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Effects of Sex Steroid Hormones on Sertoli Cells Metabolic Pathways

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Dissertação para obtenção do Grau de Mestre em
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O conteúdo do presente trabalho é da exclusiva responsabilidade do autor:

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(Ana Catarina Dias Martins)

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Publications

Martins AD, Alves MG, Simões VL, Dias TD, Rato L, Moreira, PI, Socorro S, Cavaco JE, Oliveira PF (2012) 17β-estradiol and 5α-dihydrotestosterone modulate transporters and enzymes of glucose metabolism in cultured immature rat Sertoli cells. (Submitted)

Simões VL, Alves MG, Martins AD, Dias TD, Rato L, Socorro S, Oliveira PF (2012) Regulation of Apoptotic Signaling Pathways by 5α-dihydrotestosterone and 17β-estradiol in Immature Rat Sertoli Cells. (Submitted)

Resumo

As células germinativas em desenvolvimento utilizam lactato, um produto do metabolismo da glicose das células de Sertoli (SCs), como a principal fonte de energia. O papel dos androgénios e estrogénios na modulação do metabolismo energético das células testiculares tem vindo a ser estudado, particularmente nas SCs. O presente estudo tem como objetivo explorar o efeito de hormonas esteróides sexuais sobre as vias envolvidas no metabolismo da glicose em SCs de ratos. Foram analisados os níveis de mRNA de transportadores de glicose (GLUT1 e GLUT3), fosfofrutoquinase-1 (PFK1) e lactato desidrogenase isoforma C (LHD C) por RT-PCR, e por *Western Blot* foram analisados os níveis proteicos de GLUTs, PFK-1, LDH e transportador de ácidos monocarboxílicos 4 (MCT4). Foram utilizadas para este estudo culturas primárias de SCs de ratos imaturos tratadas com 17β-estradiol (E2) ou 5α -dihidrotestosterona (DHT). Os resultados obtidos demonstram que tanto o E2 como o DHT regulam os níveis de transcrição da PFK1, GLUT1 e GLUT3. No entanto, apenas as células tratadas com DHT apresentam uma diminuição nos níveis de transcrição da LDH C. Curiosamente, os níveis de proteína destas enzimas e transportadores permaneceram inalterados, exceto em células tratadas com DHT que apresentaram uma diminuição significativa nos níveis proteicos de GLUT1, pondo em evidência uma possível via para a regulação do metabolismo da glicose em SCs por androgénios. Em conjunto, estes resultados demonstraram uma relação entre a ação das hormonas esteróides sexuais e metabolismo energético das SCs, facultando novas evidências sobre os mecanismos através dos quais o E2 e a DHT exercem a sua função como moduladores do metabolismo da glicose em SCs de rato.

Palavras-chave:

Células de Sertoli; metabolismo energético, androgénios, estrogénios, transportadores de glicose

Resumo Alargado

O metabolismo dos carboidratos em células de Sertoli (SCs) tem vindo a ser debatido desde a década de 80 do século passado, pois este processo bioquímico apresenta algumas características únicas. De facto, foi descrito que as SCs podem adaptar o seu metabolismo, a fim de assegurar uma concentração de lactato adequada no microambiente onde as células germinativas se desenvolvem. As células germinativas em desenvolvimento utilizam lactato, um produto do metabolismo da glicose das SCs, como a principal fonte de energia. O papel dos androgénios e estrogénios na modulação do metabolismo energético das células testiculares tem vindo a ser estudado, particularmente no das SCs.

O presente estudo tem como objetivo explorar o efeito de hormonas esteróides sexuais sobre as vias envolvidas no metabolismo da glicose em SCs de ratos. Foram analisados os níveis de mRNA de transportadores de glicose 1 e 3 (GLUT1 e GLUT3), fosfofrutoquinase-1 (PFK1) e lactato desidrogenase isoforma C (LHD C) por RT-PCR, e por *Western Blot* foram analisados os níveis proteicos de GLUT1 e GLUT3, PFK1, LDH e transportador de ácidos monocarboxílicos 4 (MCT4). Foram utilizadas para este estudo culturas primárias de SCs de ratos imaturos tratadas com 17β-estradiol (E2) ou 5α -dihidrotestosterona (DHT).

No presente estudo, foi possível observar que tanto o E2 e a DHT diminuíram os níveis de mRNA de enzimas-chave relacionadas com a glicólise e de transportadores de glicose, embora os níveis de proteína nem sempre refletiram essas alterações. A diminuição dos níveis mRNA pode ser atribuída a alterações nas taxas de síntese ou degradação de mRNA ou a ambos. Assim, existe a possibilidade de que, em SC de ratos, a modulação das quantidades de mRNA analisadas pelo E2 e a DHT seja exercida ao nível da transcrição e/ou pós-transcricional ou que a modulação da expressão proteica seja regulada por outros mecanismos ou numa janela temporal distinta. Os resultados obtidos demonstram que tanto o E2 como o DHT regulam os níveis de transcrição da PFK-1, GLUT1 e GLUT3. No entanto, apenas as células tratadas com DHT apresentam uma diminuição nos níveis de transcrição da LDH C. Curiosamente, os níveis de proteína destas enzimas e transportadores permaneceram inalterados, exceto em células tratadas com DHT que apresentaram uma diminuição significativa nos níveis proteicos de GLUT1, pondo em evidência uma possível via para a regulação do metabolismo da glicose em SCs por androgénios.

Em conclusão, e embora as culturas SCs primárias possam não representar exatamente a situação *in vivo*, a sua utilização permite um melhor conhecimento sobre o funcionamento do conjunto de eventos associados ao metabolismo destas células, que são cruciais para o desenvolvimento as células germinativas e, portanto, para a espermatogénese. Em conjunto, estes resultados demonstraram uma relação entre a ação das hormonas esteróides sexuais e metabolismo energético das SCs, facultando novas evidências sobre os mecanismos através

dos quais o E2 e a DHT exercem a sua função como moduladores do metabolismo da glicose em SCs de rato, com influência direta sobre a espermatogénese e a fertilidade masculina.

Abstract

Developing germ cells use lactate, derived from glucose metabolism of Sertoli cells (SCs), as their main energy source. Androgens and estrogens have been implicated in the modulation of testicular cells energy metabolism, particularly in SCs. The goal of the present study was to shed light on the effects of sex steroid hormones on glucose metabolic pathways in rat SCs. The mRNA levels of glucose transporters 1 and 3 (GLUT1 and GLUT3), phosphofructokinase 1 (PFK1) and lactate dehydrogenase chain C (LHD C) were analyzed by RT-PCR, and protein levels of GLUTs, PFK1, LDH and monocarboxylate transporter 4 (MCT4) were analyzed by Western Blot, in enriched primary cultures of immature rat SCs treated with 17β-estradiol (E2) or 5α-dihydrotestosterone (DHT). Our results show that both E2 and DHT downregulated the gene transcript levels of PFK-1, GLUT1 and GLUT3. However, only DHT-treated cells presented a downregulation of LDH C gene transcript levels. Interestingly, the protein levels of these enzymes and transporters remained unaltered except in DHT-treated cells that presented a significant decrease on GLUT1 protein levels evidencing a possible pathway for the regulation of glucose metabolism in SCs by androgens. Taken together, these results demonstrated a relationship between the action of sex steroid hormones and energy metabolism of SCs, providing evidences for the mechanisms by which E2 and DHT exert their function as modulators of rat SCs glucose metabolism.

Keywords:

Sertoli cells; energy metabolism; androgens; oestrogens; glucose transporters

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Abbreviations

AMPK – AMP-activated Protein Kinase

ARKO - Androgen Receptor Knock-out

ARs – Androgen Receptors

bFGF – Fibroblast Growth Factor

BSA - Bovine Serum Albumin

BTB – Blood-Testis Barrier

cAMP - Cyclic Adenosine Monophosphate

cDNA – Complementary Deoxyribonucleic Acid

DAB – 3,3' Diaminobenzidine Hydrochloride

DHT - 5-α-dihydrotestosterone

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

DNA - Deoxyribonucleic Acid

E2 – 17-ß-Estradiol

EDTA - Ethylene Diamine Tetra Acetic acid

EGF – Epidermal Growth Factor

ERKO - Estrogen Receptor Knock-out

ERs – Estrogen Receptors

ERα – Estrogen Receptor α

ERαKO - Estrogen Receptor α Knock-out

ERß – Estrogen Receptor ß

ERßKO - Estrogen Receptor ß Knock-out

EtOH – Ethanol

F-1,6-BP – Fructose-1,6-Biphospate

FBS - Fetal Bovine Serum

FSH – Follicle-Stimulating Hormone

FSHRKO - Follicle-Stimulating Hormone Receptor Knock-out

GLUT1 – Glucose Transporter 1

GLUT14 – Glucose Transporter 14

GLUT3 – Glucose Transporter 3

GLUT8 – Glucose Transporter 8

GLUTs – Glucose Transporters

GnRH – Gonadotrophin-Releasing Hormone

GnRH-R – Gonadotrophin-Releasing Hormone Receptor

HBSS - Hank's Balanced Salts Solution

IGF-I – Insulin Growth Factor I

ITS supplement - Insulin-Transferrin-Sodium Selenite supplement

LDH - Lactate dehydrogenase

LDH A - Lactate dehydrogenase A

LDH B - Lactate dehydrogenase B

LDH C - Lactate dehydrogenase C

LDH C4 - Lactate dehydrogenase C4

LH – Luteinizing Hormone

LHRKO - Luteinizing Hormone Receptor Knock-out

MCT1 – Monocarboxylate Transporter 1

MCT2 – Monocarboxylate Transporter 2

MCT3 – Monocarboxylate Transporter 3

MCT4 – Monocarboxylate Transporter 4

MCTs – Monocarboxylate Transporters

M-MLV RT - Moloney Murine Leukemia Virus Reverse Transcriptase

mRNA - Messenger Ribonucleic Acid

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PFK – Phosphofructokinase

PFK1 – Phosphofructokinase 1

RIPA - Radio-Immunoprecipitation Assay

RNA - Ribonucleic Acid

RNAt - Total Ribonucleic Acid

SCARKO – Sertoli Cells Androgen Receptors Knock-out

SCs – Sertoli Cells

SHs – Steroid Hormones

T - Testosterone

TBS - Tris-Buffered Saline Solution

I. Introduction

1. Sertoli cells

1.1 . General Aspects

Sertoli cells (SCs) represent the main somatic component of the tubular compartment of the testes. The testes are the primary reproductive organs in the male that have two basic functions: the production of spermatozoa and the production of hormones (Mikos et al. 1993; Foley 2001; Rato et al. 2010). In each of the testes, the testicular parenchyma is composed by seminiferous tubules (Figure 1A) and interstitial tissue, and is enclosed by the tunica albuginea (Figure 1B).

