Here they form a plug-like structure with a narrow lumen functioning as a valve preventing reflux of substances from the rete testis back into the tubular lumen (Kerr et al., 2006). The interstitial spaces between the seminiferous tubules contain the blood and lymph vessels, macrophages, and interstitial cells, also called Leydig cells (figure 1b). Macrophages

may influence the function of the Leydig cells (Hutson, 1992, Miller et al., 1983),which are primarily involved in the secretion of androgens, notably testosterone (T), as well as other steroids including estrogen (O'Donnell et al., 2001). The seminiferous epithelium consists of germ cells that form numerous concentric layers penetrated by a single type of somatic cell, the Sertoli cell.

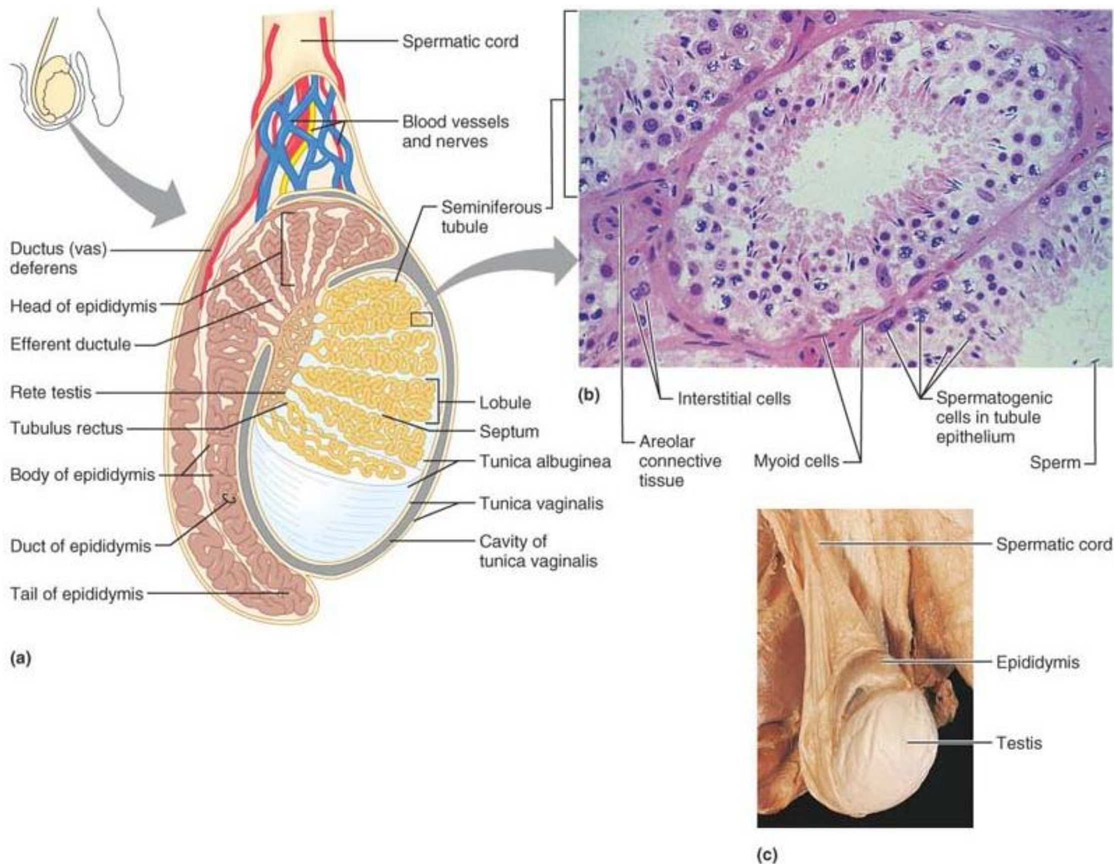

Figure 1: **Structure of the testis.** (a) Partial sagittal section through the testis and epididymis. (b) Seminiferous tubule in cross section. (c) External view of a testis. Adapted from Marieb and Hoehn (2007).

1.2. Spermatogenesis

Spermatogenesis is defined as the process whereby germ cells develop, differentiate, and metamorphose into maturing spermatozoa in the Sertoli cells *milieu*. It is an orderly and well-defined process occurring in seminiferous tubules of the testis, and a temporal event whereby undifferentiated spermatogonial germ cells evolve into maturing spermatozoa over a period of several weeks (Hermo et al., 2009). Spermatogenesis is a cyclic process which can be divided into 14 stages in rats (figure 2), 12 stages in mice, and 6 stages in men (Cheng et al., 2010). The process of

spermatogenesis in rat occurs over 50 days, 35 days in mice and 64 days in men (Adler, 1996). Mammalian spermatogenesis is characterized by three specific functional phases: in the first - the proliferative or mitotic phase - primitive germ cells or spermatogonia undergo a series of mitotic divisions; in the second - the meiotic phase - the spermatocytes undergo two consecutive divisions to produce the haploid spermatids; in the third - spermiogenesis - spermatids differentiate into spermatozoa (Shaha et al., 2008). The outermost tubule cells, which are in direct contact with the epithelial basal lamina, are stem cells called spermatogonia (Marieb and Hoehn, 2007) and include type A, Intermediate (found only in rodents) and type B forms. In all mammals, both type A and B spermatogonia undergo a series of mitotic divisions to produce a large number of germ cells, available for entry into meiosis, and thus the size of the spermatogonial population is a key determinant of the number of mature sperm eventually produced. The size of this population is likely controlled by a balance of proliferation and anti-apoptotic events (O'Donnell et al., 2006). The type A spermatogonia remains outside the blood-testis-barrier (BTB) and continue to multiply from puberty until death in order to maintain germ cell line (Saladin, 2001). The type B spermatogonia are committed to differentiate into slightly larger cells, called primary spermatocytes. These type of spermatogonia undergo several mitotic divisions before they enter a lengthy meiotic prophase as pre-leptotene spermatocytes (Cooke and Saunders, 2002). During first meiotic prophase spermatocytes undergo morphological transitions (table 1) and, at the end of meiosis I, two smaller haploid cells called secondary spermatocytes are formed. The secondary spermatocytes continue on rapidly into meiosis II, and their daughter cells, called spermatids, are small round cells with large spherical nuclei seen closer to the lumen of the tubule (Marieb and Hoehn, 2007). At the end of the second meiotic division, the four spermatids produced are interconnected – their cytoplasm does not completely pinch off at the end of each division (De Graaff, 2002). The differentiation of round spermatids into the mature elongated spermatid form takes place, with no further division, during the process known as spermiogenesis. Spermiogenesis involves formation and development of the acrosome and flagellum, condensation of the chromatin, reshaping and elongation of the nucleus, and removal of the cytoplasm before release of the spermatid during spermiation (Russell, 1993, Leblond and Clermont, 1952). The differentiation of spermatids proceeds through at least 4 prolonged steps (or phases): Golgi, capping, acrosomal, and maturation (Shaha et al., 2008). Spermiation is the final step of spermiogenesis and involves the release of the mature elongated spermatid from the Sertoli cell into the lumen of the seminiferous tubule (Russell and Griswold, 1993). Once into the lumen of the seminiferous tubule, the sperm pass through a series of

spermatic ducts to reach the urethra. These include: Efferent ductules (ED), Epididymis, Vas deferens and Ejaculatory duct (Saladin, 2001).

Table 1: Cellular changes and progression of germ cells through meiosis.

Phase	Cellular changes
Pre-leptotene	DNA replication occurs
Leptotene	Individual chromosomes become visible, telomeres begin to attach to the inside of the nuclear envelope and paired sister chromatids begin to condense
Zygotene	Homologues come together in a process called synapsis, the synaptonemal complex assembles and telomeres cluster together at a spot on the nuclear envelope, giving rise to the 'bouquet' arrangement of chromosomes
Pachytene	Synapsis is complete and homologues are joined by the synaptonemal complex
Diplotone	The synaptonemal complex disintegrates, chromosomes decondense, only to recondense again and sister chromatids remain closely associated, but homologous chromosomes are only held by chiasmata
Diakineses	Homologous chromosomes again shorten and condense, getting ready to align at the metaphase plate

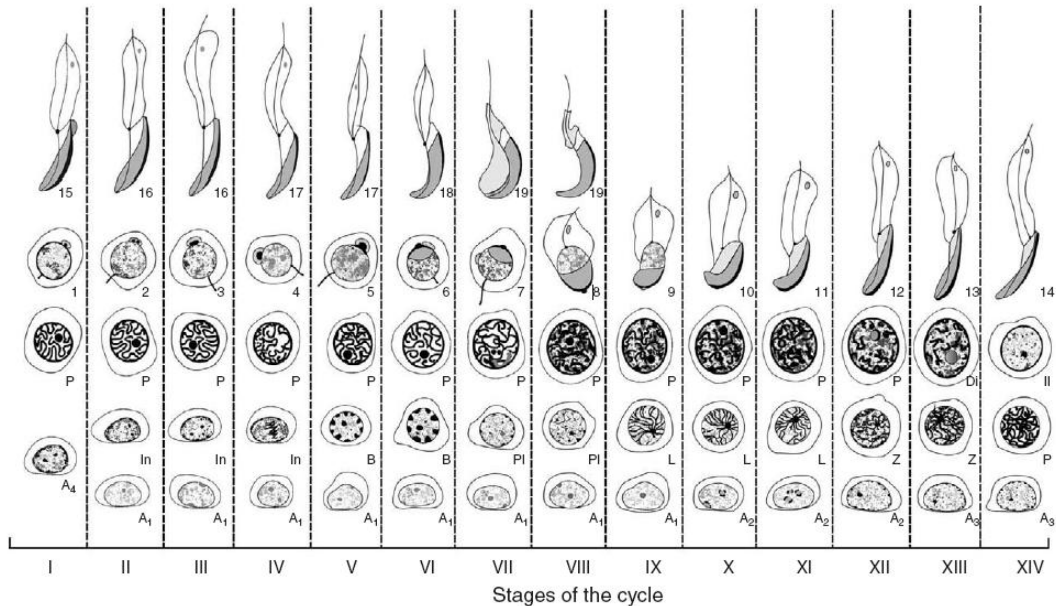

Figure 2: **Diagram of the cycle of rat spermatogenesis.** The 14 stages of the rat spermatogenic cycle, denoted I–XIV, are shown in the vertical columns. Germ cell development is shown horizontally. A(1-4), type A1 to A4 spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte; PS, pachytene spermatocyte; Di, diploid pachytene spermatocyte; II, secondary spermatocyte. The steps of spermiogenesis (1–19) are indicated beside each spermatid. Adapted from O'Donnell et al. (2006).

1.2.1. Hormonal Regulation of Spermatogenesis

The endocrine regulation of spermatogenesis is accomplished via a classic negative feedback loop (figure 3), involving interactions between the hypothalamus, pituitary, and testis (the hypothalamic-pituitary testis axis, or HPT) (O'Donnell et al., 2006). The two gonadotropins released from the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) stimulation, the luteinizing hormone (LH) and follicle stimulating hormone (FSH), are the major endocrine regulators of spermatogenesis (Amory and Bremner, 2003, Marc Luetjens et al., 2005, Weinbauer and Nieschlag, 1995). Luteinizing hormone acts on the Leydig cell to stimulate the secretion of androgens, namely T, which in turn acts on androgen receptors (AR) in the seminiferous epithelium to control spermatogenesis (Shaha et al., 2008). Follicle stimulating hormone receptors on the Sertoli cells react to FSH and regulate spermatogenesis by stimulating the production of numerous Sertoli cell factors (Shaha et al., 2008), among them androgen-binding protein that prompts the spermatogenic cells to bind and concentrate T, which in turn stimulates spermatogenesis (Marieb and Hoehn, 2007) and Inhibin that seems to have a dual, endocrine (regulation of FSH secretion in pituitary) and para/autocrine role in testicular function (Shaha et al., 2008). Moreover, testicular hormones will exert negative feedback at HPT. Testosterone inhibits GnRH release and acts directly on the anterior pituitary to inhibit LH release in response to any given level of GnRH (Marieb and Hoehn, 2007). Inhibin acts on anterior pituitary suppressing FSH output, without reducing LH and T secretion (Saladin, 2001).

