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Obesogens and Male Fertility: a Threat to Sertoli Cell Function?

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Resumo

Nas últimas décadas, diversos estudos têm evidenciado uma correlação inversa entre o aumento da esperança de vida e a qualidade espermática dos indivíduos residentes nos países desenvolvidos. Embora a etiologia desta tendência na fertilidade masculina ainda seja um assunto que suscite grande debate, os obesogénicos, compostos ambientais que predispõem para o ganho de peso, têm sido apontados como importantes causadores, sobretudo devido à sua ação enquanto desreguladores endócrinos.

Os obesogénicos podem ser encontrados praticamente em todo o lado, inclusive em dietas altamente calóricas ou no meio ambiente. O tributilestanho surge como o obesogénico modelo, sendo mesmo considerado um dos compostos mais tóxicos alguma vez introduzidos no ecossistema. Este apresenta caraterísticas lipofílicas e revela uma grande afinidade para se acumular em tecidos com elevado teor lipídico, como é o caso dos testículos. Uma vez armazenado nestes órgãos, o tributilestanho pode afetar a fisiologia e o próprio metabolismo testicular, função fulcral para a espermatogénese. A desregulação destas vias metabólicas pode estar na base molecular de efeitos reprodutivos adversos, como é o caso do aumento do *stress* oxidativo testicular ou de defeitos espermáticos.

O adequado desenvolvimento das células germinativas é altamente dependente do suporte nutricional fornecido pelas células de Sertoli, cujo metabolismo revela características particulares. As células de Sertoli metabolizam a maioria da glucose a lactato que, por sua vez, constitui a principal fonte de energia das células germinativas em desenvolvimento. Assim, a regulação do metabolismo glicolítico das células de Sertoli tem um papel central no processo da espermatogénese. Curiosamente, já se evidenciou que estas células são um alvo preferencial para tóxicos ambientais capazes de alterar a sua estrutura e/ou função.

O objetivo deste estudo passou por avaliar o impacto do tributilestanho no metabolismo das células de Sertoli, com um foco particular no metabolismo glicolítico. Para tal, recorreu-se a três concentrações do composto: 0.1 nM, uma dose considerada subtóxica, mas para a qual o ganho de peso e, particularmente, a ativação do heterodímero do recetor de retinoide X/recetor ativado pelo proliferador de peroxissoma γ foram demonstrados; 10 nM, pertencente ao intervalo de concentrações descritas no soro e tecidos de alguns indivíduos; e 1000 nM, uma concentração a partir da qual foram demonstrados efeitos citotóxicos.

Os resultados obtidos demonstraram que a exposição à concentração mais elevada de tributilestanho (1000 nM) induz efeitos citotóxicos severos nas células de Sertoli de rato, reduzindo a sua proliferação para 28%, em comparação com o grupo controlo. Dada a ausência de efeitos citotóxicos nos grupos expostos às restantes concentrações de

tributilestanho, investigaram-se eventuais alterações na expressão de marcadores de células de Sertoli maduras, através da análise da inibina B e do recetor de androgénios. Para as concentrações utilizadas, não se observaram alterações significativas na expressão dos transcritos destes marcadores.

Contudo, em termos metabólicos, ambas as doses revelaram afetar as vias relacionadas com a glicólise e com a produção de lactato. De facto, a via glicolítica foi favorecida nas células de Sertoli expostas a 10 nM de tributilestanho, visto que o aumento do consumo de glucose e piruvato foi acompanhado por um aumento na produção de lactato. No entanto, não se verificou qualquer alteração na expressão dos transportadores de glucose 1, 2 e 3, ao passo que a expressão da enzima lactato desidrogenase se revelou diminuída em relação ao grupo controlo. Adicionalmente, constatou-se também que as células de Sertoli expostas a esta concentração de tributilestanho (10 nM) apresentavam uma maior expressão da isoforma 4 do transportador de monocarboxilatos, o que sugere um contributo para uma exportação de lactato mais elevada. Relativamente às células de Sertoli expostas à concentração mais baixa de tributilestanho (0.1 nM), estas não apresentaram diferenças no consumo da glucose, apesar de ter sido evidenciada uma diminuição da expressão dos transportadores de glucose 1 e 2 neste grupo experimental. De forma idêntica, também o consumo de piruvato foi significativamente inferior em comparação ao grupo de células expostas a 10 nM de tributilestanho. Não se verificaram alterações significativas na produção de lactato, facto que poderá ter resultado da diminuição da expressão da lactato desidrogenase. Paralelamente, verificou-se, em ambos os grupos, uma diminuição significativa dos níveis de alanina, o que favorece um aumento do estado redox citosólico, sujeitando as células a um possível ambiente oxidativo.