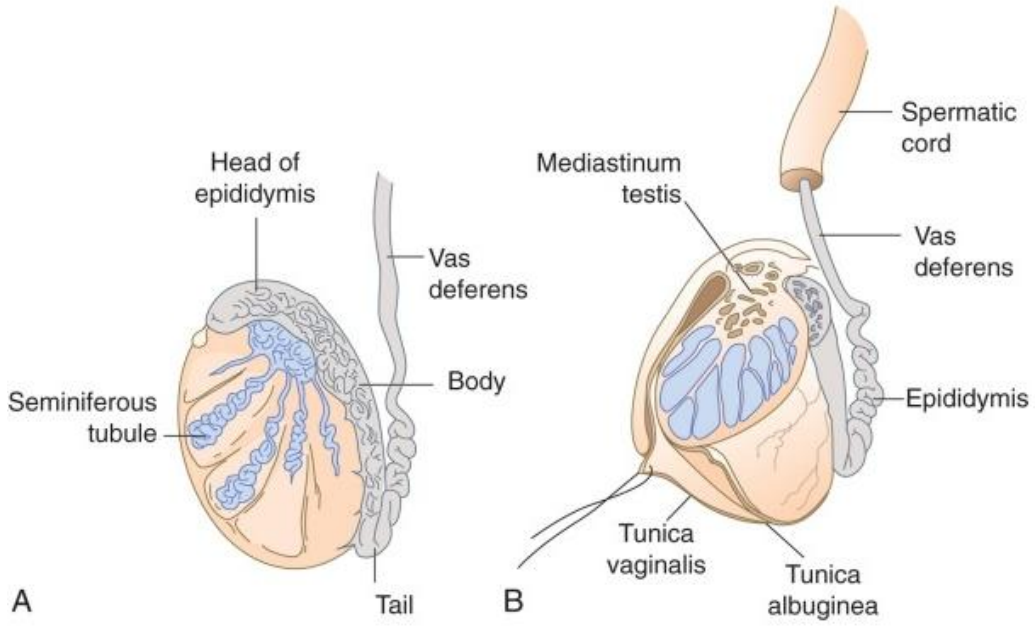

Figure 1: Testis and epididymis. **A**, One to three seminiferous tubules fill each compartment and drain into the rete testis in the mediastinum. Efferent ductules become convoluted in the head of the epididymis and drain into a single coiled duct of the epididymis. The vas is convoluted in its first portion. **B**, Cross section of the tunica vaginalis, showing the mediastinum and septations continuous with the tunica albuginea. The parietal and visceral tunica vaginalis are confluent where the vessels and nerves enter the posterior aspect of the testis (Brooks 2007)

In each testis of the rat, the seminiferous tubules are organized as longitudinally oriented coils that are arranged in funnel shape geometry and stacked within each other (Figure 1A). At the end of each seminiferous tubule, short areas of transitional epithelium are joined to form the rete testis. The germ cells are located in the seminiferous tubules, associated with somatic SCs that are responsible for the formation of the simple columnar epithelium, resting on the basal lamina and extending complex processes to enclose the germ cells throughout the epithelium (Figure 2) (Rodriguez-Sosa and Dobrinski 2009). The areas

between the seminiferous tubules, called interstitial space, vary from specie to specie (Foley 2001). Normally, the interstitial tissue contains the blood and lymphatic vessels that are the main responsibles for the movement of hormones and nutrients into and out of the testis (O'Donnell et al. 2001). Within the interstitial compartment are located the Leydig cells (Figure 2) that were firstly described in 1850, but, only in 1903, their endocrine role in the control of male sexual characteristics was disclosed (Bouin P. 1903). The importance of these cells for male sex differentiation and fertility is unquestionable as they produce testosterone (T), which is the key hormone for a normal sex differentiation and male reproductive function (Martin and Tremblay 2010). The orientation and density of Leydig cells are also species-dependent. For example, rats have few Leydig cells, and they can be found within lymphatic spaces, clustered around blood vessels and largely bathed in lymph fluid; on the other hand interstitial areas of monkeys and dogs have discrete lymphatic channels and Leydig cells are embedded in the connective tissue (Fawcett et al. 1973; Foley 2001). In the rat testis, besides Leydig cells, a resident tissue of macrophages can also be found, as part of interstitial cell population (Dirami et al. 1991; Foley 2001).

Within the seminiferous tubules, closely associated to the basement membrane and surrounded by the peritubular myoid cells, we can find the SCs (Figure 2) (Dym and Fawcett 1970; O'Donnell et al. 2001; Johnson et al. 2008). The SCs are arranged in a columnar shape, with long and thin mitochondria and at within their cytoplasm they possess lipofuscin and lipid droplets. The nuclei of these cells may have a variety of shapes, but normally they are oval or pear-shaped, with an irregular nuclear membrane. In face of its high metabolic rates, they possess an appropriate nuclear envelope, euchromatic nucleoplasm and large distinctive nucleolus as essential features (Johnson et al. 1991; Johnson et al. 2008). These cells have large dimensions, so they can support more than one germ cell. In fact this is a very important characteristic of SCs, not only to allow them to support multiple germ cells per each SC, but also to allow the movement of germ cells during the spermatogenesis (Mruk and Cheng 2004). SCs are the major responsibles for the regulation of spermatogenesis and for the different rates of spermatozoa production (Orth et al. 1988; Walker and Cheng 2005). These cells, known as “nurse cells” (Foley 2001), have many functions important not only for the development of the testicular function, but also to the expression of the male phenotype (Sharpe et al. 2003; Mruk and Cheng 2004). The main functions of the SCs are: (1) provide structural support and nutrition to developing of germ cells; (2) phagocytosis of residual bodies and degenerating germ cells; 3) production of a host proteins that regulate the release of spermatids; and 4) influence of mitotic activity of spermatogonia controlling the response to pituitary hormone release (Dym and Raj 1977; Feig et al. 1980; Jutte et al. 1982; Johnson et al. 2008).

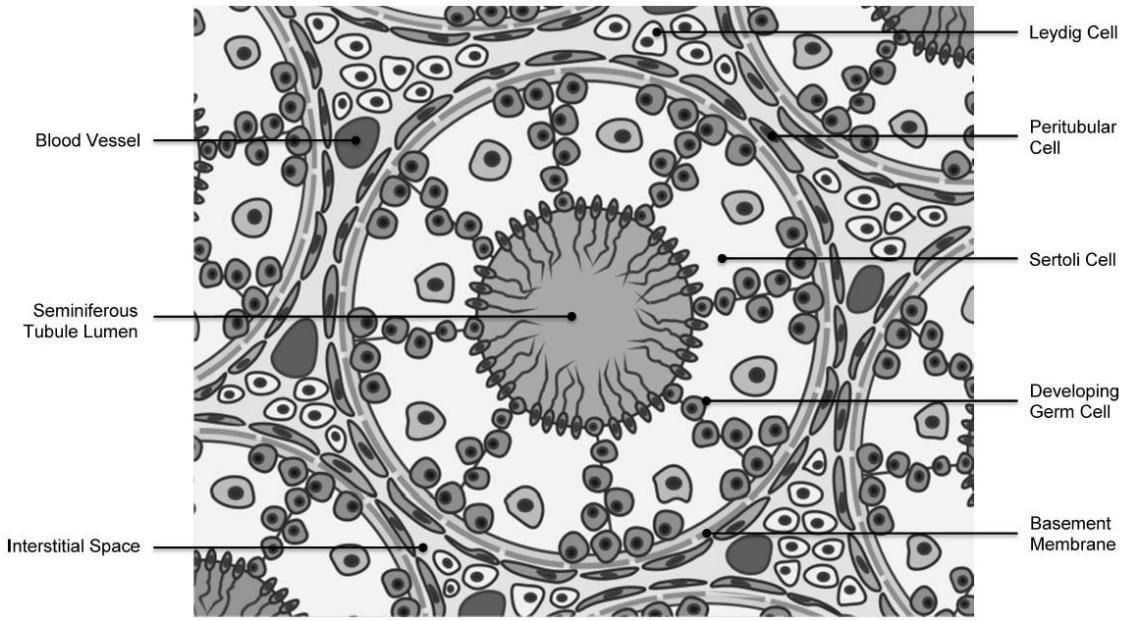

Figure 2: Schematic illustration of the seminiferous tubule and the blood-testis barrier (BTB). The BTB is a physical barrier between the blood vessels and the seminiferous tubule lumen and is formed by tight connections between Sertoli cells (SCs). Outside the BTB is the basal compartment where spermatogonial renewal occurs and inside the BTB is the apical compartment where meiosis, spermiogenesis and spermiation take place. At the interstitial space are located the blood vessels and the Leydig cells. The cytoplasmic extensions that enwrap the developing germ cells are responsible for the structural support through a microtubular filament present in the cytoplasm of SCs. External to the basement membrane are several layers of modified myofibroblastic cells, termed peritubular cells, responsible for the irregular contractions of the seminiferous tubules, which propel fluid secreted by the SCs. Adapted from Rato et al. (2011)

The adjacent SCs form tight junctions with each other, creating a tight barrier known as the blood-testis barrier (BTB) (Figure 2). These junctions have a porosity of approximately 1000 Daltons, hence nothing with higher weight can pass to the tubule interior (Walker and Cheng 2005; Lie et al. 2009; Siu et al. 2009). The BTB creates a specialized microenvironment in the apical compartment of the seminiferous epithelium and segregates the entire event of post-meiotic germ cell development from the systemic circulation (Mruk and Cheng 2004; Wong et al. 2007; Li et al. 2009). Hence, BTB can also act as an immunological barrier since it separates the mature germ cells (spermatocytes and spermatids) from the immune system. This immune barrier continues into the epididymal ducts that transport and store spermatozoa (Johnson et al. 2008). Since the BTB is the barrier between germ cells localised in the basal and the adluminal compartments, molecular events of junctional disassembly and assembly in SCs membranes are responsible for the movement of the germ cells from the basal to the adluminal compartments of the seminiferous epithelium (Wong and Cheng 2005; Li et al. 2006; Johnson et al. 2008).