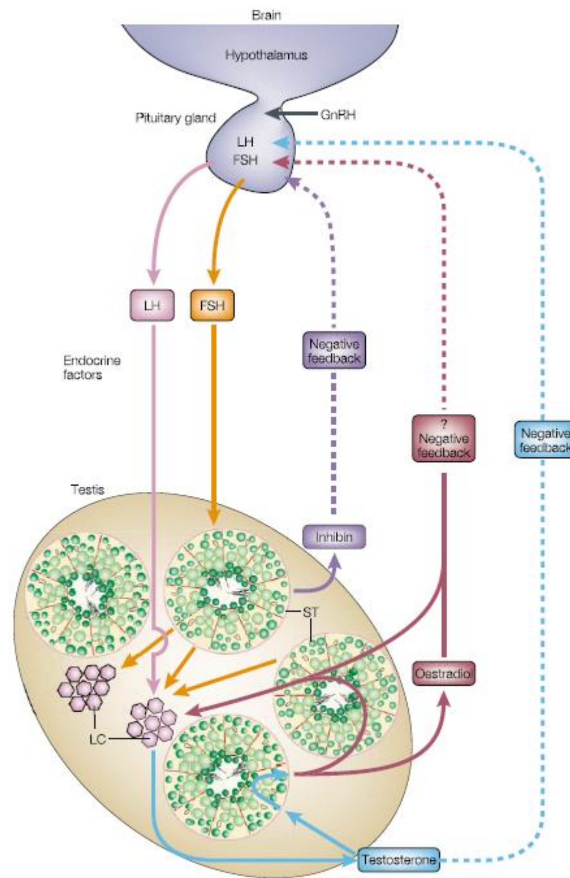

Figure 3: **The endocrine regulation of spermatogenesis.** Endocrine factors secreted by the pituitary gland (LH; FSH) and local regulators of testis function (such as androgens, estrogens and inhibins), as well as modulators of gene expression in the pituitary gland (the negative-feedback loops), are involved in the regulation of spermatogenesis. Receptors for LH and FSH are expressed in selected testicular cell populations. LC, Leydig cells; ST, Sertoli cells, adapted from Cooke and Saunders (2002).

1.3. Sertoli cell

The Sertoli cells play a major role in regulation of spermatogenesis and altering rates of spermatozoa produced (Johnson et al., 2008). Sertoli cells are the first cells to differentiate in the indifferent fetal gonad and this differentiation results in seminiferous cord formation, prevention of germ-cell entry into meiosis (Sharpe et al., 2003) by sequestering the germ cells (gonocytes) inside of newly formed seminiferous tubules (Griswold, 1998), and differentiation and function of the other somatic cells of the testis (Sharpe et al., 2003). The Sertoli cells also ensure regression of the Mullerian ducts via secretion of anti-Müllerian hormone (AMH) (Mackay, 2000). Thus, Sertoli cells can influence testis formation in the embryo. Such process requires the expression of specific genes on the Y-chromosome (Griswold, 1998), the *Sry* gene, the male sex-

determining gene, which is expressed by Sertoli cells (Petersen and Soder, 2006). In the rat, Sertoli cells proliferate from day 16 of fetal life and reach a maximum 2 days before birth. Approximately 1 million Sertoli cells are present in the rat testis at birth (Orth, 1982) and, with the continued proliferative rate of these cells the number increases to a maximum of around 40 million at day 15 of postnatal life (Wang et al., 1989), then proliferation ceases and returns in prepupertal period (Sharpe et al., 2003). Thus, Sertoli cells proliferate during two periods of life: in fetal or neonatal life and in the prepubertal life of mammals (Sharpe et al., 2003).

The proliferation of Sertoli cells is likely controlled by numerous factors, including intratesticular factors and pituitary hormones, namely FSH that is a mitogenic factor for neonatal Sertoli cells (Gondos B, 1994). The immature Sertoli cells differs extensively from the mature cell with respect to both morphology and biochemical activity (Petersen and Soder, 2006). There is a notable production of estrogens during the period division, leading the suggestion that estrogen is involved in this process (O'Donnell et al., 2001). This considerable production of estrogens is due to aromatase activity that is highest in immature Sertoli cells and declines as Sertoli cells mature, and is hormonally regulated, principally by FSH (Shaha et al., 2008). After Sertoli cells stop proliferation, they undergo maturational changes in both morphology and function at the time of puberty (Griswold and McLean, 2006).

The adult morphology consists of a larger irregularly shaped nucleus and a tripartite nucleolus, abundant smooth and rough endoplasmic reticulum and tight junctional complexes with adjacent Sertoli cells (Sharpe et al., 2003). These junctional complexes, also known to be on the basis of the formation of the BTB, divide the seminiferous epithelium into basal and apical (or adluminal) compartments, and are pivotal to spermatogenesis, since it creates an immunologic barrier by isolating the more advanced germ cell types (spermatocytes and spermatids) from the immune system, so that their antigens do not stimulate autoimmunity (Wong and Cheng, 2005). Mature Sertoli cells begin to produce seminiferous fluid, which results in the transformation of the testis cords into seminiferous tubules possessing a lumen (Petersen and Soder, 2006), allowing the release and transport of spermatozoa (Griswold, 1998). Sertoli cells synthesize specific products that are necessary for germ cell survival and those products combine to form a unique and essential environment in the adluminal compartment. Amongst those products, Sertoli cells secrete several glycoproteins that can be placed in several categories based on their known biochemical properties. The first category includes the transport or bioprotective proteins that are secreted in relative high abundance and include metal ion transport proteins such as transferrin and ceruloplasmin. The second category of secreted

proteins includes proteases and protease inhibitors, which allegedly are important in tissue remodeling processes that occur during spermiation and movement of preleptotene spermatocytes into the adluminal compartment. The third category of Sertoli cell secretions includes the glycoproteins that form the basement membrane between the Sertoli cells and the peritubular cells. Finally, the Sertoli cells secrete a class of regulatory glycoproteins that can be made in very low abundance and still carry out their biochemical roles. These glycoproteins function as growth factors or paracrine factors and include products such as AMH, *c kit* ligand and inhibin. In addition, Sertoli cells secrete bioactive peptides such as prodynorphin and nutrients or metabolic intermediates (Griswold, 1998).

Sertoli cells are also responsible for the processes of endocytose and degradation of the residual bodies and apoptotic spermatogenic cells, which are necessary for healthy spermatogenic cells to advance through spermatogenesis. A explanation for this fact is that the number of spermatogenic cells that Sertoli cells can support for maturation is limited, and the cells above this number should be removed, to provide space in the seminiferous epithelium for the development of healthy spermatogenic cells (Xiong et al., 2009). This support function makes Sertoli cells the major producers of adenosine triphosphate (ATP) in testis (Xiong et al., 2009). According to Cheng and collaborators (2010) Sertoli cell supports 30–50 germ cells at different stages of development. Originally described by Enrico Sertoli in 1865, the somatic Sertoli cell fills a crucial nursing function in connection with spermatogenesis. Failure of a Sertoli cell to mature functionally will presumably render it incapable of supporting the survival and development of the various germ cells that appear after puberty (Sharpe et al., 2003).

1.3.1. Sertoli cell metabolism: production and secretion of lactate

Among Sertoli cell functions that might be of interest to germ cell development is the provision of adequate levels of energy substrates. Studies on the metabolism of glucose have shown that Sertoli cells actively metabolize glucose but the majority of it is converted to lactate and is not oxidized via the citric acid cycle (Robinson and Fritz, 1981). On the other hand, germ cells (particularly post-meiotic germ cells) are unable to use glucose for their energy metabolism and they do prefer lactate as an energy source. The importance of lactate for normal spermatogenesis was recently highlighted

in a report showing that spermatogenesis in adult cryptorchid rat testis is improved by intratesticular infusion of lactate (Riera et al., 2002).

A contribute to an increase in lactate secretion in a cell is the import of the main carbon source for lactate production, *viz.*, glucose. The transport of this sugar through the plasma membrane (a rate-limiting step in glucose metabolism) is mediated by glucose transporters (GLUT). So far, only messenger ribonucleic acid (mRNA) for GLUT1, GLUT3, and GLUT8 have been observed in identified Sertoli cell preparations (Galardo et al., 2008). After internalization by the Sertoli cells glucose is then metabolized via cytosolic glycolysis to lactate, which is used by germ cells as a substrate for ATP production in mitochondrial oxidative phosphorylation (Robison and Fritz, 1981). Lactate is produced from pyruvate following lactate dehydrogenase A (LDHA) catalysis in Sertoli cells and is transported across the plasma membrane to the germ cells by specific proton/ monocarboxylate transporters (MCT, figure 4). In Sertoli cells MCT1 and MCT4 mediates this transport. Gallardo and collaborators (2007) reported that MCT1 has a role in lactate import from the extracellular *milieu* whereas MCT4, which has a much lower affinity for lactate than MCT1, has been proposed to serve as lactate exporter.

It has been shown that hormones, certain growth factors and cytokines are involved in the regulation of glucose transport in Sertoli cell, namely – epidermal growth factor (EGF), tumor necrosis factor α (TNF- α), interleukin 1 (IL-1), fibroblast growth factor (bFGF) and FSH . Moreover, EGF, TNF- α, IL-1, IL-1β, FSH, insulin, insulin like growth factor-1 (IGF-1) and PMods in rat Sertoli cells act as regulators of lactate secretion (reviewed by Riera et al., 2002). Galardo et al. (2008) suggests that GLUT1 constitutes the glucose transporter molecule responsible for the hormonal up-regulation of glucose entrance into the cell. In regard to sex steroids, reports from Jutte and collaborators (1982) showed that testosterone did not increase the basal lactate production by Sertoli cells from 4-week-old rats or from 6-week-old rats. In addition, lactate production was decreased in Sertoli cells from rat testes exposed in utero to flutamide (Goddard et al., 2003). An *in vitro* investigation in the lizzard *Hemidactylus flaviviridis* demonstrates the inhibitory effect of 17β-estradiol (E_2), T and dihydrotestosterone (DHT) on lactate production by Sertoli cells (Khan and Rai, 2004). A work done by Mullaney and collaborators (1994) showed that lactate production increases as the Sertoli cell differentiates during pubertal development. The reasons why Sertoli cells preferentially export lactate and pyruvate for germ cells are not known (Boussouar and Benahmed, 2004), however there are evidences that lactate might have a crucial role in spermatogenesis, showing an anti-apoptotic effect on germ cells (Erkkila et al., 2002).

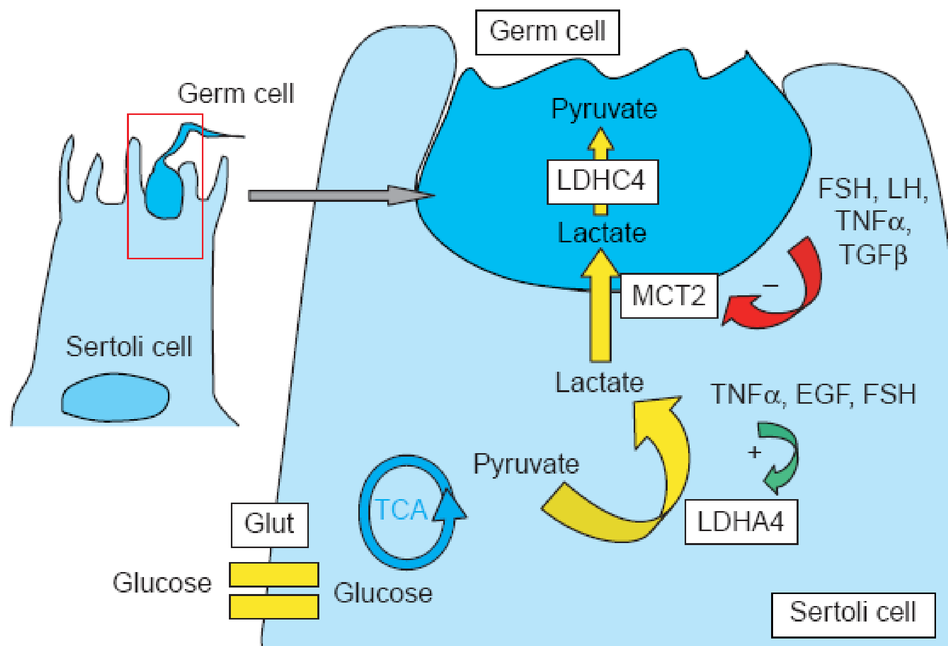

Figure 4: **Illustration of the central role that lactate plays in Sertoli cell–germ cell metabolic cooperation.** Systemic glucose is taken up by Sertoli cells via the specific GLUT1. Glucose is processed glycolytically into lactate, favored by the induction of somatic LDHA4 in Sertoli cells. Lactate leaves the Sertoli cell by MCT4 and is captured by meiotic and postmeiotic germ cells via their own MCT2. Lactate is then oxidized to pyruvate, a reaction catalyzed by the testis-specific lactate dehydrogenase LDHC4, before entering the tricarboxylic acid cycle, adapted from Boussouar and Benahmed (2004).