Em conclusão, este estudo destacou que o tributilestanho, para além de induzir efeitos citotóxicos significativos nas células de Sertoli de rato quando administrado numa dose elevada, promove diversas alterações numa das principais funções das células de Sertoli diferenciadas, o metabolismo glicolítico, podendo, desta forma, afetar a espermatogénese e consequentemente a fertilidade masculina.

Palavras-chave

Obesogénicos; Tributilestanho; Fertilidade Masculina; Células de Sertoli; Metabolismo Glicolítico.

Resumo Alargado

A chave para a sobrevivência do ser humano reside numa plena função reprodutiva. No entanto, nas últimas décadas, tem-se verificado um declínio na saúde reprodutiva masculina, tornando-se a infertilidade um problema que afeta milhões de casais em todo o mundo. Um terço dos casos de infertilidade têm sido atribuídos a problemas de causa masculina, sendo muitas vezes difícil estabelecer um diagnóstico claro para as anomalias observadas. De facto, é comum o homem apresentar uma saúde reprodutiva aparentemente normal, mas com alterações severas na qualidade e/ou quantidade do esperma, o que dificulta a escolha do tratamento a adotar. Desta forma, torna-se importante um maior compromisso entre a investigação e a clínica, no sentido de se escrutinar a etiologia associada a cada caso de infertilidade masculina.

Nos últimos anos, o crescimento acentuado das taxas de infertilidade masculina tem revelado particular incidência nos países desenvolvidos, onde o aumento da obesidade e de outras doenças metabólicas resultantes de hábitos alimentares erróneos e sedentarismo têm sido apontados como as principais causas. Isto é explicado em parte pela estreita relação entre a regulação metabólica sistémica e o sistema reprodutor masculino. Contudo, veio a perceber-se que o aumento exponencial nos casos de obesidade e infertilidade masculina não pode ser justificado unicamente através de hábitos diários, mas também por uma exposição permanente a fatores ambientais, como é o caso dos obesogénicos. A estes compostos é associada não só uma predisposição para o ganho de peso, mas especialmente uma tendência de atuação como potentes desreguladores endócrinos. Estes revelam-se capazes de: (1) aumentar o número de adipócitos e/ou o armazenamento de tóxicos nos mesmos; (2) alterar a quantidade de calorias armazenadas; e/ou (3) alterar os mecanismos moleculares através dos quais são regulados a saciedade e o apetite. Desta forma, para além de exacerbarem os efeitos dos hábitos diários, os obesogénicos induzem desregulações endócrinas que, por conseguinte, levam a severas disfunções metabólicas.

De entre as várias substâncias que predispõem os indivíduos para o aumento de peso, destaca-se o tributilestanho, o obesogénico modelo, considerado mesmo um dos compostos mais tóxicos alguma vez introduzidos de forma deliberada no ambiente. Inicialmente utilizado apenas como algicida e moluscicida na indústria da navegação, o tributilestanho chega-nos hoje não só através da cadeia alimentar, mas também pelo contacto com pó doméstico e produtos de preservação de madeiras. Efeitos biológicos adversos em diversas espécies têm vindo a ser atribuídos à contaminação por tributilestanho, nomeadamente ao nível do sistema reprodutor. Isto ocorre porque o tributilestanho tem características lipofílicas, acumulando-se facilmente em tecidos com elevado conteúdo lipídico, como é o caso dos testículos.

Nos mamíferos, os testículos são os elementos centrais do sistema reprodutor masculino, estando envolvidos na produção de espermatozóides, que determina a fertilidade masculina. A espermatogénese é o processo de expansão e desenvolvimento das células germinativas e, para que possa ocorrer de forma adequada, requer uma regulação eficaz das células de Sertoli. Estas células somáticas são fundamentais para o suporte da espermatogénese, através da formação da barreira hemato-testicular, que funciona simultaneamente como uma barreira anatómica, imunológica e fisiológica. De facto, para além de constituir um suporte físico para a espermatogénese, esta barreira permite igualmente o desenvolvimento de um ambiente imunológico adequado à ocorrência deste processo. Aliado a isto, as células de Sertoli são igualmente responsáveis por fornecer um apropriado suporte nutricional às células germinativas.