1.2 . Sertoli cells and Spermatogenesis

Spermatogenesis is the maturation process of germ cells that undergo division, meiosis and differentiation to generate haploid elongated spermatids. For the success of this process, which takes place within seminiferous tubules, it is necessary a close association of germ cells with the SCs (Figure 3) (O'Donnell et al. 2001; Rato et al. 2010).

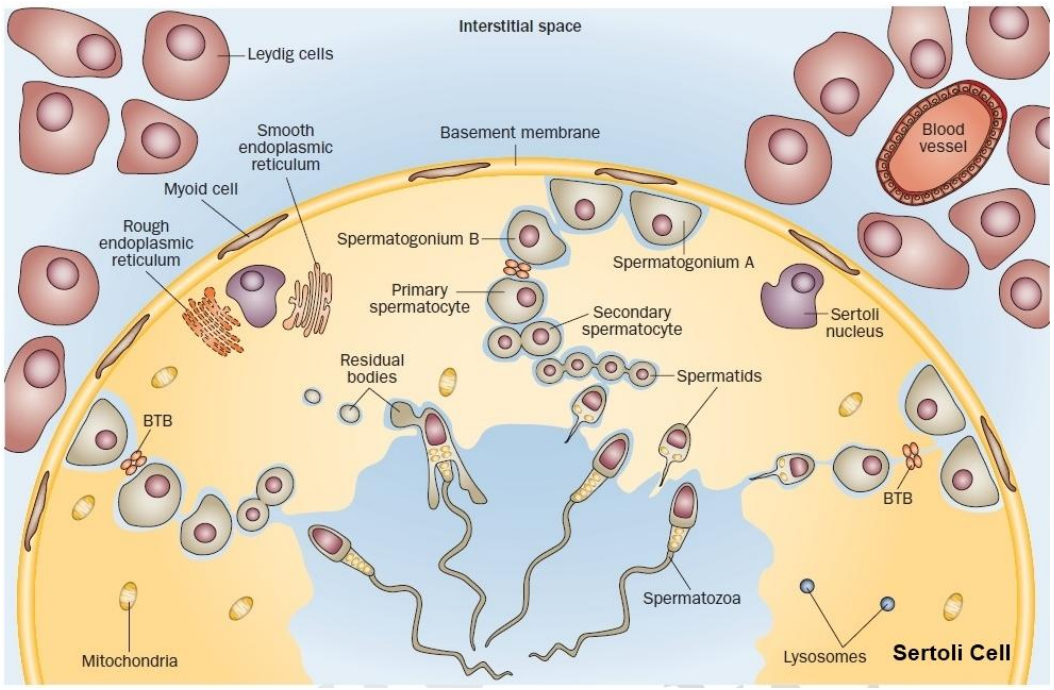

Figure 3: Schematic representation of the blood-testis barrier and of spermatogenesis. The seminiferous epithelium is composed of Sertoli (SCs) and developing germ cells at different stages. Leydig cells and blood vessels are in the interstitium. Spermatogenesis is the cellular division and transformation that produces male haploid germ cells from diploid spermatogonial stem cells. Continuous sperm production is dependent upon several intrinsic (SCs and germ cells), extrinsic (hormonal) factors. The supporting Scs adhere to the basement membrane where spermatogonia are also adherent. Spermatogonia type A divide and develop into spermatogonia type B, which enter meiotic prophase and differentiate into primary spermatocytes that undergo meiosis I to separate the homologous pairs of chromosomes and form the haploid secondary spermatocytes. Meiosis II yields four equalized spermatids that migrate toward the lumen where fully formed spermatozoa are finally released. Abbreviations: BTB, Blood-testis Barrier. Adapted from Rato et al. (2012)

The spermatogenesis in the rodent testes begins a short time after the birth and takes place within the seminiferous epithelium. The development of the germ cells is a process complex, extremely well regulated and divided in four steps, mitosis, meiosis, spermiogenesis and spermiation (O'Donnell et al. 2001; Lie et al. 2009). These events can be described as a cycle of cellular changes that can be further divided in stages. In rats there are 14 stages (Cheng et al. 2010), during which germ cells can be found from the periphery until the centre

of the seminiferous tubule, according to their degree of maturation (Figure 2) (Wang et al. 2011).

Spermatogonia are the undifferentiated germ cells that enter in mitosis originating type A, intermediate and type B spermatogonia (de Rooij and Russell 2000; Lie et al. 2009). Only type B spermatogonia differentiate into leptotene spermatocytes that cross the BTB into the adluminal compartment of the seminiferous epithelium (Wong et al. 2005; Lie et al. 2009). The next stage of the spermatogenesis is the transformation in pachytene spermatocytes and posterior entrance in meiosis I, followed by meiosis II and consequent formation of spermatids. The spermatids are localized near to tubule lumen, and suffer spermiogenesis, characterized by extensive morphological, chromosomal condensation and formation of the acrosome, tail and residual body (Lie et al. 2009). At the end of this process, the mature spermatids are released into to the tubule lumen (Figure 2), and proceed through the duct system to the epididymis where they suffer several biochemical changes, to become the motile spermatozoa capable of fertilization (O'Donnell et al. 2001).

Spermatogenesis is a complex process that is finely regulated by multiple hormones (Bull et al. 2000; O'Donnell et al. 2001). This regulation starts in the hypothalamus, by the intermittent releasing of gonadotrophin-releasing hormone (GnRH), which binds with high-affinity to the gonadotrophin releasing hormone -receptor (GnRH-R) on the anterior pituitary (Naor 1990; Bull et al. 2000; Harrison et al. 2004). In the anterior pituitary, GnRH regulates the production and releasing of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Naor 1990; Shupnik 1996; Botte et al. 1999) that stimulate the synthesis of the sex steroid hormones (SHs) and gametogenesis in the testis (Figure 4) (Botte et al. 1999; Harrison et al. 2004). Allan and collaborators (2004) have described the vital role of FSH in determining the mitotic proliferation capacity of SCs, and its important role in stimulating mitotic germ cell proliferation and meiotic germ cell development, but the limited and incomplete postmeiotic progress initiated by FSH, confirmed that LH activity is critical for the conclusion of spermatogenic progress.

In the testis, LH which is a heterodimeric glycoprotein hormone, plays crucial roles in the regulation of vertebrate reproductive functions (Chopineau et al. 1999). This hormone controls, via Leydig cells, the production of SHs, namely T (Dym and Raj 1977; McLachlan et al. 2002) and hence seems to be crucial to a normal spermatogenesis (Figure 4) (Zhang et al. 2004). Pakarainen and colaborators (2005) have described that in luteinizing hormone receptor knock-out (LHRKO) mice, spermatogenesis is arrested at round spermatids, adult-type Leydig cells are absent, and T production is dramatically decreased; although T treatments in hypogonadal LHRKO male mice restored spermatogenesis, and fertility.

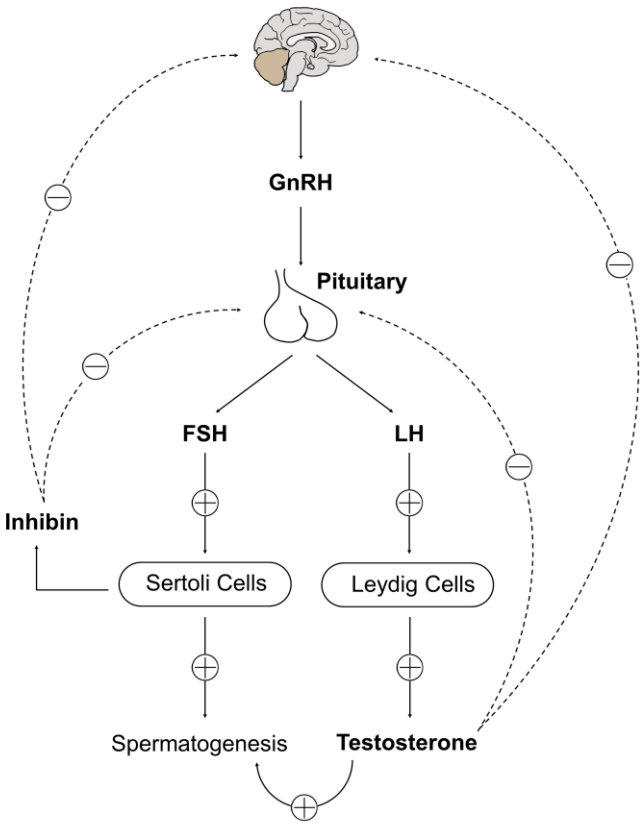

Figure 4: Simplified diagram of the hypothalamus-pituitary-testis axis control of spermatogenesis. The two pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are responsible for the connection between the brain and the testis. The production of inhibin by Sertoli Cells (SCs) and testosterone (T) by Leydig cells provide a negative feedback control that results in reduction of gonadotrophin-releasing hormone (GnRH) production in the hypothalamus and reduce LH and FSH production on pituitary, thus maintaining the homeostasis of FSH, LH, T and inhibin. All these hormones and factors have a tight control on spermatogenesis. Abbreviations: ⊕ -positive feedback; ∅ - negative feedback.