1.3.2. Tubular fluid secretion in the seminiferous epithelium: Ion transporters in Sertoli cells

Sertoli cells also control the composition of the seminiferous tubular fluid (STF), the physico-chemical *milieu* where spermatogenesis occurs. It has been shown that the composition of the fluid within the seminiferous tubules is very stable due to the existence of the BTB, which regulates the passage of various endogenous and administered substances (Koskimies and Kormano, 1973) supporting the fundamental relevance of the intraluminal fluid composition. This luminal *milieu* is markedly distinct from the interstitial fluid and plasma and is critical to the occurrence of spermatogenesis (Fisher, 2002), since meiosis can only be completed after fluid secretion has been established (Setchell, 1970). The secretion of STF commences during sexual maturation, after the formation of BTB, and is dependent on FSH (Jegou et al., 1982). Sertoli cells regulate, among others, the passage of ions, the selective flow of water, steroids, and carbohydrates into the tubular lumen (Setchell and Waites, 1975). The establishment of the ionic composition of this luminal fluid is critical to the

normal occurrence of spermatogenesis (Pace et al., 2000). This event include the net movements of water, Na^+, Cl^-, and HCO^-_3 reabsorption, K^+ secretion, and luminal acidification (Levine and Marsh, 1971, Au and Wong, 1980). There are many reports about formation and ionic composition of STF. First reports origin from Tuck and collaborators (1970) which postulated that Sertoli cells were responsible for fluid secretion in seminiferous tubules. These authors used a variation of the micropuncture technique and found that the fluid, which they called the primary fluid, contained high K^+ concentration but low Na^+ and Cl^- concentrations (Na^+ 38 mM; K^+ 112 mM; Cl^- 62 mM). The HCO_3^- concentration was estimated to be approximately 88 mM. These values were significantly different from another fluid (Na^+ 108 mM; K^+ 45 mM; Cl^- 120 mM), called the free flow fluid, which they collected spontaneously from the tubule without prior oil injection. Based on their findings, those authors suggested that the seminiferous epithelium secretes a K^+-rich solution which would be mixed in the tubule with a Na^+-rich/low K^+-containing fluid secreted by the rete testis. Jenkins and collaborators (1980) studied the electrolyte composition of the STF and their results were in agreement with those previously found by Tuck's group. However it has been shown posteriorly that the proposed composition of the fluid collected from the tubule varies depending on the method of the collection. Fisher (2002) showed by microscopic examination and alternative non-perfusion electrophysiological measurements of the electric potentials, that perfusion techniques, damaged severely the rat seminiferous epithelium. Fisher opposed to the "Tuck" hypothesis and believed that seminiferous tubules are responsible for the production of its luminal fluid. More recently, Clulow and Jones (2004) determined the most suitable approach for defining the composition of the secretions of the seminiferous epithelium is to examine frozen sections by x-ray microanalysis. Measurements of the major electrolytes in the STF of the *Japanese quail* described a fluid rich in Na^+ and Cl^- content, with a K^+ concentration of at least twice that of blood concentration (Na^+ 179 mM; K^+ 13 mM; Cl^- 170 mM) and indicated that this fluid is the main source of the luminal solutes in the extratesticular ducts (Clulow and Jones, 2004).

The control of the pH of the seminiferous fluid is crucial for male fertility and the intracellular pH (pH_i) of Sertoli cells should also play a major role in this process (Mruk and Cheng, 2004, Tuck et al., 1970). This cellular parameter is kept mainly through the net balance between production and elimination of protons and by intracellular buffers (Roos and Boron, 1981). Sertoli cells express various types of ion membrane transporters (table 2). The transporters directly involved on the movement basic and acidic particles across the membrane are classified in acid extruders, which requires energy to move H^+ from the cell or the uptake HCO_3^- (Boron, 2004), or acid loaders,

which mediates the exit of weak bases, usually HCO_3^- or CO_3^{2-} or entry of H^+ (Boron, 2004). The Na^+-driven HCO_3^- /Cl^- exchanger (NBCE) and the Na^+/ HCO_3^- co-transporters (NBCe) have been suggested in Sertoli cells (Oliveira et al., 2009b) where they must have central role on the regulation of pH_i in cells, as well as on the transport of acid-base equivalents (and/or salt) across the seminiferous epithelia responsible to maintain the slightly acidic pH of the lumen of the tubule (Levine and Marsh, 1971). Similarly to NBCE and NBCe, Na^+/H^+ exchangers (NHE) have also been described in Sertoli cells (Gorczynska-Fjälling, 2004, Oliveira et al., 2009b, Oliveira et al., 2009a) and are involved in regulation of pH_i and cell movement (Malo and Fliegel, 2006).

Table 2: Ion membrane transporters described on the plasmatic membrane of Sertoli cells.

	Type	References
Ion pumps	Na^+/K^+ ATPase	[a,b]
	Ca^{2+} ATPase	[c]
	H^+-ATPase	[d,e]
Ion Transporters	NBC	[d,e]
	NCBE	[d,e,f]
	NCX	[g]
	NHE	[d,e]
Ions channels	L-Type Ca^{2+} channel	[h]
	N-Type Ca^{2+} channel	[h]
	T-Type Ca^{2+} channel	[i]
	P/Q-Type Ca^{2+} channel	[j]
	Cl^- channel	[k,l]
	CFTR Cl^- channel	[m]
	K^+ channel	[n,o,p]

Legend: CFTR: Cystic fibrosis transmembrane conductance regulator; NBC: Sodium bicarbonate co-transporters; NCBE: Na^+-driven Cl^-/HCO_3^- exchangers; NCX: Na^+/Ca^{2+} exchanger; NHE: Na^+/H^+ exchangers; References: a: (Hinton and Setchell, 1993); b: (Byers and Graham, 1990); c: (Feng et al., 2006); d: (Oliveira et al., 2009b); e: (Oliveira et al., 2009a); f: (Boron, 2001); g: (Grasso et al., 1991); h: (Taranta et al., 1997); i: (Lalevee et al., 1997); j: (D'Agostino et al., 1992); k: (Auzanneau et al., 2003); l: (Babenko et al., 1998); m: (Boockfor et al., 1998); n: (Von Ledebur et al., 2002); o: (Loss et al., 2004); p: (Wassermann and Loss, 2004).

2. Sodium/Proton Exchanger Isoform 3

2.1. Structure and Function

Carrier-mediated transport of sodium in exchange for protons across biological membranes has been described universally in organisms throughout the various phyla, from simple prokaryotes such as bacteria to more complex eukaryotes of the plant, fungi, and animal kingdoms (Orlowski and Grinstein, 2004). There are nine known Na^+/H^+ exchanger (NHE) isoforms in the mammalian genome (Orlowski and Grinstein, 2004) each with unique, tissue and cellular distribution, inhibitor sensitivities, regulatory elements and ensuing physiological roles (Malo and Fliegel, 2006). The NHE isoforms can be grossly subdivided into two groups: those that reside and function predominantly in the plasma membrane (NHE1–5) and those that are found largely or exclusively in endomembrane organelles (NHE6–9) (Alexander and Grinstein, 2009).
Na^+/H^+ exchanger isoform 3 (NHE3, figure 5) is one of nine isoforms of the mammalian NHE gene family. NHE3 was identified by Orlowski and collaborators (1992) and is found almost exclusively in the epithelia of renal and gastrointestinal tissue (Orlowski et al., 1992), however, it was suggested that there NHE3 is also expressed at a low level in human thymus, prostate, ovary and testis (Brant et al., 1995). In the male reproductive male tract NHE3 expression is well documented, namely in rat epididymis (Pastor-Soler et al., 2005, Kaunisto et al., 2001), rat ED (Kaunisto and Rajaniemi, 2002, Oliveira et al., 2002, Hess, 2003) and mice ED (Lee et al., 2001, Zhou et al., 2001). NHE3 is encoded by *Slc9a3* gene which is localized in rat chromosome 1 at p11 (figure 6a) (Szpirer et al., 1997). The *Slc9a3* gene spans 40 kilobases and contains at least 17 exons (figure6b). The exons are evenly distributed along the genomic DNA with the exception of exon 1 which is separated from the other exons by a large intron that is estimated to be 25 kilobases. The protein-coding exons generally range in size from 71 to 302 nucleotides, with the exception of exon 17 which is 1700 nucleotides and contains the TGA stop codon (starting at position 2 of the exon) and a large segment of the 3'-untranslated region (Kandasamy and Orlowski, 1996). NHE3 shares with the other NHE isoforms a bipartite structure: an N-terminal, 12 pass transmembrane domain (residues 1–454 in the case of NHE3), followed by a relatively unstructured cytosolic C terminal domain (residues 455–831), moreover, in contrast to the NHE1 isoform in the rat, NHE3 is not glycosylated (Alexander and Grinstein, 2009). As said, NHE3 mediates the exchange of extracellular Na^+ for intracellular H^+ with a stoichiometry of 1:1. Sodium–proton exchange through NHE3 is therefore electroneutral. The driving force of this crossmembrane exchange is the large inward

gradient of Na^+ generated by the Na^+/K^+-ATPase. Consequently the transport via this exchanger is secondarily active. NHE3 is also capable of functioning in reverse (exchanging intracellular Na^+ for extracellular H^+) if the chemical gradients are inverted (Alexander and Grinstein, 2009).

Even if the function of NHE3 on the seminiferous tubules has not been elucidated, it has been shown that this transporter is important to the overall function of the testis. NHE3-knockout mice have been shown to exhibit tubular fluid accumulation associated with infertility (Zhou et al., 2001). These results demonstrated the relevance of NHE3 on tubular fluid secretion and the role of the expression of this membrane transporter on the physiology of the male reproductive tract. Nevertheless, it should not be overlooked the fact that some of these transporters are also present on the plasma membrane of developing spermatogenic cells (Wang et al., 2003, Wang et al., 2007) and that its malfunction could also be associated with the reproductive disorders and for the abnormal tubular fluid secretion reported.

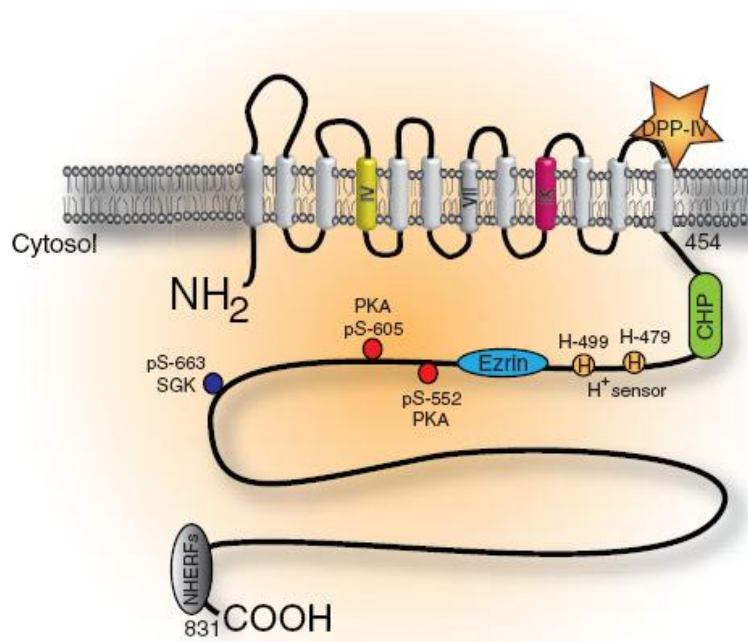

Figure 5: **NHE3 structure.** The structure of NHE3 is divided into 12 transmembrane domains (residues 1–454) and a large cytosolic C terminal domain (residues 455–831). The transmembrane domains implicated in ion transport are depicted in yellow and the domain responsible for inhibition by amiloride is depicted in pink (transmembrane domain IX). The diagram also indicates the putative binding sites for CHP (calcineurin homologous protein), ezrin and the NHERFs, the proton modifier sites (H^+ sensor) and the sites that are phosphorylated by either PKA and serum and glucocorticoid kinase (SGK). DPP, dipeptidyl peptidase, adapted from Alexander et al. (2009)