O metabolismo das células de Sertoli revela caraterísticas únicas, já que é através deste que a maioria da glucose produzida é convertida a lactato, a principal fonte de energia utilizada pelas células germinativas em desenvolvimento. Desta forma, a regulação do metabolismo glicolítico das células de Sertoli revela-se central para a espermatogénese e, consequentemente, para a fertilidade masculina. Este suporte metabólico fornecido pelas células de Sertoli pode, no entanto, ser facilmente perturbado por alterações metabólicas que ocorrem ao nível sistémico. Para além disso, estas células já foram identificadas como sendo um alvo preferencial dos tóxicos ambientais, que se revelam capazes de alterar a sua estrutura e/ou função.

Assim, dada a importância do metabolismo da glucose para a fertilidade masculina, o objetivo deste trabalho passou por avaliar o efeito do tributilestanho no metabolismo glicolítico de células de Sertoli obtidas a partir de culturas primárias de ratos de 20 dias. Para isso, testaram-se três concentrações de tributilestanho: 0.1 nM, uma dose que se encontra abaixo dos níveis fisiológicos, mas para a qual o ganho de peso e, particularmente, a ativação do heterodímero do recetor de retinoide X/recetor ativado pelo proliferador de peroxissoma γ já foram demonstrados; 10 nM, uma concentração que se encontra no intervalo dos níveis fisiológicos; e 1000 nM, uma concentração superior aos níveis fisiológicos e, inclusivamente, a partir da qual já foram evidenciados efeitos citotóxicos.

Os resultados obtidos mostraram que a exposição à concentração mais elevada de tributilestanho (1000 nM) induz severos efeitos citotóxicos nas células de Sertoli de rato, reduzindo a sua proliferação para 28%, em comparação com o grupo controlo. Dada a ausência de efeitos citotóxicos nos grupos expostos a concentrações mais baixas de tributilestanho, investigaram-se eventuais alterações na expressão de marcadores de células de Sertoli maduras, através da análise da inibina B e do recetor de androgénios. Para as concentrações utilizadas, não se observaram alterações significativas na expressão dos transcritos destes marcadores.

Contudo, em termos metabólicos, ambas as doses revelaram afetar as vias relacionadas com a glicólise e com a produção de lactato. De facto, a via glicolítica foi favorecida nas células de Sertoli expostas a 10 nM de tributilestanho, visto que o aumento do consumo de glucose e piruvato foi acompanhado por um aumento na produção de lactato e por uma diminuição da produção de alanina. No entanto não se verificou qualquer alteração na expressão dos transportadores de glucose 1, 2 e 3, ao passo que a expressão da enzima lactato desidrogenase estava diminuída em relação ao controlo. Adicionalmente, verificou-se também que as células de Sertoli expostas a esta concentração de tributilestanho (10 nM) apresentavam uma maior expressão da isoforma 4 do transportador de monocarboxilatos em relação ao grupo controlo o que sugere um contributo para uma exportação de lactato mais elevada. Relativamente ao grupo de células de Sertoli expostas à concentração mais baixa de tributilestanho (0.1 nM), estas não apresentaram diferenças significativas no consumo da glucose, apesar de ter sido evidenciada, neste grupo experimental, uma diminuição da expressão dos transportadores de glucose 1 e 2. De forma idêntica, também o consumo de piruvato foi significativamente inferior em comparação com o grupo de células expostas a 10 nM de tributilestanho. Não se verificaram alterações significativas na produção de lactato, facto que poderá ter resultado da diminuição da expressão da lactato desidrogenase. Paralelamente, verificou-se também uma diminuição significativa nos níveis de alanina em ambos os grupos, o que favorece um aumento do estado redox citosólico, sujeitando as células a um possível ambiente oxidativo.

Em conclusão, este estudo mostrou que o tributilestanho, para além de induzir efeitos citotóxicos significativos nas células de Sertoli de rato quando administrado numa dose elevada, também promove diversas alterações numa das principais funções das células de Sertoli diferenciadas, o metabolismo glicolítico, podendo desta forma afetar a espermatogénese e, consequentemente, a fertilidade masculina.

Abstract

In the last decades, several studies evidenced a negative correlation between life expectancy and sperm quality in developed countries. Although the etiology of this trend in male fertility remains a matter of debate, environmental compounds that predispose to weight gain, namely obesogens, are appointed as pivotal contributors due to their action as endocrine disruptors.