FSH is also a glycoprotein that regulates the development, growth, puberty and the reproductive function (Grigorova et al. 2010). In the testes, FSH receptors are exclusively located on SCs (Figure 4), and this hormone has an important role in the development of the immature testis, acting predominantly on SCs proliferation (McLachlan et al. 2002; Walker and Cheng 2005). Krishnamurthy and collaborators (2000) have described that follicle-stimulating hormone receptor knock-out (FSHRKO) male mice presented a reduction in testis and epididymis weight, smaller seminal vesicles and seminiferous tubular diameter, and a reduction in serum T levels, that most likely accounts for the lowering of the weight of sex accessories. It has also been described that FSHRKO-Sertoli cell androgen receptor knock-out (SCARKO) mice presented reduced total germ cell numbers, and that although those cells entered meiosis, their development stopped at early pachytene (Abel et al. 2008).

It has been demonstrated that the direct effects of FSH are independent of androgens (O'Shaughnessy et al. 2010). FSH acts only during the initial stages of spermatogenesis to optimise germ cell number and cannot stimulate completion of meiosis, which is entirely dependent on androgen action. FSHRKO mice are fertile, although their testis weight is reduced (Abel et al. 2000), showing that the inability to respond to FSH does not impair fertility, even if testicular weight is reduced and if the feedback regulation of pituitary gonadotrophins and intratesticular paracrine connections is disturbed. Used in infertility treatments of aligospermic or teratozzospermic men, FSH increases the number of sperm with normal ultrastructural characteristics (Acosta et al. 1992; Bartoov et al. 1994; Glander and Kratzsch 1997).

1.3 . Sertoli cells and Sex Steroid Hormones

Sex SHs are lipophilic molecules derived from cholesterol and synthesized in the adrenal cortex (glucocorticoids, mineralocorticoids, and adrenal androgens), in the testes (testicular androgens and estrogens), and in the ovary and placenta (estrogens and progestagens or progestins). The bloodstream delivers these hormones, via a carrier protein and due to their lipophilic nature they can cross the cellular membrane freely by simple diffusion (Beato and Klug 2000).

Androgens are known as the male sex hormone and include a number of steroids, such as T, androstenediol and 5-α-dihydrotestosterone (DHT), amongst others. The testes are responsible for 90-95% of the androgens production and the remaining are produced in the adrenal glands (Liang and Liao 1992; Chuu et al. 2011). They have a central role in masculinisation of the reproductive tract, genitalia, and other organ systems during the sexual differentiation process (Hughes 2001; Sultan et al. 2001). During the adulthood, the androgens action on seminiferous tubules has a crucial role for the maintenance of a normal spermatogenesis and fertility. These androgen-related effects are essentially mediated by SCs, although their exact mechanisms remain unclear (Hill et al. 2004; Tan et al. 2005).

The androgen receptors (ARs) are responsible for mediating the action of androgens. They are members of the nuclear receptor super family, encoded by an X chromosomal gene (Lubahn et al. 1988; Quigley et al. 1995). The main circulating androgen is T, that once in the cells may be converted to DHT by 5α-redutase (Grossmann et al. 2001; Le et al. 2006; Thackare et al. 2006) in an irreversible reaction (Thackare et al. 2006). DHT has approximately three times more affinity for ARs than T and has 15-20 times more affinity than adrenal androgens (Liang and Liao 1992; Grossmann et al. 2001; McLachlan et al. 2002; Le et al. 2006).

De Gendt and collaborators (2004) described that the adult male androgen receptor knock-out (ARKO) mice presented a phenotype consistent with complete androgen insensitivity syndrome. When compare with the ARKO males, SCARKO males displayed normal development of epididymis, ductus deferens, coagulating gland, seminal vesicles, and prostate. Morphological analysis of SCARKO testes described apparently normal germ cells that entering meiosis, but as the spermatogenic cycle progresses the efficiency of a normal spermatogenesis was lost. The serum levels of T and LH in SCARKO animals revealed no significant difference, that were reflected in similar organ weights of most androgen target tissues (De Gendt et al. 2004). Nevertheless, Chang and collaborators (2004) have described that SCARKO mice have smaller testis, hypotestosteronemia, and are infertile whit azoospermia, with spermatogenesis arrested at the pre-meiotic diplotene stage. Abel and collaborators (2008) have showed that SCARKO mices presented less germ cells and there was an apparent loss of pachytene spermatocytes, with a clear decrease in the number of secondary spermatocytes and few round spermatids.

Estrogens also play an important role in the development and maintenance of the male reproductive function and fertility (Nilsson et al. 2001; O'Donnell et al. 2001; Carreau et al. 2008). Estrogen biosynthesis is catalysed by a microsomal member of the cytochrome P450 superfamily, the aromatase cytochrome P450 (O'Donnell et al. 2001). The capacity of the mammalian tissues to express aromatase and synthesize estrogens can be observed in the ovaries and testes, the placenta and fetal liver, adipose tissue, chondrocytes and osteoblasts of bone, and the hypothalamus, limbic system, and cerebral cortex (O'Donnell et al. 2001). In rats, aromatase and the capacity to synthesize estrogens is a characteristic of the adipose tissue (Monteiro et al. 2008), the corpus luteum (Doody et al. 1990) and the brain (Lephart 1996). In the testes, aromatase is also expressed, particularly in immature SCs (Fritz et al. 1976; Abney 1999), in Leydig cells of adult male rodents (Valladares and Payne 1979; Abney 1999), in round spermatids, in elongating spermatids and in the late spermatids (Janulis et al. 1996).

Estrogens have two action pathways, the classic genomic pathway of ligand via the interaction of specific estrogen receptors (ERs) with target genes (Kuiper et al. 1997) or by changing the intracellular concentration of calcium or cyclic adenosine monophosphate (cAMP) second messengers (Morley et al. 1992; Aronica and Katzenellenbogen 1993). In the genomic pathway, estrogens actions involve ligand binding to estrogen receptor, dissociation of chaperone complexes and receptor phosphorylation; receptor dimerization; nuclear translocation; DNA binding and interaction with cofactors; and modulation of transcriptional activity (O'Donnell et al. 2001). The estrogens can also rapidly induce increases in the concentration of calcium or cAMP second messengers (Morley et al. 1992; Aronica and Katzenellenbogen 1993) in what seems to be a non genomic mechanism of action (Revelli et al. 1998), apparently via receptors on the plasma membrane. Additionally, physiological

concentrations of estradiol can also induce a rapid release of nitric oxide in endothelial cells via membrane-bound receptors (Caulin-Glaser et al. 1997).

ERs are members of the large ligand-activated nuclear receptor super-family (O'Donnell et al. 2001; Boukari et al. 2007). The classic action of estrogens is mediated by the activation of two specific receptors in target cells, estrogen receptor α (ERα) and estrogen receptor ß (ERß), with highly homologous ligand-inducible transcription factors being responsible for regulation of the expression of specific genes (Boukari et al. 2007). The ERα are expressed in various cellular types of the testicular tissue (Cavaco et al. 2009), namely SCs (Taylor and Al-Azzawi 2000), Leydig cells (Pelletier and El-Alfy 2000; Taylor and Al-Azzawi 2000), spermatocytes (Pentikainen et al. 2000), spermatids (Durkee et al. 1998) and spermatozoa (Durkee et al. 1998; Aquila et al. 2004; Solakidi et al. 2005). The ERß are also expressed in the various cell types of the testicular tissue (Cavaco et al. 2009), namely in SCs (Pelletier and El-Alfy 2000; Saunders et al. 2001; Saunders et al. 2002), Leydig cells (Pelletier and El-Alfy 2000; Saunders et al. 2001), myoid peritubular cells (Saunders et al. 2001), spermatogonia (Makinen et al. 2001; Saunders et al. 2001; Saunders et al. 2002), spermatocytes (Pentikainen et al. 2000; Makinen et al. 2001; Saunders et al. 2002), spermatids (Pentikainen et al. 2000; Makinen et al. 2001; Saunders et al. 2002; Lambard et al. 2004) and spermatozoa (Pentikainen et al. 2000; Aquila et al. 2004; Lambard et al. 2004; Solakidi et al. 2005).

In mice, it has been demonstrated that estrogen receptor knock-out (ERKO) males presented a reduced mating frequency, low sperm numbers, and defective sperm function (Eddy et al. 1996). Animal models with ERKO presented compromised spermatogenesis, steroidogenesis and fertility (Eddy et al. 1996; Dupont et al. 2000; Lazari et al. 2009). Weiss and collaborators (2008) demonstrated that the seminiferous epithelium of ERKO mice was thinner and spermatogenesis was decreased. Lee and collaborators (2000) have described that in estrogen receptor α knock-out (ERαKO) mice the concentration of sperm in the caudal epididymis was reduced, and that those animals had a disruption of spermatogenesis with dilated seminiferous tubules and rete testis. It has also been showed that the testis weight of ERαKO mice was significantly reduced (Gould et al. 2007), and that these animals presented disrupted seminiferous tubules with a partial or complete loss of germ cells, spermatogonia, spermatocytes and spermatids, and also plasma and testicular T concentrations significantly increased; they have described, as well, in estrogen receptor ß knock-out (ERßKO) mice, that the number of Leydig cells and spermatogonia per testis was significantly increased, although the increase of Leydig cells number was not followed by a significant increase in testicular or plasma T concentrations.