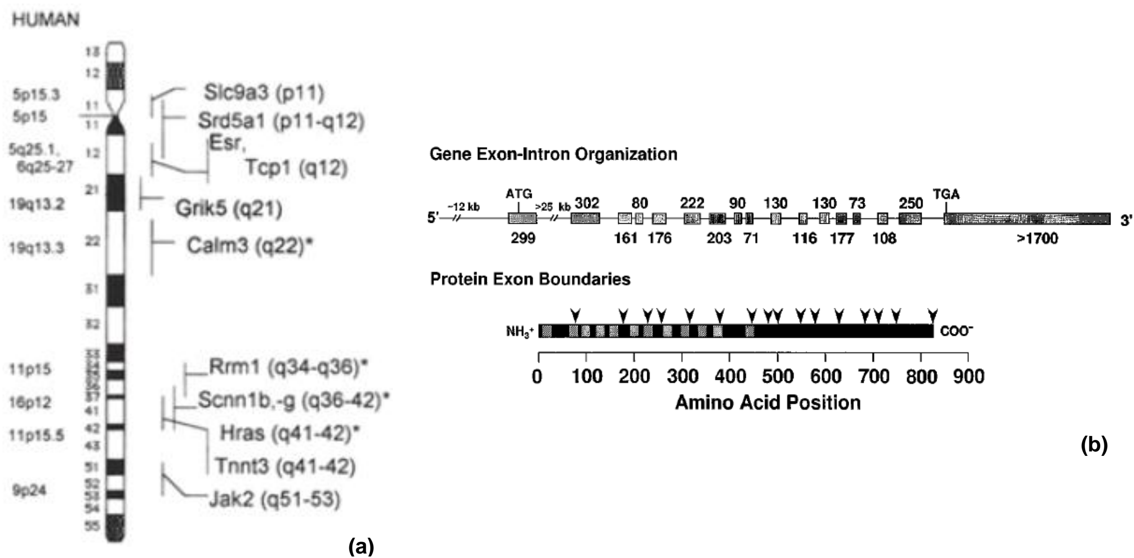

Figure 6: **Cytogenetic location and genomic organization of the rat *Slc9a3* gene. (a)** The left column indicates the position of the human homologs of the rat genes whose cytogenetic location is shown on the right of the rat Chr 1 diagram, adapted from Szpirer et al., (1997). **(b)** The relative locations of the NHE3 exons within the gene, depicted by *gray bars*, and their sizes (in bp) are illustrated. The positions of the boundaries between exons in the NHE3 protein are indicated by the *arrows*. The *hatched boxes* represent the proposed membrane spanning segments, adapted from Kandasamy and Orlowsky (1996).

2.2. Sex steroid regulation of NHE3

It has been reported the hormonal regulation of NHE3 by sex steroids. Previous studies has shown that NHE3 is the major protein regulated by estrogen (Zhou et al., 2001, Oliveira et al., 2002, Hess, 2003) in ED. Oliveira and collaborators (2002) showed that estrogen receptor α knockout (ERKOα) rats and ICI 182,720 (ICI) treated rats presented testicular atrophy and infertility. Oliveira's group reported that the mechanism by which estrogen regulates fluid reabsorption is related to the expression of NHE3. This data are corroborated by Zhou and collaborators (2001) which suggested that NHE3 is among the proteins involved in fluid transport in the ED. Loss of NHE3 activity in ERKOα could have been a primary cause of fluid accumulation in mice ED. Through Northern blot analysis Zhou and collaborators (2001) revealed that only NHE3 mRNA was significantly decreased in ERKOα. The 4-fold reduction in $^{22}Na^{+}$ uptake via the NHE3 pathway was consistent with estrogen's regulation of NHE3 expression and indicated a possible physiological role of estrogens in the ED epithelium. However, ERKOα data are based on a mouse that lacks estrogen receptor α (ERα) throughout development. Therefore, the observed abnormalities could represent developmental defects rather than direct regulation of ERα in the adult (Zhou

et al., 2001). The results obtained from wild type (WT) rodents treated with pure antiestrogen ICI demonstrated that ICI-induced pathological changes were nearly identical to those in ERKOα ED (Zhou et al., 2001). Conversely, Lee and collaborators (2001) suggested that ICI treatments do not produce a significant change in mRNA levels.

An examination of the published sequence for rat NHE3 promoter region, (GenBank accession number S83406), revealed a palindromic estrogen response element with only 1-bp change from the consensus (GtTCAgtcTGACC), plus an additional consensus estrogen response element half-site. Thus, transcriptional regulation of NHE3 by estrogen would be expected (Zhou et al., 2001). Moreover Snyder and collaborators (2009) identified by real time reverse transcriptase polymerase chain reaction (RT-PCR) that NHE3 gene in ED is responsive to testosterone propionate in treated animals. NHE3 had a 9-fold increase in testosterone-propionate treated animals respectively compared with the castrated animals. In addition, rat NHE3 promoter presents the TGTTCT half-site which is recognized, among others, by AR (Kandasamy and Orlowski, 1996).

II. Aim of the project

There are several reports which provided confirmation that estrogens and androgens play an important role in male fertility, and regulate fluid transport on the male reproductive tract. These results lead us to speculate that sex steroids probably modulates fluid secretion in the reproductive tract by regulating the expression of ion transporters involved in the movement of charged particles such as Na^+ and basic/acidic ions on Sertoli cells. Another important feature of Sertoli cells is the role of its metabolism in germ cells fate. As there is a growing awareness that androgens and estrogens have general metabolic roles that reach far beyond reproductive processes.

Specifically, using primary cultures of rat Sertoli cells, we aimed to:

1. Identify the expression of NHE3 (mRNA and protein);
2. Quantify the expression of NHE3 mRNA in 7 experimental groups: E_2-treated, ICI-treated, E_2/ICI-treated, DHT-treated, Flutamide-treated and Flutamide/DHT treated cells and control;
3. Quantify the production or consumption of glucose, lactate, pyruvate and alanine, and the respective production rate, in E_2-treated, DHT-treated cells and control.

III. Materials and Methods

1. Establishement of Rat Primary Culture Sertoli Cell

Male Wistar rats (20-day-old) were sacrificed by cervical dislocation, the testis were immediately excised in aseptic conditions and washed twice in a 50 mL conical tube in 30 ml of ice cold HBSS (Hanks Balanced Salt Solution Ca^{2+}/Mg^{2+} free; Sigma) containing 10000 U/mL of penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B (pH 7.4). After adherent epididymis and vas deferens removal, testis were decapsulated in HBSS, and the loosen tissue was washed three time in same solution. Sertoli cells were isolated by a modification of a method described previously by Meroni and colaborators (2002) which consist in two treatment types: mechanic and enzymatic treatment. Briefly, tissue from decapsulated testes was placed in a Petri dishes containing glycine medium (HBSS plus 1 M glycine, 2 mM etilene diamine tetra acetic acid (EDTA), 0.002% (w/v) Soybean Trypsin Inhibitor; pH 7.2). This procedure allowed the removal of contaminating peritubular cells. The tubules were washed again in a conical tube and dispersed in same solution containing 10 µg/mL DNAse during 10 minutes at room temperature, to remove residual peritubular cells. The dispersed tubules were forced through a large-pore Pasteur pipette to "unravel" the tubules and further release the interstitial tissue/cells. The washed tubular pellet was then digested with 4.5 mg/20 mL collagenase type I (C0130, Sigma) and 1 mg/20 mL DNase (250U; D4263, Sigma) in HBSS for 15-20 minutes at room temperature. It was important that collagenase digestion did not fragment the seminiferous tubules, because if the tubules were severely fragmented resulted in poor purity. The Sertoli cell suspension, collected (by centrifugation 300x*g* for 3 minutes), was washed three times in HBSS and resuspended in Sertoli culture medium which consisted of a 1:1 mixture of Ham F12 and Dulbecco's modified Eagle's medium (DMEM: F12 Ham, Sigma), 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 fetal bovine serum (FBS; Sigma). This cellular suspension was then forced through a 20G needle, in order to disaggregate large Sertoli clusters. For cell culture, the concentration of clusters on the cellular suspension obtained from the procedure described above was adjusted to 5000 clusters/ml plated on 25 cm^2 culture flasks (Cell+; Sarstedt), and incubated at 37ºC in an atmosphere of 5% CO_2, 95% O_2. The day of plating was considered day 0 of culture. The cultures were left undisturbed until day 2.

1.1. Hormonal treatments

After Sertoli cells cultures showed 90-95% of confluence, cells were detached using trypsin-EDTA (Sigma) and divided to 7 culture flasks. Cells were plated at a density of approximately of 1 x 10^6 cells per culture flask in a 1:1 mixture of Ham F12 and DMEM, 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% FBS and incubated in an atmosphere of 5% CO2, 95% O_2 at 37ºC. After 72 hours of culture, medium was replaced by serum-free medium supplemented with insulin, transferrin and sodium selenite supplement (ITS supplement, pH 7.4, Sigma). In order to evaluate the effects of hormones on NHE3 expression Sertoli cells were treated with 100 nM of E_2 (Sigma), 100 nM DHT (Sigma), 100 nM ICI 182,720 (Tocris Bioscience, USA) a pure estrogen antagonist, 100 nM Flutamide (Sigma) androgen antagonist, 100 nM ICI 182,720/E_2, 100 nM DHT/ Flutamide. Control groups were treated with 100 nM of ethanol (EtOH). Treatments were performed during 50 hours in an atmosphere of 5% CO2, 95% O_2 at 37ºC.

2. RNA Total Extraction

Sertoli cells were detached from the culture flasks using trypsin-EDTA solution (Sigma). Culture flasks were washed with 3 mL of phosphate buffered saline (PBS) to remove residual cells. Sertoli cells were centrifuged for 5 minutes at 3000x*g* in Sigma 3K18C centrifuge. Total ribonucleic acid (RNAt) was extracted from isolated Sertoli cells by TRI reagent™ (Sigma) according to the manufacturer's instructions. After cell isolation by centrifugation, the pellet was homogeneized with 500 µL TRI. TRI lyses cells and dissociates nucleoprotein complexes. To ensure complete dissociation of nucleoproteins complexes, samples standed for 5 minutes at room temperature and then 100 µL of chloroform were added for phase separation. The samples were shaken vigourosly for 15 seconds and allowed to stand for 5 minutes at room temperature and then centrifugated at 12000x*g* in Hettich Mikro 200R centrifuge for 15 minutes at 4ºC. Centrifugation separates the mixture into 3 phases: colorless upper aqueous phase (containing RNA), an interphase (containing DNA) and a red organic phase (containing proteins). After centrifugation aqueous phase was transferred to a fresh tube and used tom isolate RNAt. To this aqueous phase 250 µL of 2-propanol were added to precipitate RNA. Then the mixture was centrifugated at 12000x*g* for 10 minutes at 4ºC to pellet RNA. After centrifugation the supernatant was discarded and RNA pellet was

washed with 500 μL of 75% EtOH (in DEPC-H_2O at -20ºC). This step was repeated after a centrifugation at 7500x*g* for 5 minutes at 4ºC. After final centrifugation step the supernatant was discarded and the RNA pellet was dried for 5-10 minutes by air-drying. RNA pellet was dissolved by repeated pipetting with a micropipette in 10 μL DEPC-H_2O. RNA concentration was determined by measuring absorbance at A260 and A280 (Nanophotometer^{TM}, Implen, Germany). The integrity of ribosomal RNA was checked by ethidium bromide stained agarose gel electrophoresis.

3. RT-PCR

For the complementar deoxyribonucleic acid (cDNA) synthesis, RNAt (1 μg) was denatured for 5 minutes at 65ºC in a reaction containing 10 mM of deoxynucleotide triphosphates (Amersham, GE Healthcare, Portugal) and 125 ng/μL of random hexamer primers (Invitrogen, USA). After the denaturation step, samples were centrifugated briefly. The samples were incubated at 37º C for 2 minutes with First-Strand Buffer (Invitrogen) and 0.1 M of DTT (Invitrogen) in the thermal cycler (Px2 ThermoHybaid, UK). 200 U of M-MLV reverse transcriptase (200U/μL; Invitrogen) was then added and samples were incubated for 10 min at 25ºC followed by 50 minutes at 37ºC. The reaction was inactivated by heating samples for 15 minutes at 70ºC. Reactions in the absence of reverse transcriptase were also included for each RNA tested in order to check for genomic contamination. The cDNA synthesized was stored at -20ºC.