Obesogens can be found virtually everywhere, including in high-energy diets or in the surrounding environment. Tributyltin arises as the obesogen model, being considered one of the most toxic compounds ever introduced into the environment. Tributyltin presents lipophilic characteristics and high affinity to accumulate in tissues with high lipid contents, as is the case of testes. Once stored in these organs, tributyltin can affect testicular physiology and metabolism, which are crucial for spermatogenesis. Disruption of these tightly regulated metabolic pathways may be the molecular basis of adverse reproductive outcomes, such as increased oxidative stress or even sperm defects.

The appropriate development of germ cells is highly dependent on the nutritional support provided by Sertoli cells, which metabolism present some unique features. Sertoli cells metabolize glucose, being the majority of it converted to lactate, the main fuel for developing germ cells. Thus, the regulation of Sertoli cell glycolytic metabolism plays a central role on spermatogenesis. Interestingly, these cells were already proven to be a target for environmental toxicants, being these compounds able to alter their structure and/or function.

Herein, we evaluated the impact of tributyltin on Sertoli cell metabolism, with a particular focus on glycolytic metabolism. In order to achieve it, we selected 3 different concentrations of tributyltin: 0.1 nM, a subtoxic dose but to which weigh gain and retinoid X receptor-peroxisome proliferator-activated receptor γ activation were already described; 10 nM, a dose within the range of concentrations reported in serum and tissues of humans; and 1000 nM, a dose from which broad cytotoxic effects have already been evidenced.

Our results evidenced that the exposure to the highest concentration of tributyltin (1000 nM) induce severe cytotoxic effects in rat Sertoli cells, decreasing their proliferation to 28%, when compared with the control group. Since the lower concentrations of tributyltin (10 nM and 0.1 nM) did not induced cytotoxic effects, we investigated possible changes in Sertoli cells maturation markers through the analysis of inhibin B and androgens receptor. However, for the adopted concentrations, significant changes were not observed in the expression of these transcripts.

However, both concentrations (10 and 0.1 nM) revealed to affect glycolysis and lactate production-related events. Indeed, the glycolytic pathway was favored in Sertoli cells exposed to tributyltin 10 nM, since the increased glucose and pyruvate consumption was followed by an increase in lactate production. However, the protein expression of glucose transporters 1, 2 and 3 remain unaltered, while the expression of lactate dehydrogenase was decreased when compared to control. In addition, Sertoli cells exposed to this concentration of tributyltin revealed also an increased expression of monocarboxylate transporter isoform 4 when compared to control, which had probably contributed to a higher lactate export.

Concerning to Sertoli cells exposed to the lowest concentration of tributyltin (0.1 nM), significant changes in glucose consumption were not observed, even though a decreased expression of glucose transporters 1 and 2 was evidenced in this experimental group. Similarly, also the pyruvate consumption was significantly lower when compared with the group of Sertoli cells exposed to tributyltin 10 nM. The absence of significant changes in lactate production may be due to the decreased lactate dehydrogenase expression. Besides, we also observed a significant decrease in alanine levels in both groups of Sertoli cells exposed to tributyltin, which favors a high cytosolic oxidative redox state, predisposing the cells to a possible oxidative environment.

In conclusion, this study showed that tributyltin, in addition to induce significant cytotoxic effects in rat Sertoli cells when administered at a high dose, also promotes several changes in one of the main functions of differentiated Sertoli cells, the glycolytic metabolism. In this regard, tributyltin may affect spermatogenesis and thus male fertility.

Keywords

Obesogens; Tributyltin; Male Fertility; Sertoli Cells; Glycolytic Metabolism.