2. Sertoli Cells Metabolism

During the development of spermatogenesis, germ cells energetic needs are altered (Brauchi et al. 2005). In the early stages of development the germ cells use glucose as nutrient, which is freely available from the systemic circulation (Riera et al. 2002; Brauchi et al. 2005). In later stages of their development, germ cells loose this ability to metabolize glucose (Boussouar and Benahmed 2004). In fact, whereas spermatogonia use glucose for energy supply, spermatids and spermatocytes are dependent on lactate (Figure 5) (Jutte et al. 1982; Nakamura et al. 1984; Bajpai et al. 1998). However, spermatozoa can use glucose and fructose as their main energy sources (Jutte et al. 1982; Bajpai et al. 1998).

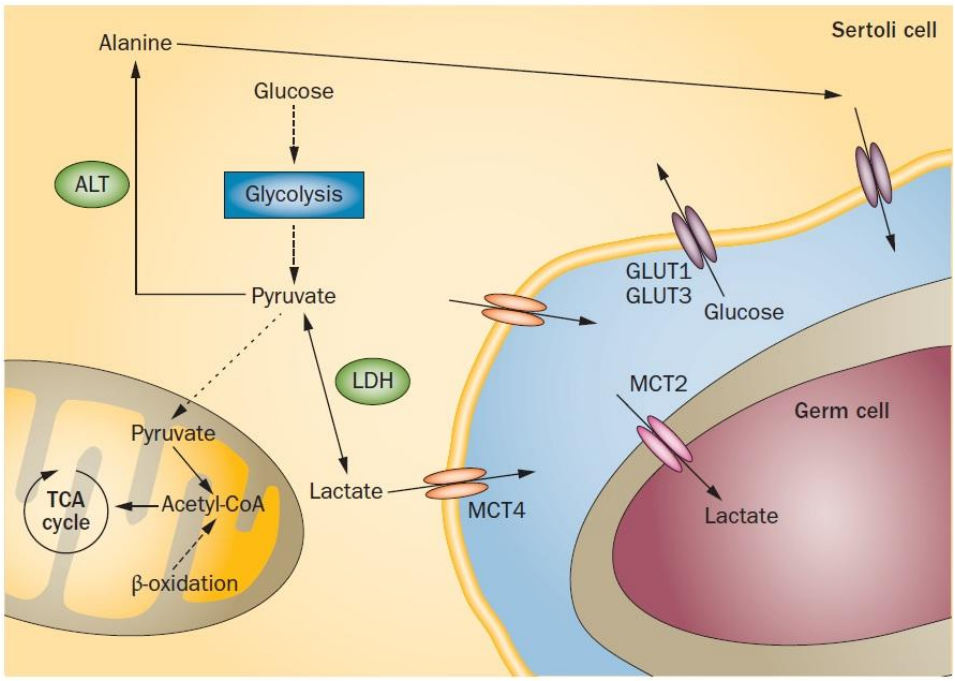

Figure 5: Schematic illustration of Sertoli cell metabolism. Sertoli Cells (SCs) are capable of consuming a variety of fuels including glucose, lactate and fatty acids. SCs preferentially metabolize glucose, the majority of which is converted to lactate. Lactate and pyruvate are transported out of SCs via the family of proton-linked plasma membrane transporters known as MCTs, while glucose is imported via the GLUT family of membrane proteins. Glucose enters the glycolytic pathway, which results in the production of pyruvate, which can be converted into lactate, or alanine, or be transported, to the mitochondrial matrix, where it is oxidized and decarboxylated by pyruvate dehydrogenase, forming acetyl-CoA, which can enter the Krebs cycle. The oxidation of these substrates is coupled with ADP phosphorylation, via the electron transport chain to form ATP. Abbreviations: ALT: Alanine aminotransferase; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3;LDH, Lactate dehydrogenase; TCA, tricarboxylic acid; MCT2, monocarboxylate transporter 2; MCT4, monocarboxylate transporter 4. Adapted from Rato et al. (2012)

SCs, due to their localization and function, have to meet the energy demands of developing germ cells. In SCs, carbohydrate metabolism presents some unique characteristics since the majority of glucose they metabolize is used to produce lactate and not directed for the Krebs cycle (Robinson and Fritz 1981; Grootegoed et al. 1986). Robinson and Fritz (1981) reported that, once in culture, these cells use the majority of glucose to produced lactate, and only a small part (approximately 25%), is used to produce pyruvate for the Krebs cycle (Figure 5) (Grootegoed et al. 1986). Furthermore, in *in vitro* conditions, SCs pentose phosphate pathway is not at a maximum rate, and the rate of oxidative activity is determined by the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation (Robinson and Fritz 1981; Grootegoed et al. 1986). It has also been reported that SCs have the capacity to adapt their cellular metabolism according to the available substrates in order to supply the germ cells with the required lactate as they still produce lactate even in absence of glucose (Riera, Galardo et al. 2009).

The SCs import glucose from the external medium (Hall and Mita 1984), using the glucose transporters (GLUTs) present on their membrane. The GLUTs are a family of structurally related glycoproteins. Until now there have been identified 14 GLUTs isoforms, named as glucose transporter 1 (GLUT1) to glucose transporter 14 (GLUT14) (Manolescu et al. 2007). Presently, GLUT1, glucose transporter 3 (GLUT3) and glucose transporter 8 (GLUT8) isoforms have been identified in SCs (Ulisse et al. 1992; Kokk et al. 2004; Carosa et al. 2005; Galardo et al. 2008; Oliveira et al. 2011; Oliveira et al. 2012). However, GLUT8 is not localized in the plasma membrane, so it is not expected to be involved in glucose transport from the extracellular milieu (Reagan et al. 2001; Piroli et al. 2002). GLUT1 and GLUT3 are present in the plasma membrane and can mediate the incorporation of glucose from the extracellular medium by the SCs (Figure 5) (Galardo et al. 2008; Oliveira et al. 2011; Oliveira et al. 2012).

Once inside the cells, glucose enters in the glycolytic process, and after a series of reactions is transformed into pyruvate, in a process catalysed by multiple enzymes. Phosphofructokinase 1 (PFK1) is a key enzyme in this process and catalyses the irreversible conversion of fructose-6-phosphate to fructose-1-6-bis-phosphate (F-1,6-BP) (Chehtane and Khaled 2010). The energy status levels of the cells is usually related with the regulation of this enzyme making this step the most important control point, and the first rate-limiting step in glucose metabolism (Jutte et al. 1982; Mor et al. 2011). At the end of the glycolytic process, the conversion of pyruvate to lactate in a step catalyzed by lactate dehydrogenase (LDH) isozyme occurs (Figure 5), with the synchronized oxidation/reduction of NADH to NAD^+ (Everse and Kaplan 1973).

The LDH isoenzymes are encoded by a multigene family in the vertebrates. The lactate dehydrogenase A (LDH A) and lactate dehydrogenase B (LDH B) genes code for protein subunits that combine to form five different tetrameric isoenzymes, expressed in several somatic tissues, with specific characteristics correlated with the metabolic conditions

(aerobic and anaerobic) prevailing within the tissue (Markert et al. 1975; Nadal-Ginard 1978). In immature testis, LDH A is the principally expressed isoform thus assuming a crucial role (Blackshaw and Elkington 1970). There is a third functional LDH gene (lactate dehydrogenase C (LDH C)) which encodes the testis-specific isoenzyme lactate dehydrogenase C4 (LDH C4), that seems to be expressed predominantly in differentiating germ cells within the seminiferous epithelium (Goldberg and Hawtrey 1967; Meistrich et al. 1977; Odet et al. 2008), although its expression as also been observed in SCs (Goldberg et al. 2010).

In SCs, after being produced, lactate is exported to germ cells via the cytoplasmic membrane by specific proton/monocarboxylate transporters (MCTs) (Figure 5) (Boussouar and Benahmed 2004), that catalyse the facilitated diffusion of the lactate together with a proton (Halestrap and Price 1999; Brauchi et al. 2005).

The MCT family has a total of 14 members, but only MCTs 1–4 have been identified to function as proton-linked MCTs (Halestrap 2012). The isoforms monocarboxylate transporter 1 (MCT1), monocarboxylate transporter 2 (MCT2) and monocarboxylate transporter 4 (MCT4) are widely expressed in all tissues while monocarboxylate transporter 3 (MCT3) is specifically expressed in the retina (Brauchi et al. 2005). The MCT1 is expressed in the head of the sperm and the epididymis (Garcia et al. 1995) and was also identified in germ cells (Goddard et al. 2003) and in SCs (Galardo et al. 2007). The MCT2 can be found in the tail of spermatozoa in the epididymis (Garcia et al. 1995) and elongated spermatids (Goddard et al. 2003). The MCT4 is expressed in SCs and seems to play an important role in these cells (Bonen 2001; Galardo et al. 2007; Oliveira et al. 2011; Oliveira et al. 2012; Rato et al. 2012), as it has been involved in the export of lactate to the extracellular medium in cells with high glycolytic capacity (Bonen 2001; Bonen et al. 2006; Galardo et al. 2007).

2.1 . Hormonal Regulation of Sertoli Cells Metabolism

Multiple factors that control the lactate production have been described, such as FSH (Mita et al. 1982), epidermal growth factor (EGF), insulin and insulin growth factor-I (IGF-I) (Oonk et al. 1989), paracrine factor P-Mod-S (Mullaney et al. 1994), tri-iodothyronine (Palmero et al. 1995), basic fibroblast growth factor (bFGF) (Schteingart et al. 1999), cytokines (Riera et al. 2002), arachidonic acid (Meroni et al. 2003) and carnitine (Palmero et al. 2000). The cellular energy homeostasis is mediated by the AMP-activated protein kinase (AMPK) that activates the signal transduction pathways influencing the SCs metabolism (Hardie 2003). The AMPK present in SCs, when activated, is responsible for the increase in lactate production, glucose uptake and GLUT1 and MCT4 expression (Galardo et al. 2007). On the other hand, the decrease of glucose levels activates the AMPK leading to an increase of glucose uptake, GLUT1 expression and decrease of GLUT3 expression (Riera et al. 2009). In absence of glucose, the production of lactate is maintained most probably due to aminoacids

or glycogen metabolism (Leiderman and Mancini 1969). It is also known that glutamine and leucine can be energy sources for the SCs (Grootegoed et al. 1986). The SCs can also actively metabolize fatty acids very actively too (Hurtado de Catalfo and de Gomez Dumm 2002).