Polymerase chain reactions (PCR) were carried out using 1 μL of cDNA in 25 μL of total volume. A mixture containing 2.5 μL of 10X PCR Buffer, minus Mg^{2+} (Invitrogen), 1.5 mM of Magnesium Chloride (Invitrogen), 10 mM of dNTP (Amersham), 50 μM of each primers (table 3 – STABVIDA, Oeiras, Portugal), 0.5 U of Platinum® *Taq* DNA Polymerase (Invitrogen) and H_2O sterile was prepared. After preparation of the mixture in PCR tubes (Sarstedt), 1 μL of cDNA was added. PCR reactions were performed in thermocycler (Px2 Thermo Hybaid). The conditions of PCR reactions were optimized and are described in table 2. PCR products were visualized in ethidium bromide stained agarose gel (1%) electrophoresis using software Molecular Imager FX Pro Plus MultiImager (Biorad, Hercules, USA) coupled to the image acquisition system Vilber Lourmat.

Table 3: Primers sequences, PCR product size and PCR conditions

Primers	Sequences	Size Product	PCR Conditions
rNHE3_FW2466 **rNHE3_RV2986**	5'-GCAACTTCCGCCGCCTGACT-3' 5'-GGGAGGGGCCACTGAGGGAG-3'	521	94ºC: 2 min 94ºC: 30 s } 40x 61ºC: 30 s 72ºC: 30 s 72ºC: 5 min
18S_FW **18S_RV**	5'-AAGACGAACCAGAGCGAAAG-3' 5'-GGCGGGTCATGGGAATAA-3'	152	94ºC: 2 min 94ºC: 30 s } 25x 56ºC: 30 s 72ºC: 30 s 72ºC: 5 min
rVIM_FW844 **rVIM_RV1041**	5'-AGATCGATGTGGACGTTTCC-3' 5'-TCCGGTATTCGTTTGACTCC-3'	198	94ºC: 2 min 94ºC: 30 s } 35x 50ºC: 30 s 72ºC: 30 s 72ºC: 5 min:
rAMH_FW51 **rAMH_RV260**	5'-GGCTGTGTTACAGGCTGACA-3' 5'-GACTCTTGGACAGCCTCCAG-3'	210	94ºC: 2 min 94ºC: 30 s } 35x 54ºC: 30 s 72ºC: 30 s 72ºC: 5 min

4. Cloning Procedure

The PCR products obtained from testis cDNA were cloned in pGEM®-T Easy vector (Promega) and sequenced (Stabvida) to confirm the identity of the amplicons.

4.1. DNA extraction from PCR products

The cDNA encoding rat NHE3 was purified from agarose gel using the kit Nucleospin extract II PCR clean-up/ Gel extraction (MACHEREY-NAGEL, Germany), according to the manufacturer's instructions. After running the agarose gel, the fragment was excised from the gel and the weight of the gel slice was determined. For each 100 mg of gel, 200 µL of Binding buffer NT was added. After incubation of the mixture at 50ºC to dissolve the gel, the column was placed into the collecting tube; the sample was loaded with 600 µL of wash buffer NT3 and centrifuged for 1 min at 11.000x*g*. The flow was discarded and the column was placed back into collecting

tube. The centrifugation was repeated for 2 minutes to completely dry membrane. Finally, the column was placed into a clean tube, 20-25 μL of elution buffer NE was added and the sample was incubated at room temperature for 1 minute and centrifuged for 1 minute at 11.000x*g*. The column was discarded and the DNA was stored at 4ºC. The product of this purification was confirmed by electrophoresis on a 1% agarose gel.

4.2. Ligation of inserts into pGEM®-T Easy Vector

The ligations of inserts were carried out by addition of 0.5 μL of pGEM®-T Easy Vector (Promega, figure 7), 2.5 μL of 2X Buffer, 1.5 μL of purified DNA, 0.1-1 U of T4 DNA ligase (Promega) and sterile water to a final volume of 10 μL. These reactions were incubated at 4ºC over night.

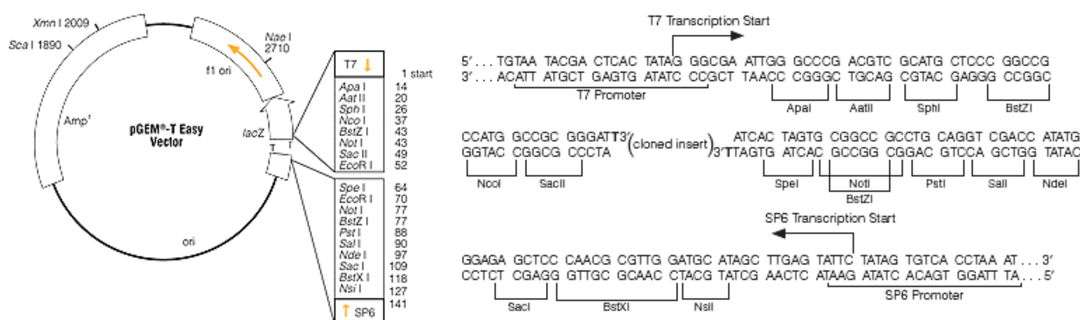

Figure 7: pGEM®-T Easy vector circle map. Promoter and multiple cloning site of the pGEM®-T Easy vector (Adapted from Promega technical manual).

4.3. Transformation of competent bacteria *E.Coli*

1 μL of the ligation was added to 100 μL of the competent bacteria and incubated on ice during 35-40 minutes. The next step was to perform heat shock incubating for 2 min at 42ºC. The sample was plated immediately in agar (USB) plates with IPTG (0.5mM), X-Gal (80 μg/mL) and Ampicilin (100 μg/mL), in sterile conditions. The plates were incubated at 37ºC over night.

4.4. Culture of bacteria in liquid broth

White colonies were removed from the plates and placed in 15 mL falcon tubes containing approximately 4 mL of LB Broth with amplicilin (100 μg/mL), in sterile

conditions. These falcon tubes were incubated over night at 37ºC in an orbital shaking incubator (250 RPM; ARABAL Agitorb 200E).

4.5. Purification of plasmid DNA

To purify plasmid DNA was used the Alkaline lysis method. After culture in LB broth, bacterias were collected by centrifugation at 18620x*g* in Hettich Mikro 200R centrifuge. Pellet was resuspended in 100 µL in GET buffer (50mM glucose, 10mM EDTA, 25mM Tris, pH 8.0) followed by an addition of 200 µL NaOH (2M)/SDS (10%) (freshly made) and incubation on ice for 10 minutes. Then 200 µL of Potassium Acetate (3M) were added and incubated on ice for 5 minutes. After incubation it was performed a centrifugation at room temperature for 10 minutes at 18620x*g*. The supernatant was transferred to 1.5 mL fresh tubes and 2 µL RNase A were added. This mixture was incubated at least 20 minutes at 37ºC to degrade contaminant RNA. After incubation 350 µL of 2-propanol were added and the mixture was incubated for 30 min-2 hours. Then samples were centrifugated at 4ºC for 30 minutes at 18620x*g* and supernatant was discarded. The pellet was washed with 500 µL 70% EtOH and centrifuged at 18620x*g* again at room temperature for 10 minutes. EtOH was removed, and the resulting pellet was dried at 50ºC and then dissolved in 50 µL of sterile H_2O.

4.6. Digestion of plasmid DNA

To confirm the insert presence in plasmid, DNA was digested with Eco RI enzyme (Takara Bio Inc., Japan). The digestion reaction contained: 2.5 µL of purified DNA, 1 µL of 10X Buffer, 7.5 U of Eco RI (Takara Bio Inc.) and sterile water to a final volume of 10 µL. After incubation of 2 hours at 37ºC, these products were separated by electrophoresis on a 1% agarose gel with ethidium bromide.

4.7. DNA sequencing

The sequencing of the bands corresponding to the NHE3 was performed by STAVIDA using the T7 fwd pGEM primer (5'- GTA TAC GAC TCA CTA TAG GGC -3'). The results were analysed by NCBI's BLASTN software (www.ncbi.nlm.nih.gov, 2010).

5. Semi-quantitative PCR

The expression of NHE3 mRNA was evaluated semiquantitatively by RT-PCR, quantifying the expression of the NHE3 gene and of the housekeeping gene 18S. The reagents and conditions used for both RT-PCR were described in point 4 and table 3. The PCR products of 18S gene and NHE3 gene were run separately in ethidium bromide stained 1% agarose gel electrophoresis at 180V for 30 minutes. The gels were charged with total of 17μL (15μL of sample and 2μL of loading buffer).

6. Immunocytochemistry

Sertoli cell culture purity was revealed by the immunoperoxidase technique using a specific polyclonal antibody against vimentin (anti-Vimentin, Clone V9, Invitrogen). Briefly, cells were grown on 6 well culture plates and processed by the labeled streptavidin–biotin method using an ExtrAvidin-Peroxidase Staining Kit (Sigma) giving a brown coloration to the Sertoli cells after reaction with 3,3' diaminobenzidine hydrochloride (DAB, Sigma). Medium was removed, the cells were rinsing 3 times in phosphate-buffered saline (PBS; 4ºC). Cells were fixated with methanol (-20ºC) for 5 minutes and rinsing 3 times in PBS (4ºC). Endogenous peroxidase activity was blocked by incubating the sections with 0.1% H_2O_2 for 10 minutes. Cells were rinsing three times in PBS (4ºC). Then, cells were permeabilized with 0.2% Tripton X-100 in PBS and rinsing 3 times in PBS to remove the excess of detergent. Nonspecific antibody binding was minimized by incubation with blocking solution (PBS containing 1% bovine serum albumin (BSA)) for 1 hour at room temperature. Cells were rinsing 3 times in PBS. Cells were incubated 4ºC overnight with primary antibody against vimentin (rat anti-Vimentin, Clone V9, Invitrogen) diluted in PBS, pH 7.4, and 1% BSA with 0.1% sodium azide (NaN_3). After incubation with primary antibody, cells were washed 3 times in PBS (4ºC) and then were incubated with second antibody in 1:500 dilution solution of

PBS, 1% BSA with 0.1% NaN_3 for 90 minutes at room temperature. Cells were rinsing 3 times in PBS. Antibody binding was detected using ExtrAvidin-Peroxidase staining kit (Sigma) and 3.30-diaminobenzidine tetrahydrochloride (Sigma) according to the manufacturer's instructions. Cells were incubated with 150 µL ExtrAvidin-Peroxidase diluted in PBS, 1% BSA for 30 minutes at room temperature. After 3 washes in PBS cells were incubated with 150 µL of DAB solution, until brown colour was easily detected. Cells were rinsed 3 times in PBS. The cell nuclei were then stained with Mayers haematoxylin solution. Negative-control staining was performed by substituting the primary antibody by PBS.

7. Western Blot

After removal Sertoli cells from culture flasks, cells were centrifuged for 5 minutes at 3000x*g* in Sigma 3K18C centrifuge. Then Sertoli cells were homogeneized in appropriate volume of RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, with freshly added 100 ng/ml PMSF 30 µL/ml aprotinin, 10 µL/mL sodium orthovanadate and 10 µL/mL protease inhibitor cocktail) and allowed to stand 15 minutes on ice. The homogenates was centrifuged at 14000x*g* in Hettich Mikro 200R centrifuge for 20 minutes at 4ºC. After centrifugation, the pellet was discarded. The total protein concentration was measured using the Bradford assay. All sample volumes were adjusted with RIPA buffer in order to have same final protein concentrations. Proteins extracted from Sertoli cells were resolved on a 12% SDS-PAGE at 30 mA/gel for 1 hour. After electrophoresis, proteins were electrotransferred to a PVDF membrane (Amershan) at 750 mA for 1 hour and 15 minutes. The membrane were blocked in Tris-buffered saline (TBS) with 0,05% Tween 20 (TBS-T, Applichem, Darmstadt, Germany) contained 5% skimmed dried milk (Regilait, France) for 1 hour. The membrane was then incubated overnight with (1:200) rabbit polyclonal primary antibody against NHE3 (200 µg/mL, SantaCruz, Biothecnology, USA) at 4ºC. After washing in TBS-T, the membrane was incubated with (1:2000) goat polyclonal antibody against rabbit IgG for 1 hour and 30 minutes. The membrane was again washed in TBS-T, air dried, and incubated with ECF substrate (Amershan) for at least 5 minutes and visualized on the Molecular Imager FX (Biorad, Hercules, USA).