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Abbreviations

^{1}H-NMR	Proton Nuclear Magnetic Resonance
AMPK	5’ Adenosine Monophosphate-Activated Protein Kinase
AR	Androgen Receptor
ß2MG	ß2-Microglobulin
BMI	Body Mass Index
BTB	Blood-Testis Barrier
CAMs	Cell Adhesion Molecules
cDNA	Complementary Deoxyribonucleic Acid
DMEM:F12	Dulbecco’s Modified Eagle Medium Ham’s Nutrient Mixture F12
dNTPs	Deoxynucleotide Triphosphates
EDTA	Ethylene Diamine Tetra Acetic Acid
EtOH	Ethanol
FSH	Follicle-Stimulating Hormone
GLUT1	Glucose Transporter 1
GLUT2	Glucose Transporter 2
GLUT3	Glucose Transporter 3
GLUT8	Glucose Transporter 8
GLUTs	Glucose Transporters
GnRH	Gonadotropin-Releasing Hormone
HBSS	Hank’s Balanced Salts Solution
HED	High-Energy Diets
HPT	Hypothalamic-Pituitary-Testicular
ITS	Insulin-Transferrin-Sodium Selenite
LCs	Leydig Cells
LDH	Lactate Dehydrogenase
LH	Luteinizing Hormone
MCT4	Monocarboxylate Transporter 4
mRNA	Messenger Ribonucleic Acid
PBS	Phosphate-Buffered Saline
PFK	Phosphofructokinase
PFK1	Phosphofructokinase 1
PPARy	Peroxisome Proliferator-Activated Receptor Gamma
PVDF	Polyvinylidene Difluoride
qPCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
RXR	Retinoid X Receptor
SCs	Sertoli Cells
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SRB	Colorimetric Sulforhodamine B
T	Testosterone
TBT	Tributyltin
WHO	World Health Organization

Introduction

Testicular Anatomy and Histology

The mammalian testes are paired complex organs. They are divided into compartments, called testicular lobules, which are separated by the septum - fibrous inner extensions of the tunica albuginea (Figure 1) [1]. A human testis contains from 250 to 300 lobules, each one enclosing one to four highly coiled seminiferous tubules [2]. Seminiferous tubules are the testicular functional unities [3], containing the Sertoli cells (SCs) and the germ cells in different development stages. Surrounding each seminiferous tubule are contractile myoid cells that promote the movement of mature sperm and testicular fluids through the tubules [2]. The interstitial spaces between the seminiferous tubules contain all the blood and lymphatic vessels essential for the movement of hormones and nutrients into and out of the testes [4]. Besides, in this space we can find nerves, macrophages and also Leydig cells (LCs), which are responsible for the synthesis of sex steroid hormones [4]. The seminiferous tubules converge upon a plexus of channels, the rete testis, from which 15 to 20 ductuli efferents conduct spermatozoa to the epididymis [2].

Testes essentially perform two major functions: synthesis of steroid hormones, primarily testosterone (T), and formation of haploid germ cells, sperm [5]. The main hormonal control system of these functions is the hypothalamic-pituitary-testicular (HPT) axis, since it regulates the spermatogenic process through the interaction between the hypothalamus, pituitary and testes [6]. Hypothalamus releases the gonadotropin-releasing hormone (GnRH) into the hypophyseal-portal circulation, which stimulates gonadotrophic cells of the anterior pituitary to secrete the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [1, 7]. LH acts on the T-producing LCs, while FSH acts on SCs [7, 8]. Nevertheless, a chain of complex local interactions among the various testicular cell types such as germ, Sertoli, peritubular and LCs are involved in spermatogenesis control [7, 9]. Spermatogenesis is the process by which immature germ cells undergo division, differentiation and meiosis to originate spermatozoa. This process occurs in seminiferous tubules, through close association of germ cells with epithelial somatic cells, the SCs. SCs play a central role on the development of functional testes and, consequently, on the expression of a male phenotype [10, 11]. In fact, these cells influence testes formation in the embryo and spermatogenesis in the adult, by regulating the surrounding environment of the developing germ cells [8]. Within the seminiferous tubules, SCs extend from the basement membrane to the lumen, directly interacting with the developing germ cells (Figure 2) [10]. Adjacent SCs form tight junctional complexes, the basis of the formation of the blood–testis barrier (BTB), dividing the seminiferous epithelium into the basal compartment, where spermatogonia and spermatocytes are found, and the apical (or adluminal) compartment, containing different stages of meiotic spermatocytes, round spermatids, elongated spermatids and spermatozoa (Figure 2) [10, 12]. From Enrico Sertoli works, in 1865, came out the

concept that SCs act as “nurse cells” [13] since, in addition to physical support, these cells also provide nutrients and regulatory factors crucial to germ cell sustenance [10, 14].