It has been described that DHT modulates glucose consumption and lactate production and that both DHT as well as 17β-estradiol (E2) decrease messenger ribonucleic acid (mRNA) expression levels of metabolism associated genes, in cultured SCs. In rat (Rato et al. 2012) and human (Oliveira et al. 2011) cultured SCs, glucose consumption was found to be stimulated after DHT treatment. Interestingly, that increase in glucose consumption is not followed by an increase in lactate production thus, it is probably related with a reduced transport of lactate to the extracellular medium, via MCTs, or a decrease of the conversion of pyruvate into lactate catalyzed by LDH A (Rato et al. 2012). In fact, the mRNA levels of MCT4 decrease after DHT treatment, which is concomitant with the lower lactate production and export. The decrease of lactate production by DHT-treated cells can also be a consequence of a lower cellular conversion of pyruvate to lactate catalyzed by LDH A, as the mRNA levels of LDH A were found to be decreased. The role of E2 in rat (Rato et al. 2012) and human (Oliveira et al. 2011) SCs metabolism was also investigated and E2-treated cells produced high amounts of alanine. This fact is remarkable as the appearance of high alanine content can be associated with a reduced redox cytosolic state (Oliveira et al. 2011; Rato et al. 2012).

Recently, Oliveira and collaborators (2012) have described, for human SCs-enriched primary cultures, that insulin deprivation markedly modulated glucose consumption, lactate secretion and the expression of metabolism-associated genes involved in lactate production and export. Those authors reported that in human SCs cultured *in vitro* the first hours of insulin-deprivation are critical (Oliveira et al. 2012). Noteworthy, an adaptation on the glucose uptake was reported in these insulin-deprived SCs, that differentially modulated the expression of GLUT1 and GLUT3 (Oliveira et al. 2012), and also presented a significant decrease in the mRNA levels of MCT4 and LDH A, suggesting that lactate interconversion from pyruvate and the export of lactate are modulated by insulin. That hypothesis was concomitant with the lower lactate concentration detected in extracellular media of insulin-deprived SCs.

II. Aim of the Project

In this project, we aimed to disclose the SCs' metabolic pathways modulation associated to sex hormone levels. Under this perspective, we evaluated mRNA and protein expression of membrane metabolite transporters (GLUTs, MCTs) and metabolic enzymes (LDH for lactate production and PFK-1 for glycolysis), in rat SCs, in order to identify changes associated with the levels of androgens (DHT) and oestrogens (E2).

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, E2, DHT, Bovine Serum Albumin (BSA), ExtrAvidin-Peroxidase Staining Kit, 3,3' Diaminobenzidine Hydrochloride (DAB), trypsin-EDTA, Insulin-Transferrin-Sodium Selenite supplement (ITS supplement), TRI reagent and other drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (FBS) was obtained from Biochrom AG (Germany). Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and random hexamer primers were obtained from Invitrogen (CA, USA). dNTPs were obtained from GE Healthcare (Buckinghamshire, UK). 1x Buffer and Taq DNA Polymerase were obtained from Fermentas Life Sciences (Ontario, Canada).The LDH C, GLUT1, GLU3, PFK-1 primers were obtained from STABVIDA, Oeiras, Portugal. Tween 20 was obtained Applichem, Darmstadt, Germany. Dried milk was Regilait, France. Polyclonal antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany), Millipore (Temecula, USA), Sigma-Aldrich (Roedermark, Germany) and Abcam (Cambridge).

2. Primary Cultures of Rat SCs

Male Wistar rats (20-day-old) were sacrificed by cervical dislocation, the testis were immediately excised in aseptic conditions and washed two times in a 50 mL conical tube in 30 mL 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 testis were decapsulated in HBSS, and the loosen tissue was washed three times in HBSS. SCs were isolated by slight modifications of the method previously described by Oliveira and collaborators (2012), which consists in two types of treatment: mechanic and enzymatic. Briefly, to remove contaminating peritubular cells, the tissue from decapsulated testes were 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 during 10 minutes at room temperature. To "unravel"

the tubules and further release the interstitial tissue/cells, the dispersed tubules were forced to pass several times through a large-pore Pasteur pipette. The tubular pellet was then digested for 15-20 minutes at room temperature with 0,225 mg/ml collagenase type I and 0,05 mg/mL DNase in HBSS. After the collagen 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: F12 Ham, 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.

3. Hormonal Treatments

When the SCs cultures presented a 90-95% confluence, culture medium was replaced by serum-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 E2 or 100 nM DHT prepared in 0,025% ethanol (EtOH). Sex steroid hormone concentrations were 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 (Setchell et al. 1983; Turner et al. 1984; Hess 2000; Jarow and Zirkin 2005; Roth et al. 2010). Control group was treated with 0,025% EtOH. Treatments were performed during 50 hours in an atmosphere of 5% CO_2, 95% O_2 at 33ºC.

4. RNA Extraction

SCs were detached from the culture flasks using a trypsin-EDTA solution. To remove residual trypsin, detached cells were washed with 3 mL of phosphate buffered saline (PBS),

by centrifugations 5 minutes at 3000.*g*. Total ribonucleic acid (RNAt) was extracted from isolated SCs with TRI reagent, following the manufacturer's instructions. Briefly, after the last centrifugation, the cellular pellet was homogenised with 500 µL of TRI for lysing the cells and dissociating the nucleoprotein complexes. The samples were left to stand for 5 minutes at room temperature and then 100 µL of chloroform were added for phase separation, to ensure a complete dissociation of nucleoproteins complexes. The samples were shaken vigorously for 15 seconds, left to stand for 5 minutes at room temperature and centrifuged at 12000.*g* for 15 minutes at 4ºC, to separate the mixture into 3 phases: a colorless upper aqueous phase (containing RNAt), an interphase (containing deoxyribonucleic acid (DNA)) and a red organic phase (containing proteins). To isolate RNAt, the aqueous phase was transferred to a fresh tube, 250 µL of 2-propanol were added to precipitate ribonucleic acid (RNA), and the mixture was centrifuged at 12000.*g* for 10 minutes at 4ºC. The supernatant was discarded and the RNA pellet was washed with 500 µL of 75% EtOH at -20ºC. This mixture was centrifuged at 7500.*g* for 5 minutes at 4ºC. This washing step was repeated once more. The supernatant was discarded and the RNA pellet was air-dried for 5-10 minutes. The RNA pellet was dissolved in 10 µL DEPC-H_2O. RNAt concentration and absorbance ratio (A260/A280) were spectrophotometry determined (Nanophotometer^{TM}, Implen, Germany).

5. RT-PCR

The RNAt obtained was reversely transcribed in a mixture containing 0,5 mM of each dNTP, 250 ng of random hexamer primers, 1 µg of RNAt and H_2O sterile until a volume of 20 µl, and incubated 5 minutes to 65ºC. Then, 200 U of M-MLV RT and Reaction Buffer were added and incubated 60 minutes at 37ºC. The resulting complementary deoxyribonucleic acid (cDNA) was used with exon-exon spanning primer sets designed to amplify LDH C, GLUT1, GLU3, PFK-1 cDNA fragments and the housekeeping gene 18S. 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, 1.5 mM of Magnesium Chloride, 10 mM of dNTP, 50 µM of each primers (Table 1), 0.5 U of Taq DNA Polymerase and H_2O sterile was prepared. After preparation of the mixture in PCR tubes, 1 µL of cDNA was added. The conditions of PCR reactions, performed in a thermocycler, were optimized and are described in Table 1. PCR products were visualized in ethidium bromide stained 1% agarose gel electrophoresis at 120V for 30 minutes. The gels were charged with total of 11 µL (10µL of sample and 1µL of loading buffer). Finally, 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 densities for each band were obtained with BIO-PROFIL Bio-1D Software from Quantity One, according to standard methods.

Table 1: Oligonucleotides and Cycling Conditions for PCR Amplification of Glucose transporter 1 (GLUT1), Glucose transporter 3 (GLUT3), Phosphofructokinase 1 (PFK1) and Lactate Dehydrogenase C (LHD C) and 18S.

Gene	Sequence (5'- 3')	AT (°C)	Amplificon Size (bp)	C
LDH C AN: NM_017266.2	Sense: ATG TGG GCA TGG CGT GTG CC Antisense: CCC AGC CAT GGC AGC TCG AA	65	477	40
GLUT1 AN: NM_138827.1	Sense: TCC GGC GGG AGA CGC ATA GT Antisense: CCC GCA TCA TCT GCC GAC CC	61	842	35
GLUT3 AN: NM_017266.2	Sense: GCG CAG CCC TTC CGT TTT GC Antisense: CCC CTC GAA GGC CCG GGT AA	63	806	35
PFK1 AN: NM_013190.4	Sense: GAG TGC TGA CAA GCG GCG GT Antisense: GTG GCC CAG CAC GGT CAC TC	61	839	35
18 S AN: NR_046237.1	Sense: AAG ACG AAC CAG AGC GAA AG Antisense: GGC GGG TCA TGG GAA TAA	56	149	25

Abbreviations: AT - annealing temperature; C - Number of cycles during exponential phase of amplification; AN – Genbank Accession Number

6. Total Protein Extraction

Once detached from culture flasks, SCs were washed in PBS and centrifuged for 5 minutes at 3000.*g*. Cells were lysed in an appropriate volume (2,5 mL for each culture flask) of Radio-Immunoprecipitation Assay (RIPA) buffer (1x PBS, 1%NP-40, 0,5% sodium deoxycholate, 0,1% SDS, 1 mM PMSF) supplemented with 1% protease inhibitor cocktail, aprotinin and 100 mM sodium orthovanadate. The lysed cells were 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 measured using the Bradford assay.