8. NMR spectroscopy

During cell culture, 250 µL of the preservation solution were collected at 5, 10, 25, 35, and 50 hours, for nuclear magnetic resonance (NMR) analysis. To each sample 40µL of a 10 mM sodium fumarate solution in 99.9% D_2O were added. ^{1}H-NMR spectra of the collected samples were acquired at 14.1 Tesla, 25 ºC, using a Varian 600 MHz spectrometer equipped with a 3 mm indirect detection probe with z-gradient (Varian, Palo Alto, CA). Solvent-suppressed ^{1}H-NMR spectra were acquired with 6 kHz sweep width, using 14 seconds delay for allowing total proton relaxation, 3 seconds water pre-saturation, 45° pulse angle, 3.5 seconds acquisition time, and at least 64 scans. The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSpro™ NMR spectral analysis program (Acorn, NMR Inc, Fremont, CA).

9. Statistical Analysis

The statistical significance of differences in NHE3 expression among experimental groups was assessed by one-way ANOVA, followed by Dunnett's test. The statistical significance of differences in glucose, pyruvate consumption, lactate and alanine production among experimental groups was assessed by two-way ANOVA, followed by Bonferroni post-test. All experimental data are shown as mean ± S.E.M. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). $P<0.05$ was considered significant and those results are signalized.

IV. Results

1. Sertoli cell culture

Sertoli cells isolated from male rat (20 days old) were assessed for purity in whole-mount preparations in a phase contrast microscope Olympus CKX41. Cell contaminants were below 5% after 96 h in culture as examined by phase contrast microscopy. Purity was estimated by the immunoperoxidase technique using a specific polyclonal antibody against vimentin (figure 8), a specific Sertoli cell marker. To reinforce these results it was performed a RT-PCR (figure 9) in order to evaluate the expression of specific Sertoli cell markers, vimentin and AMH.

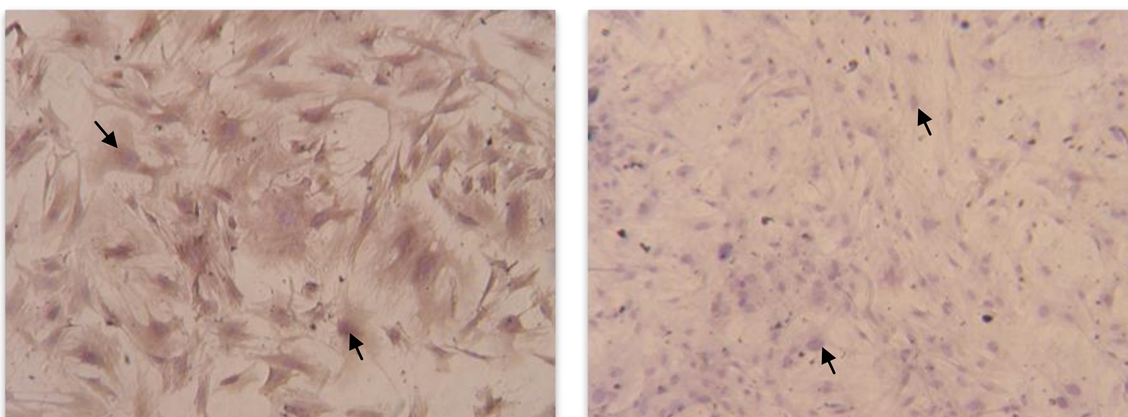

Figure 8: **Immunocytochemical staining for vimentin in rat Sertoli cells.** a) rat Sertoli cells with round nuclei (arrows; x10); b) negative control: nuclei stained with hematoxylin-eosin (arrows; x10).

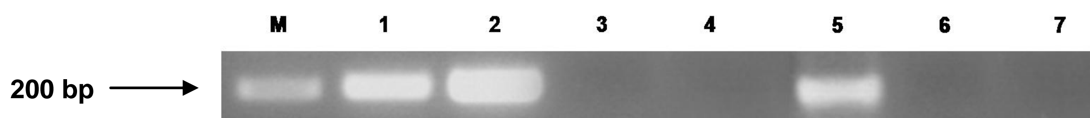

Figure 9: **mRNA Vimentin and AMH obtained by RT-PCR. M:** Gene Ruler DNA Ladder Mix; **1:** positive control for vimentin; **2:** Vimentin (198bp); **3:** control without reverse transcriptase for vimentin; **4:** negative control; **5:** AMH (210bp); **6:** control without reverse transcriptase for AMH; **7:** negative control.

2. mRNA expression of NHE3 in Sertoli cells

Analysis of mRNA NHE3 expression was carried out by RT-PCR (figure 10). After cloning, sequencing and sequence analysis, the insert obtained had the expected size (521bp) and when its sequence was analyzed with NCBI's BLASTN software

(www.ncbi.nlm.nih.gov, 2010), it showed more than 99% alignment with mRNA sequence of NHE3 from *Rattus Norvegicus* (figure 11).

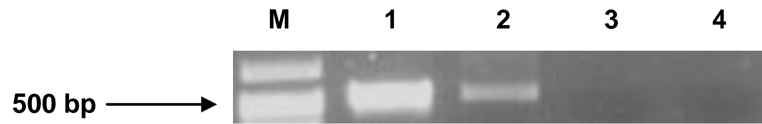

Figure 10: **mRNA NHE3 expression in Sertoli cells. M**: Gene Ruler DNA Ladder Mix; **1:** positive control for NHE3 (521bp); **2:** NHE3 (521bp); **3:** control without reverse transcriptase for NHE3; **4:** negative control.

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NM_012654       2466  GCAACTTCCGCCGCCTGACTCCATTCCGCCTCAGCAACAAATCAGTGGATTCCTTCCTGC
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NHE3 (521 bp)   521   GCAACTTCCGCCGCCTGACTCCATTCCGCCTCAGCAACAAATCAGTGGATTCCTTCCTGC

NM_012654       2526  AGGCTGATGGCCCCGAGGAACAGCTCCAACCCGCTTCCCCTGAGTCCACACACATGTGAT
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NHE3(521bp)     461   AGGCTGATGGCCCCGAGGAACAGCTCCAACCCGCTTCCCCTGAGTCCACACACATGTGAT

NM_012654       2586  ACAGGCTCTGGCACACCACTTACTGGACGCTTGTCTCGTCAGTCATCTGCTGCTGCCCAG
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NHE3(521bp)     401   ACAGGCTCTGGCACACCACTTACTGGACGCTTGTCTCGTCAGTCATCTGCTGCTGCCCAG

NM_012654       2646  CACG-CATTGCTCCCTGCAGGGCTTGCCCACCCACGTGCCCCAAGCCCCGCCCCACCTGA
                      || | |||||||||||||||||||||||||||||||||||||||||||||||||||||||
NHE3(521bp)     341   CA-GCCATTGCTCCCTGCAGGGCTTGCCCACCCACGTGCCCCAAGCCCCGCCCCACCTGA

NM_012654       2705  GCGCACTTCGACTGTCACTTTTGCCTCTGGTGTTCCTGGGACAGAGCCAGAGCACCGGGC
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
NHE3(521bp)     282   GCGCACTTCGACTGTCACTTTTGCCTCTGGTGTTCCTGGGACAGAGCCAGAGCGCCGGGC

NM_012654       2765  CCCACCCAGTGCCCACGCTCGGTCGGCGGCTGCGCGCCGGGTACACAGTAGGCGCTCATT
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NHE3(521bp)     222   CCCACCCAGTGCCCACGCTCGGTCGGCGGCTGCGCGCCGGGTACACAGTAGGCGCTCATT

NM_012654       2825  CCAAAAGCCTCGCCCACGCCGACGCCCAGCTCTGCTAAAACTCGCTTCTCTTCCGGGCTC
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NHE3(521bp)     162   CCAAAAGCCTCGCCCACGCCGACGCCCAGCTCTGCTAAAACTCGCTTCTCTTCCGGGCTC

NM_012654       2885  TACAGAACTGCCACTGCACGCTCTGGAGCAAGAAGAGACGCCGCTGTGATCGCTGCCTGG
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
NHE3(521bp)     102   TACAGAACTGCCACTGCACGCTCTGGAGCAAGAAGAGACGCCGCTGTGATCGCTGCCTGG