7. Western Blot

Western Blot procedure was performed as previously described by Alves and collaborators (2011). Briefly, proteins samples (50 µg) 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 rabbit anti-GLUT1 (1:300, Millipore, Temecula, USA, CBL242), or rabbit anti-GLUT3 (1:500, Abcam, Cambridge, MA, ab41525), or rabbit anti-PFK-1 (1:1000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 67028), or rabbit anti-MCT4 (1:1000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 50329), or rabbit anti-LDH (1:10000, Abcam, Cambridge, MA, ab52488). Mouse anti-actin was used as protein loading control (1:1000, Sigma, Roedermark, Germany, A 5441). The immune-reactive proteins were detected separately with goat anti-rabbit IgG-AP (1:5000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 2007) or goat anti-mouse IgG-AP (1:5000, Santa Cruz Biotechnology Heidelberg, Germany, Sc 2008). 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.

8. Statistical Analysis

The statistical significance of protein variation and mRNA expression among the experimental groups was assessed by two-way ANOVA, followed by Bonferroni post-test. 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. E2 decreases mRNA levels of GLUT1, GLUT3 and PFK

To analyse the possible effect of E2 on the expression of glucose metabolism associated enzymes and transporters, SCs were cultured for 50 h in media containing 100 nM of E2 (E2 group), or not (Control group). SCs viability was not altered by culture conditions as evaluated by trypan blue exclusion. The possible effect of E2 on mRNA transcript levels of GLUT1 and GLUT3 was evaluated by a semi-quantitative RT-PCR. The mRNA expression of GLUT1 and GLUT3 in E2-treated cells was significantly lower when compared with the control group (0.53 ± 0.11 and 0.40 ± 0.05 fold reduction, respectively) (Figure 6B). This mRNA decrease in GLUT1 and GLUT3 in E2-treated cells was not followed by a significant decrease in the protein expression levels of these transporters as determined using a western blot analysis (Figure 7B).

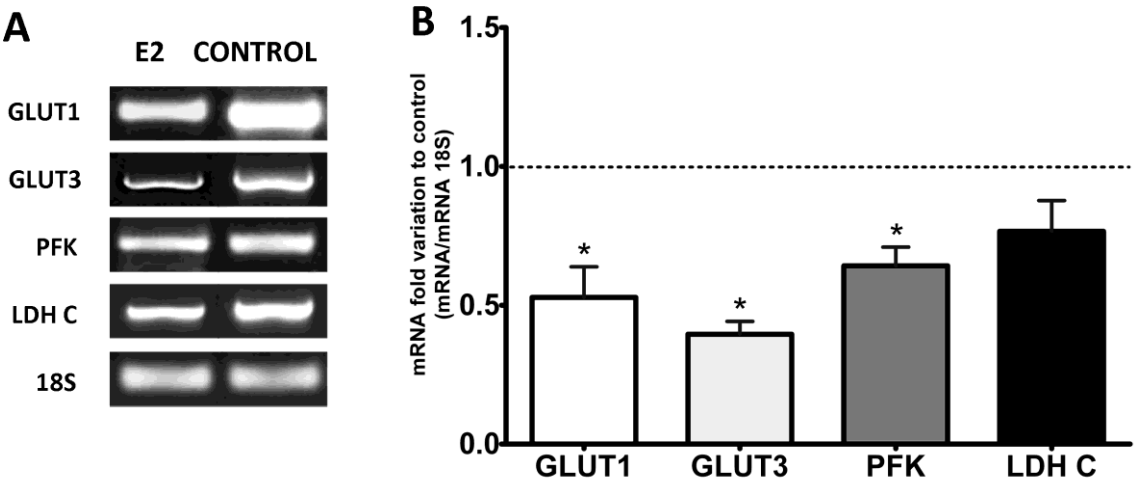

Figure 6: Effect of 17-β-Estradiol (E2) on Glucose transporter 1 (GLUT1), Glucose transporter 3 (GLUT3), Phosphofructokinase (PFK) and Lactate Dehydrogenase C (LHD C) mRNA levels in rat Sertoli cells. Panel A shows a representative agarose gel electrophoresis. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA levels found in cultures with 100 nM E2 when compared with cultures on control condition (dashed line). Results are expressed as means ± SEM (n=5 for each condition). Significantly differently results ($p < 0,05$) are indicated: * relatively to control.

Usually, the first rate-limiting step in glucose metabolism is PFK1 activity thus, we analysed the mRNA and protein levels expression of this enzyme. The mRNA levels of PFK1 were also significantly decreased in E2-treated cells (0.64 ±0.07 fold reduction to control) (Figure 6B). However the protein expression levels did not present significant differences relatively to the control group (Figure 7B). Although it has been reported that E2 has an effect on gene expression levels of LDH A in rat cultured SCs (Rato et al. 2012), we found no

differences regarding LDH C mRNA expression levels relatively to control group (Figure 6B). Also, the protein expression of LDH was not altered by E2 treatment (Figure 7B). It has also been reported that E2 treatment decreases the mRNA transcript levels of MCT4 (Rato et al. 2012). However, the protein levels of MCT4 did not suffers an alteration on in E2-treated SCs when compared to control (Figure 7B).

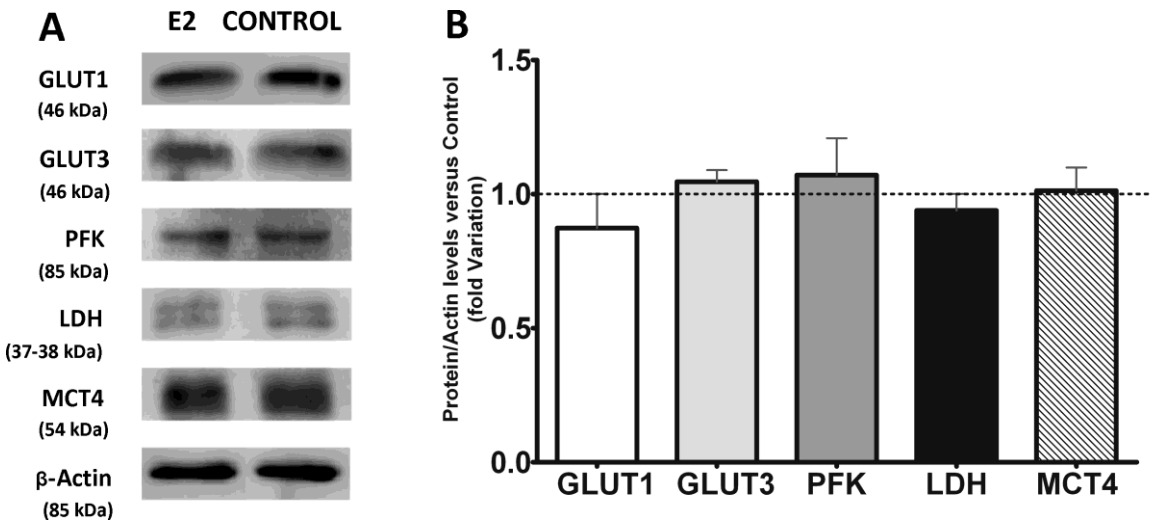

Figure 7: Effect of 17-β-Estradiol (E2) on Glucose transporter 1 (GLUT1), Glucose transporter 3 (GLUT3), Phosphofructokinase (PFK) and Lactate Dehydrogenase (LHD) and Monocarboxylate Transporter 4 (MCT4) protein levels in rat Sertoli cells. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of protein levels found in cultures with 100 nM E2 when compared with cultures on control condition (dashed line). Results are expressed as means ± SEM (n=5 for each condition). Significantly differently results ($p < 0,05$) are indicated: * relatively to control.

2. DHT modulates glucose transporters in cultured Sertoli Cells

One of the major functions of SCs is to produce lactate for the developing germ cells, from glucose. Thus, the effects of DHT on glucose membrane transport proteins in rat cultured SCs were analysed. DHT-treated cells presented a significant decrease on GLUT3 and GLUT1 mRNA expression levels, 0.31 ± 0.02 fold and 0.44 ± 0.12 fold relatively to control, respectively (Figure 8B). Following the noted decrease on these glucose transporters mRNA levels, we also investigated the possibility of an alteration on the protein levels. We found that only GLUT1 presented a significant decrease on protein expression, 0.52 ± 0.05 fold

variation relatively to control (Figure 9B), while GLUT3 protein expression levels remained unchanged.