NM_012654       2945  CCGATGGCCCAGCTCCGAGGGTCTCCCTCAGTGGCCCCTCCC  2986
                      ||||||||||||||||||||||||||||||||||||||||||
NHE3(521bp)     42    CCGATGGCCCAGCTCCGAGGGTCTCCCTCAGTGGCCCCTCCC  1
```

Figure 11: **Sequence obtained from the cloning of a PCR fragment from NHE3 (NM_012654)**. The sequencing result was aligned using the program BLASTN software (www.ncbi.nlm.nih.gov, 2010).

3. Semi-quantitative PCR

In order to quantify the mRNA of NHE3 the values were normalized with the 18S expression in the Sertoli cells for each group. The results of electrophoresis of PCR products for 18S are represented in figure 12. The results of electrophoresis of PCR products for NHE3 are represented in figure 13.

Figure 12: Electrophoresis of the PCR products for the 18S gene in Sertoli cells. **M**- Gene Ruler DNA Ladder Mix; **E_2**- 17β estradiol; **DHT**- dihydrotestosterone; **ICI**- ICI 182,780; **N**- negative control. Fragment size (152bp).

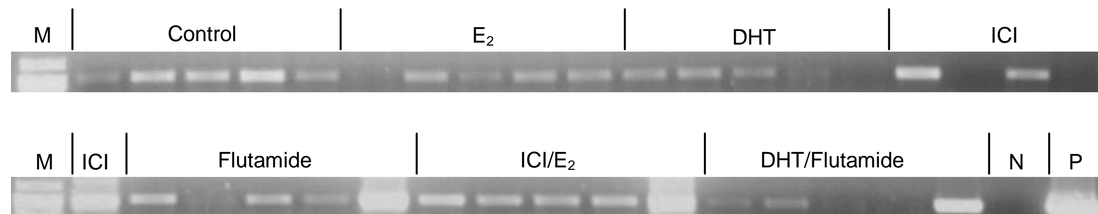

Figure 13: Electrophoresis of the PCR products for the NHE3 gene in Sertoli cells. **M**- Gene Ruler DNA Ladder Mix; **E_2**- 17β estradiol; **DHT**- dihydrotestosterone; **ICI**- ICI 182,780; **N**- negative control; **P**- positive control. Fragment size (521bp).

The results of semi-quantification of NHE3 were obtained performed by dividing the intensity of each band of NHE3 by the intensity of the corresponding band of the housekeeping gene 18S and are represented in figure 14. There are no significant statistically differences among experimental groups.

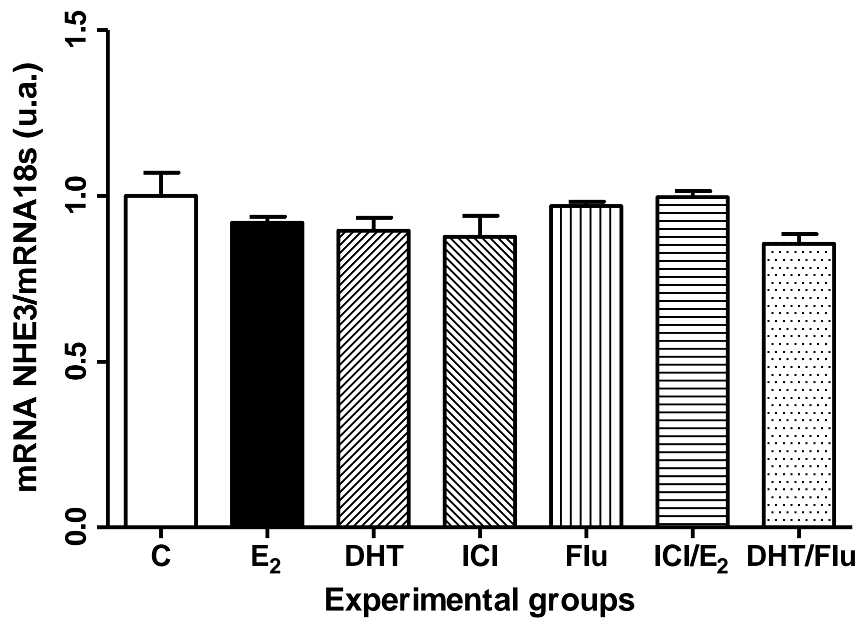

Figure 14: **Semi-quantification of NHE3 expression in Sertoli cells** under hormonal treatment (100nM). The groups are not significantly different ($p<0.05$), One way ANOVA, followed by Dunnett's multiple comparison test. n=5. **C**- control; **E_2**-17β estradiol; **DHT**- dihydrotestosterone; **ICI**- ICI 182,780; **Flu**- flutamide;

4. Western Blot

The presence of NHE3 protein was confirmed by western blot analysis which showed an immunoreactive band of 85 kDa in positive control (kidney) and in Sertoli cells from diverse samples (figure 15).

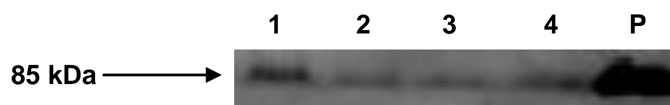

Figure 15: **Western blot analysis of NHE3 in Sertoli cells using a anti-NHE3 policlonal antibody (1:200)**. **1**: ICI-treated cells; **2**: Flutamide-treated cells; **3**: ICI/E_2-treated cells; **4**: Flut/DHT-treated cells; **P**:positive control.

5. NMR results

Hydrogen nuclear magnetic resonance spectroscopy was performed to determine lactate production, glucose consumption and variations in other substrates

during the 50 hours of cell incubation in ITS medium. Sodium fumarate (final concentration of 2 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution. The following metabolites were determined whenever present: lactate, doublet located at 1.33 ppm; alanine, doublet at 1.45 ppm; pyruvate, singlet at 2.36 ppm; and H1-α glucose, doublet at 5.22 ppm (figure 16).

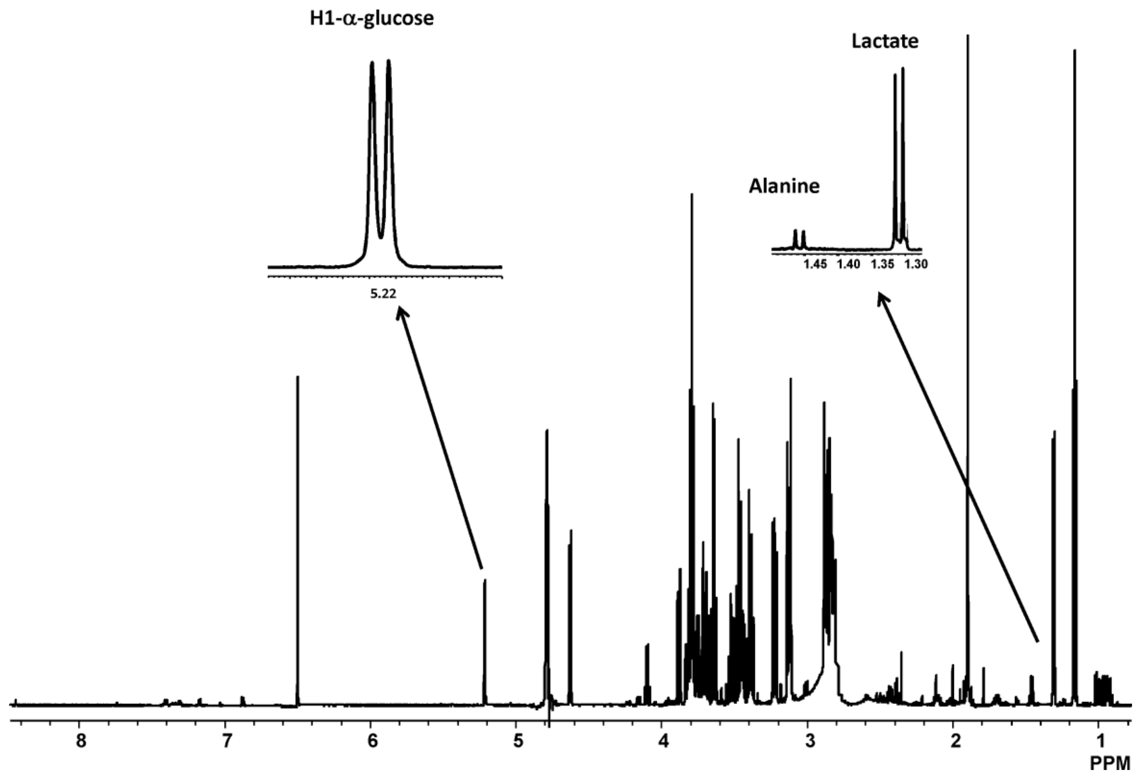

Figure 16: **NMR spectrum.** Localization of alanine, lactate and H1-α-glucose peaks.

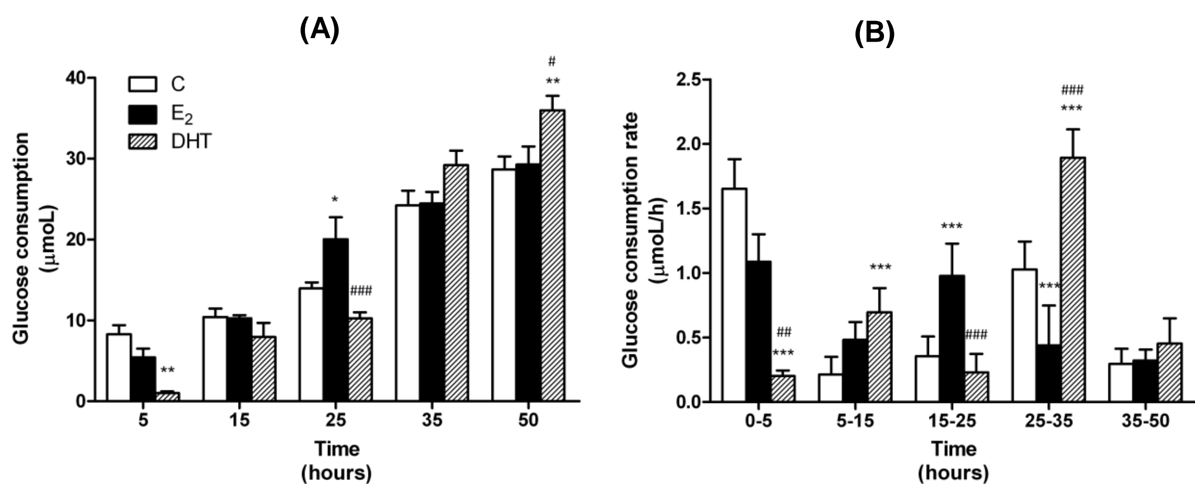

Figure 17: **Glucose consumption by Sertoli cells.** A) Glucose consumption; B) Glucose consumption rate. C- control; E_2- 17β estradiol; DHT- dihydrotestosterone. (n=5). * significantly different when compared to control * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); # significantly different when compared to E_2. # ($p<0.05$); ## ($p<0.01$); ### ($p<0.001$).

The glucose consumption in the first hours is lower in DHT-treated Sertoli cells however after 50 hours the Sertoli cells cultured in control conditions presented a glucose consumption of 28 ± 2 μmoles while those cells treated with E_2 and DHT consumed 29 ± 2 and 36 ± 2 μmoles of glucose, respectively. The treatment of cultured Sertoli cells with DHT resulted in significantly higher glucose consumption after 50 hours though they just increase glucose consumption after 25 hours treatment (figure17A). The glucose consumption rate is smaller in the 5 first hours in DHT-treated Sertoli cells (0.20 ± 0.04 μmol/h) when compared with E_2-treated (1.09 ± 0.21 μmol/h) and control condition (1.65 ± 0.23 μmol/h). Between 25 and the 35 hours of incubation, the glucose consumption rate highly increases in DHT-treated cells which presented a rate of 1.89 ± 0.22 μmol/h, while cells in the control condition and those treated with E_2 consumed glucose at a rate of 1.03 ± 0.22 μmol/h and 0.44 ± 0.30 μmol/h, respectively (figure17B).

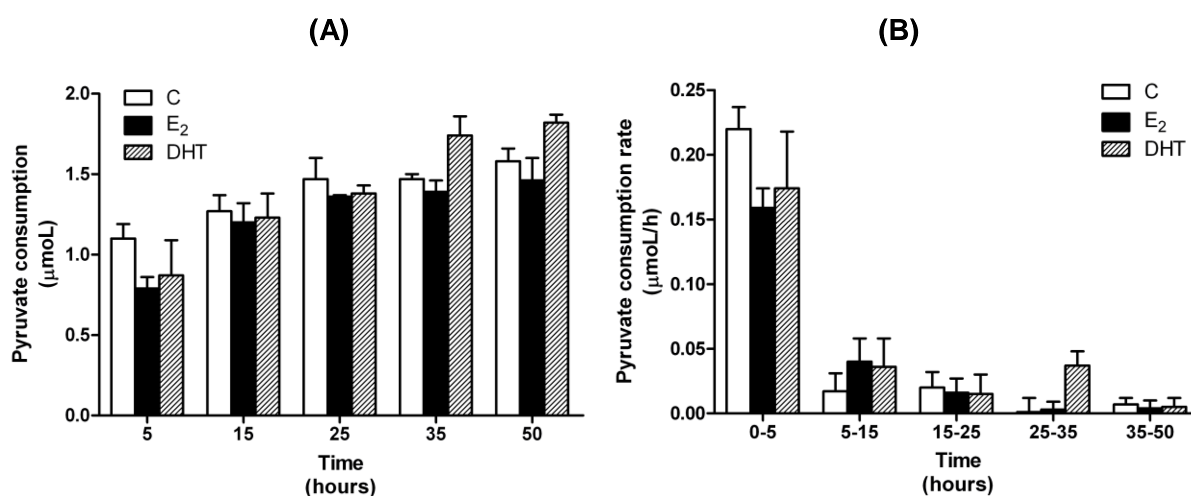

Figure 18: **Pyruvate consumption by Sertoli cells.** A) Pyruvate consumption; B) Pyruvate consumption rate. C- control; E_2- 17β estradiol; DHT-dihydrotestosterone. (n=5). * significantly different when compared to control * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); # significantly different when compared to E_2. # ($p<0.05$); ## ($p<0.01$); ### ($p<0.001$).

There are no hormonal-related effects in pyruvate consumption during 50 hours culture. The pyruvate consumption was not dependent on the hormonal treatment. After 50 hours, the pyruvate consumption was 1.58 ± 0.08, 1.46 ± 0.14 and 1.82 ± 0.05 μmoles in control, E_2-treated and DHT-treated cells, respectively (figure 18A). There were also no differences in pyruvate consumption rate between control, E_2-treated and DHT-treated Sertoli cells during the 50 hours (figure 18B).

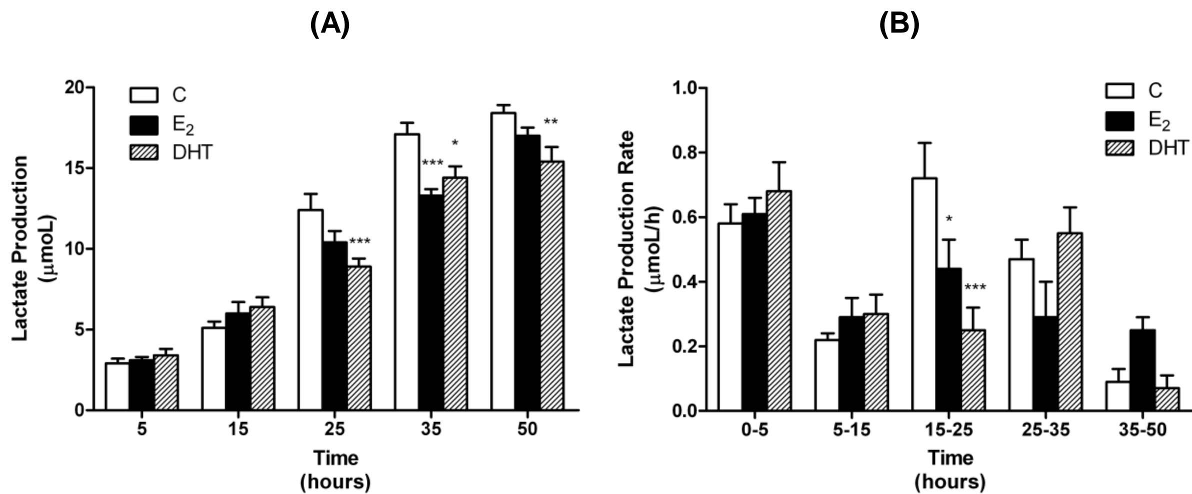

Figure 19: **Lactate production by Sertoli cells.** A) Lactate production; B) Lactate production rate. C- control; E_2- 17β estradiol; DHT- dihydrotestosterone. (n=5). * significantly different when compared to control * (p<0.05); ** (p<0.01); *** (p<0.001); # significantly different when compared to E_2. # (p<0.05); ## (p<0.01); ### (p<0.001).

DHT treatment decreases lactate production after 50 hours. The amount of lactate produced during the 50 hours was similar for control cells and cells treated with E_2, 18 ± 0.5 and 17 ± 0.5 µmoles, respectively. During the first 15 hours, Sertoli cells in all the conditions produced similar amounts of lactate however after the 50 hours treatment with DHT the cells secreted significantly lower amounts of lactate (15 ± 0.9 µmoles, figure 19A). The lactate production rate is similar in the first 15 hours in Sertoli cells cultured in all conditions. Between 15 and 25 hours of culture, the lactate production rate highly increases in control cells (0.72 ± 0.11 µmol/h) when compared with E_2-treated (0.44 ± 0.09 µmol/h) and DHT-treated cultures (0.25 ± 0.07 µmol/h). Between the 25 and 35 hours of culture, the lactate production rate highly increases in DHT-treated cells which presented a rate (0.55 ± 0.08 µmol/h) similar to cells in the control condition (0.47 ± 0.06 µmol/h) and higher than those treated with E_2 (0.29 ± 0.11 µmol/h, figure 19B). As for glucose consumption, in cells treated with DHT, the lactate production rate peaked between 25 and the 35 hours of incubation, nevertheless after 50 h treatment, the cells secreted significantly lower amounts of lactate than Sertoli cells from other groups.

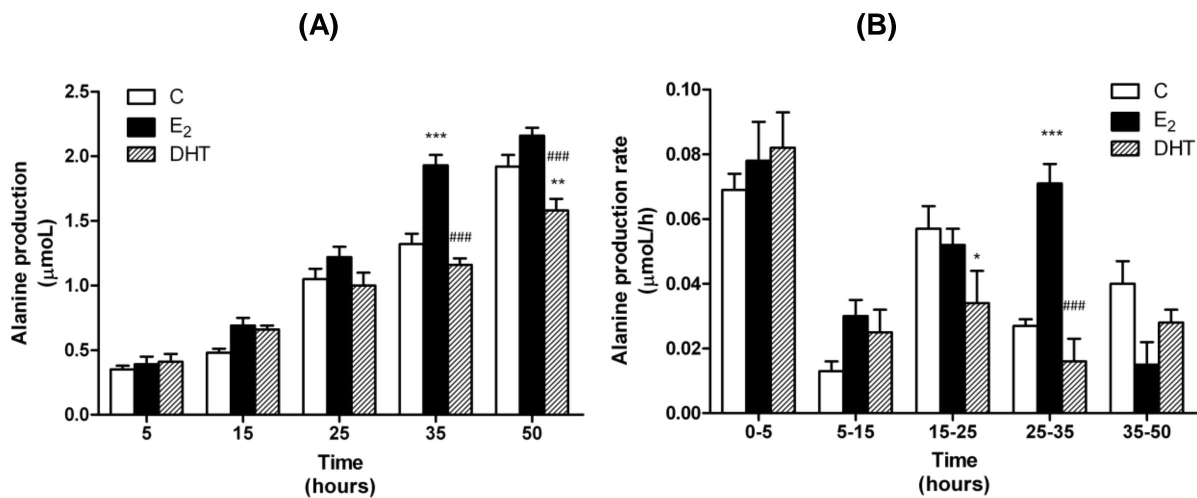

Figure 20: **Alanine production by Sertoli cells.** A) Alanine production; B) Alanine production rate. C- control; E_2- 17β estradiol; DHT- dihydrotestosterone. (n=5). * significantly different when compared to control * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$); # significantly different when compared to E_2. # ($p<0.05$); ## ($p<0.01$); ### ($p<0.001$).

E_2 treatment highly increases alanine production after 25 hours. The alanine production was very similar in all conditions during the first 25 hours of treatment. Throughout that period the control cells, the cells treated with E_2 and those treated with DHT secreted 1.05 ± 0.08, 1.22 ± 0.08 and 1.00 ± 0.10 μmoles of alanine, respectively. After the first 25 hours, the production of alanine significantly increased in Sertoli cells treated with E_2 (1.93 ± 0.08 μmoles) when compared with cells in control conditions and those treated with DHT which produced similar amounts of alanine (1.32 ± 0.9 and 1.16 ± 0.05 μmoles, respectively). At the end of the 50 hours, E_2-treated cells secreted significantly higher alanine amounts than cells from the other conditions (figure 20A). Alanine production rate was very similar in the first 5 hours in E_2-treated Sertoli cells (0.39 ± 0.06 μmol/h), DHT-treated (0.41 ± 0.06 μmol/h) and in control condition (0.35 ± 0.03 μmol/h). Between 25 and the 35 hours of incubation, the alanine production rate highly increased in E_2-treated cells which presented a rate of 0.07 ± 0.006 μmol/h, while cells in the control condition and those treated with DHT consumed glucose at a rate of 0.03 ± 0.002 μmol/h and 0.07 ± 0.007 μmol/h, respectively (figure 20B).

V. Discussion