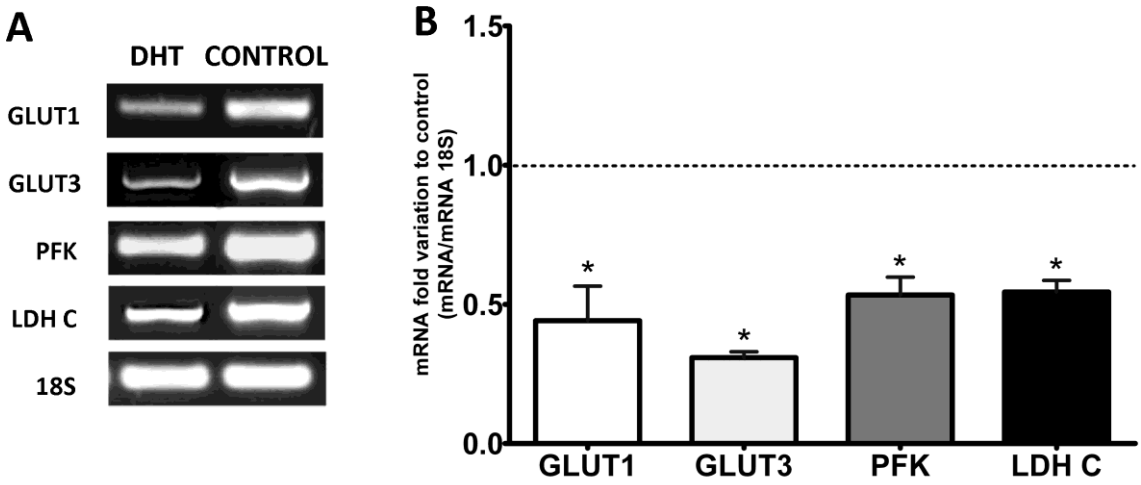

Figure 8: Effect of 5-α-Dihydrotestosterone (DHT) on Glucose transporter 1 (GLUT1), Glucose transporter 3 (GLUT3), Phosphofructokinase (PFK) and Lactate Dehydrogenase C (LHD C) mRNA levels in rat Sertoli cells. Panel A shows a representative agarose gel electrophoresis. Panel B shows pooled data of independent experiments, indicating the fold variation of mRNA levels found in cultures with 100 nM DHT when compared with cultures on control condition (dashed line). Results are expressed as means ± SEM (n=5 for each condition). Significantly differently results ($p < 0,05$) are indicated: * relatively to control.

3. DHT decreases mRNA levels of PFK and LDH C

DHT-treated cells presented a significant decrease on PFK1 mRNA transcript levels (0.53 ± 0.06 fold when compared with the control) (Figure 8B), but the protein levels of PFK-1 were not significantly altered (Figure 9B). Also, as previously reported for LDH A (Rato et al. 2012), DHT-treated cells presented significantly decreased gene transcript levels of LDH C (0.54 ± 0.04 fold) when compared to cells in control conditions (Figure 8B). Nevertheless, the protein levels of LDH remained unchanged after the 50h treatment with DHT (Figure 9B). It has been previously reported that DHT-treated cells presented a decrease in MCT4 gene transcript levels (Rato et al. 2012) however, in the present study, MCT4 protein levels were not affected by DHT treatment (Figure 9B).

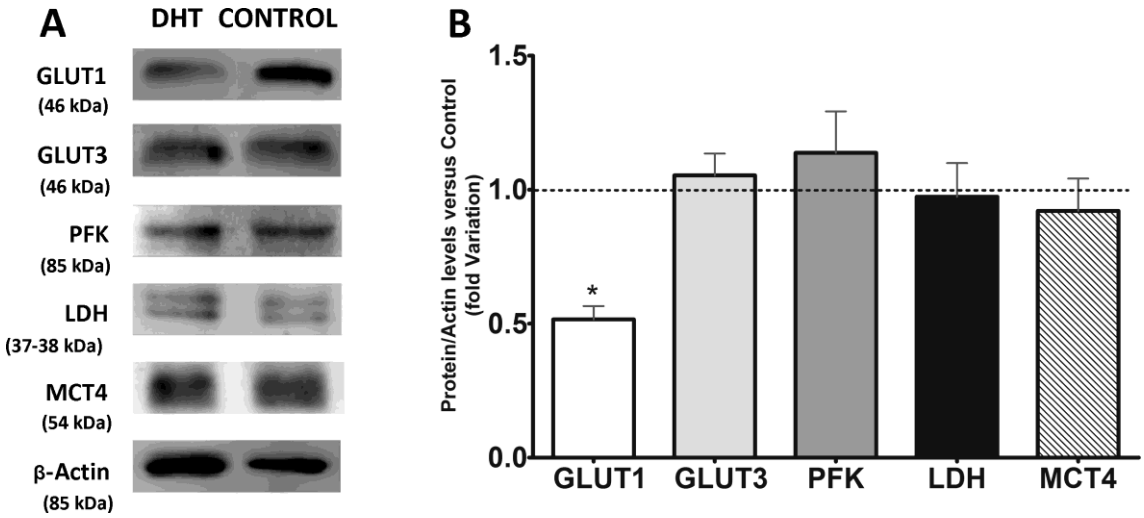

Figure 9: Effect of 17-ß-Estradiol (E2) on Glucose transporter 1 (GLUT1), Glucose transporter 3 (GLUT3), Phosphofructokinase (PFK-1) and Lactate Dehydrogenase (LHD) and Monocarboxylate Transporter 4 (MCT4) protein levels in rat Sertoli cells. Panel A shows a representative western blot experiment. Panel B shows pooled data of independent experiments, indicating the fold variation of protein levels found in cultures with 100 nM DHT when compared with cultures on control condition (dashed line). Results are expressed as means ± SEM (n=5 for each condition). Significantly differently results ($p < 0,05$) are indicated: * relatively to control.

V. Discussion

In cultured cells, glucose is one of the most reliable substrates for ATP production and cell maintenance. Carbohydrate metabolism in SCs has been under debate since the 80's (Robinson and Fritz 1981; Grootegoed et al. 1986) because these cells present some unique characteristics in these processes and germ cells depend upon lactate production by SCs (Griswold 1998). In fact, it has been reported that SCs can adapt their metabolism in order to ensure a satisfactory lactate concentration in the microenvironment where germ cells develop (Riera et al. 2009; Oliveira et al. 2012). Thus, changes in SCs carbohydrate metabolism may result in a compromised spermatogenesis. Recently, our group have focused our research on the hormonal control of SCs metabolism and the possible mechanisms behind the hormonal-related effects (Oliveira et al. 2011; Oliveira et al. 2012; Rato et al. 2012). It was reported that E2 and DHT are key modulators of in vitro rat (Rato et al. 2012) and human (Oliveira et al. 2011) cultured SCs. In both cases, lactate production, which is the preferred energy substrate for spermatocytes and spermatids (Jutte et al. 1981; Mita and Hall 1982), was severely affected by hormonal treatment. Nevertheless, the mechanisms of glucose transport and glucose metabolism remained undisclosed. In this work, was observed that mRNA transcript levels of GLUT1 and GLUT3 are under strict hormonal control. This is concomitant with previous works in human SCs where insulin regulates both transporters (Oliveira et al. 2012) and DHT-treated cells decreased GLUT3 mRNA transcript levels (Oliveira et al. 2011). However, only DHT-treated cells presented a significant decrease in GLUT1 protein levels. It has been reported that DHT stimulates glucose overall consumption (Rato et al. 2012) and thus could hypothesized that GLUTs mRNA and protein expression should be increased. Nevertheless, it was also reported that glucose consumption rate remains high until 35th hour of culture but then significantly decreases until the 50th hour culture hours (Rato et al. 2012). Others (Mahraoui et al. 1994) have reported that mRNA and protein levels of GLUTs are in close relation with glucose consumption rates thus explaining why DHT-treated cells are able to consume high glucose and present a decrease on GLUT1 mRNA and protein levels after 50 hours. It was also reported (Rato et al. 2012) that DHT decreased lactate production in rat SCs cultured under the same experimental conditions as in this study. In fact, DHT-treated cells present less mRNA transcript levels of PFK1, which is one of the most important enzymes in glucose metabolism, being responsible for the conversion of fructose-6-phosphate to F-1,6-BP after glucose enters the cells. Interestingly, the protein expression of PFK1, LDH and MCT4 remained unchanged. This is concomitant with the suggestion made by Rato and collaborators (2012) that DHT can modulate rat SCs metabolism by redirecting their normal functioning, i.e. lactate production, to Krebs cycle. This would compromise spermatogenesis thus explaining why some pathological conditions associated with altered androgen levels, such as the Klinefelter syndrome (Smyth and Bremner 1998), develop subfertility or infertility associated problems. Others (Gupta et al. 1991) have reported an androgen stimulatory effect on the activity of succinate and malate dehydrogenases in castrated estrogen and DHT-treated animals thus suggesting that sex steroids stimulate the activity and expression of enzymes involved in the Krebs cycle and in

the related metabolic pathways. Interestingly and also concomitant with this suggestion is the fact that LDH C, which converts pyruvate into lactate, was only down-regulated by DHT at mRNA level. E2-treated cells presented a significant decrease on MCT4 gene transcript levels but not on LDH C, although it was previously (Rato et al. 2012) showed that LDH A gene transcript levels are decreased under the same conditions. Nevertheless, one cannot disregard that in immature testis, LDH A is the predominantly expressed isoform (Hawtrey and Goldberg 1968; Skidmore and Beebee 1991), and although LDH C is a testis-specific isoform, we expect that LDH A better reflects changes in lactate metabolism in this situation. Indeed, it has been suggested that in cells with high glycolytic activity, such as tumour cells, LDH A could be a therapeutic target as these cells greatly depend upon LDH A activity (Granchi et al. 2010). Furthermore, stimulation of LDH A related activity, rather than LDH C, has also been shown to be a key step in the effect of EGF on lactate production in cultured SCs (Boussouar and Benahmed 1999). Thus, results our group point to a crucial role of LDH A in lactate production by immature SCs rather than LDH C. The present study show that both E2 and DHT decreased the levels of mRNA of glycolysis related key enzymes and glucose transporters in cultured immature SCs, although the protein levels did not always reflect the changes on mRNA transcript levels. 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 et al. 2002). Thus, the possibility exists that, in rat SCs, E2 and DHT 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.

In conclusion, although SCs primary cultures may not exactly represent an in vivo situation, they allow further knowledge on the functioning of these cells metabolism, which are crucial for the developing germ cells and thus for spermatogenesis. This work increases the knowledge about sex hormones metabolic control over SCs and the mechanisms by which they can exert such modulation, with direct influence over spermatogenesis and male fertility.

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