The main aim of this work was to study the effects of sex steroids in NHE3 expression by Sertoli cells. Furthermore, it was also verified the role of sex hormones in metabolism modulation of the Sertoli cells. Firstly it was established primary cultures of rat Sertoli cells. The cultures of Sertoli cells obtained, grew in monolayer and cell contaminants were below 5% after 96 hours in culture. Similar results were obtained by Meroni and collaborators (2002). In culture, Sertoli cells did not show high proliferative capacity. In addtion, Steinberger and Steinberger (1971) indicated that in serum-free testis fragment cultures, Sertoli cells cease proliferation at the equivalent of days 10–12 after birth, irrespective of the age of the rat from which the cells were isolated. This finding prompted the suggestion that Sertoli cells contain an intrinsic timing mechanism that initiates their differentiation in the absence of external signaling factors (Buzzard et al., 2002). However, Buzzard and collaborators (2002) demonstrated that Sertoli cell differentiation is not controlled by an autonomous, intrinsic maturation programme. Instead, external signals must act on these cells to initiate timely differentiation.

The establishment of the adequate composition of the STF is critical to the normal occurrence of spermatogenesis. This event include, among others, the net movements of water, ions (Na^+, Cl^-, and HCO^-_3 reabsorption, K^+ secretion, and luminal acidification) (Au and Wong, 1980, Levine and Marsh, 1971, Turner, 1984), hormones, proteins and carbohydrates into the tubular lumen (Setchell and Waites, 1975). Intracellular pH is relevant for Sertoli cell physiology and germ cells viability. It has been suggested the presence of several membrane transport systems that participate in pH regulation in cultured Sertoli cells, (Oliveira et al., 2009a, Oliveira et al., 2009b). In the literature there are few works about expression of NHE in Sertoli cells. In the present study it was identified the expression of NHE3 mRNA by RT-PCR in cultured rat Sertoli cells, and the expression of NHE3 protein by means of the immunoblotting technique. Furthermore, NHE3 PCR products were cloned and sequenced. Sequencing of PCR products confirmed the identification of the NHE3 mRNA and showed a 99% homology with rat NHE3 mRNA (GenBank accession number NM_012654). Rat NHE3 was cloned for the first time by Orlowski and collaborators (1992). They analyzed mRNA NHE3 tissue distribuition, but did not identify mRNA NHE3 expression in testes.

It has been suggested that sex steroids plays an important role in regulation of NHE3 expression in male reproductive tract (Hess, 2003, Zhou et al., 2001, Oliveira et al., 2002, Snyder et al., 2009, Kaunisto and Rajaniemi, 2002), but the results of this study demonstrated that both, estrogens and androgens, did not exert significant effects in NHE3 expression in experimental groups. E_2-treated cells and ICI-treated cells do not show differences in NHE3 expression when compared to control group.

Concordant results were describe by Lee and collaborators (2001) in cells of the ED of ICI-treated mice in which the mRNA NHE3 levels do not show reduction when compared to WT mice. Another interesting result was described in a study by Sinkevicius and collaborators (2009) that demonstrate no significant differences in NHE3 mRNA expression levels in cells of ED between WT mice and estrogen nonresponsive ERα knock-in mice. ERα can also be activated in the absence of estrogen by growth factors like IGF-1 or EGF (Shaha et al., 2008). Thus, Sinkevicius and collaborators concluded that ligand-independent ERα signaling is important for concentrating epididymal sperm via regulation of efferent ductule fluid reabsorption. However Oliveira and collaborators (2002) demonstrated that long-term exposure to ICI 182,780 in rats lead to a decrease expression in NHE3 in ED, that culminate in a similar ERKOα phenotype.

As for androgens, there are also no significant differences in DHT, flutamide and flutamide/DHT treated cells when compared to control group. Reports from Caflisch (1993) showed that flutamide treatment elevated significantly *in situ* pH in proximal caput, middle caput, middle corpus and proximal cauda epididymidis but not in seminiferous tubules, showing that $HCO3^-$ concentration did not change in this part of male reproductive tract. Together this results lead to the suggestion that control of luminal acidification in seminiferous tubules is controlled via ligand-independent nuclear receptors signaling.

Glucose is the most widely used substrate for the generation of cellular energy (ATP) in cell culture, which is necessary for cell growth and maintenance. Glucose is used either by oxidative phosphorylation (30–38 mol of ATP per mol of glucose) or by glycolysis (2 mol of ATP and 2 mol of lactate per mol of glucose). Glucose consumption and lactate production indicate which metabolic pathway the cells use to produce energy from glucose. In our experimental setting, glucose consumption was significantly higher in DHT-treated Sertoli cells after 50 hours than in control conditions or E_2-treated cells. However, this increase in glucose consumption was not followed by an increase in lactate production. Surprisingly, DHT-treated cells produced less lactate than those treated with E_2 or in control conditions. This may be due to several factors such as to a decrease in cellular production of lactate, to a delay in lactate transport to extracellular medium or even to lactate utilization as substrate by DHT-treated cells. In *lizard* Sertoli cell, it was proved that *in vitro,* lactate production was decreased by E_2 in a dose-dependent manner and DHT showed inhibitory effects in lactate production in a dose-dependent manner and in a time-dependent manner (Khan and Rai, 2004). So, glucose metabolism in cultured Sertoli cells treated with E_2 or DHT may be modulated by lactate concentration and ultimately by lactate transporters. This may be very

important since exogenous lactate is essential to support ATP production by spermatogenic cells. Further studies will be needed to confirm this hypothesis. Grootegoed and collaborators (1986) described that cultured Sertoli cells oxidized exogenous pyruvate during incubation in the presence of glucose (Grootegoed et al., 1986). In our experimental conditions, pyruvate consumption was increased not only after treatment with DHT and E_2, but also in control conditions which confirms that cultured Sertoli cells highly consume pyruvate when available. The exogenous pyruvate is a substrate in intermediary metabolism that can be converted into lactate, presumably until pyruvate/lactate reaches equilibrium. This conversion is an NADH-dependent reduction but pyruvate may also be converted into alanine via transaminase reaction, through the enzyme alanine aminotransaminase (Yang et al., 2002). The ratio of lactate to alanine is an index of redox state of the cell (O'Donnell et al., 2004) as the reduction of pyruvate into lactate or its conversion into alanine is related with the reoxidation of cytosolic NADH into NAD^+, the lactate/alanine ratio reflects the NADH/NAD^+ ratio. The presence of alanine can be associated with a reduced redox cytosolic state (low ratio NADH/NAD^+) since the conversion of lactate to pyruvate by lactate dehydrogenase is more extensive when higher levels of NAD^+ are present. In our experimental conditions, alanine production was significantly decreased in DHT-treated cells after 50 hours when compared with E_2-treated cells. The lactate production was also decreased after DHT treatment. Being so, this may suggest that DHT is redirecting glucose metabolism to krebs cycle and not to lactate or alanine production. The cells in this condition become metabolically more active. Glucose metabolism proved to be upregulated by hormonal treatment however, it cannot exclude the role of ketone bodies and fatty acids to Sertoli cells in these conditions since they are also described to be a major energy substrate for cultured rat Sertoli cells (Jutte et al., 1985) and a puzzling differentiation-related change in carbohydrate metabolism occurs during mammalian spermatogenesis (Bajpai et al., 1998).

In summary, this study demonstrates that sex steroids do not exert significant effects in NHE3 expression by Sertoli cells. It is likely that control of pH_i of the Sertoli cells and luminal acidification in seminiferous tubules does not depend on directly of estrogen mediated-ER signaling and androgen mediated-AR signaling. Glucose consumption was increased in DHT-treated cells, but the lactate production does not accompany this increase. In other hand, E_2-treated cells show the highest levels in alanine production. Thus, sex steroids seem to present an important role in modulation of Sertoli cells metabolism. However, it is important to consider the glycogenolysis pathway may also be hormonally regulated. This work was a first step to identify key

mechanisms by which hormones can regulate Sertoli cells metabolism and spermatogenesis.

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