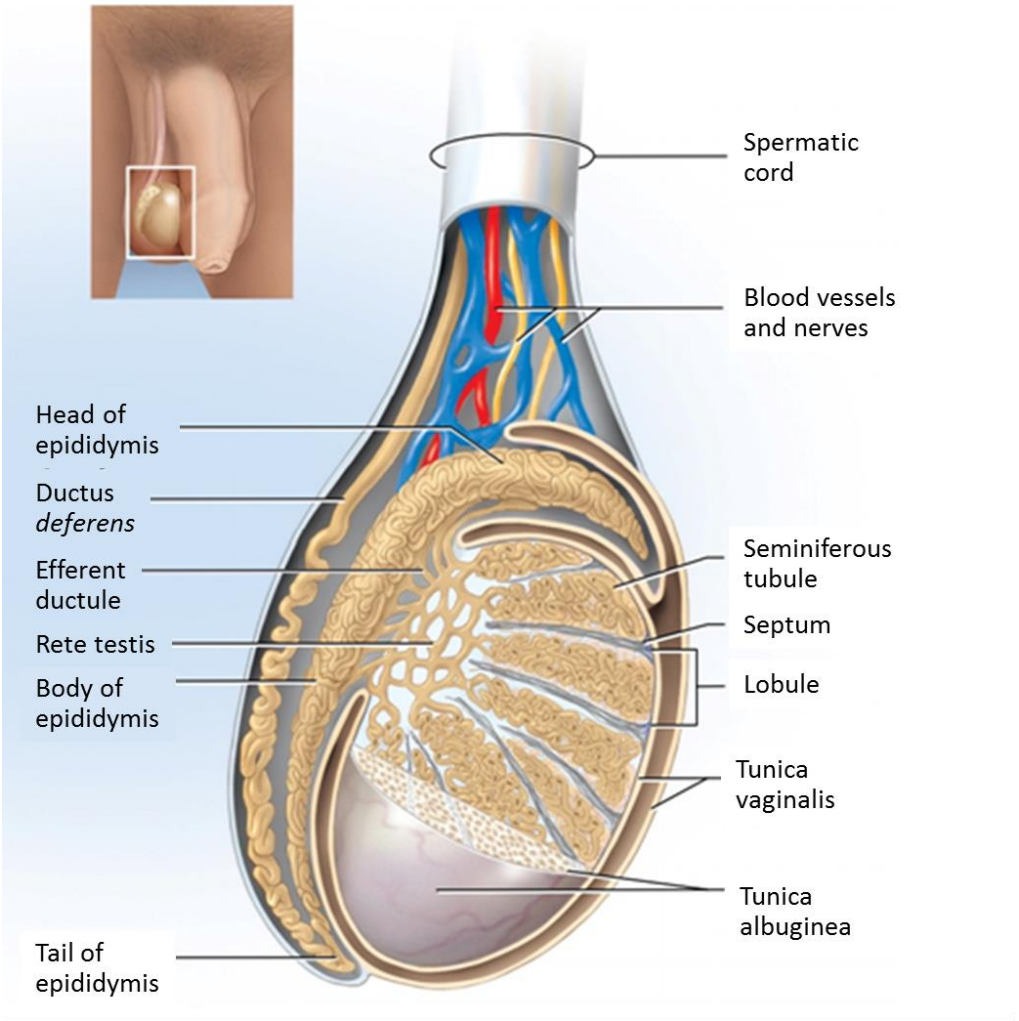

Figure 1: **Schematic representation of the mammalian testis and epididymis**. The testis is encapsulated by two layers: tunica vaginalis (the most outer tunic) and tunica albuginea. Extensions from tunica albuginea (septum) divide testis in lobules where the seminiferous tubules are located. Seminiferous tubules converge to the rete testis that is connected to the efferent ducts. The head of the epididymis is linked to the testis by several efferent ducts. Adapted from [1].

Developing germ cells form intimate associations with SCs and, at a given moment, 30 to 50 germ cells in various stages of development may be in contact with a single Sertoli cell [3]. Cell-to-cell interactions, not only between SCs and specific germ cells but also between adjacent SCs, are essential in the regulation of mammalian spermatogenesis [10, 15]. Indeed, SCs are able to adapt their production/secretion of proteins and factors involved in germ cell development to the changing needs of the germ cell, occurring in a stage-dependent manner [10]. Consequently, well-functioning SCs provide the developing germ cells with the appropriate nutrients, energy sources, hormones, and growth factors as well as protection

from harmful agents and the host's own immune system [16]. Without the physical and metabolic support of the SCs, germ cell differentiation, meiosis and transformation into spermatozoa will be impossible to occur [17, 18].

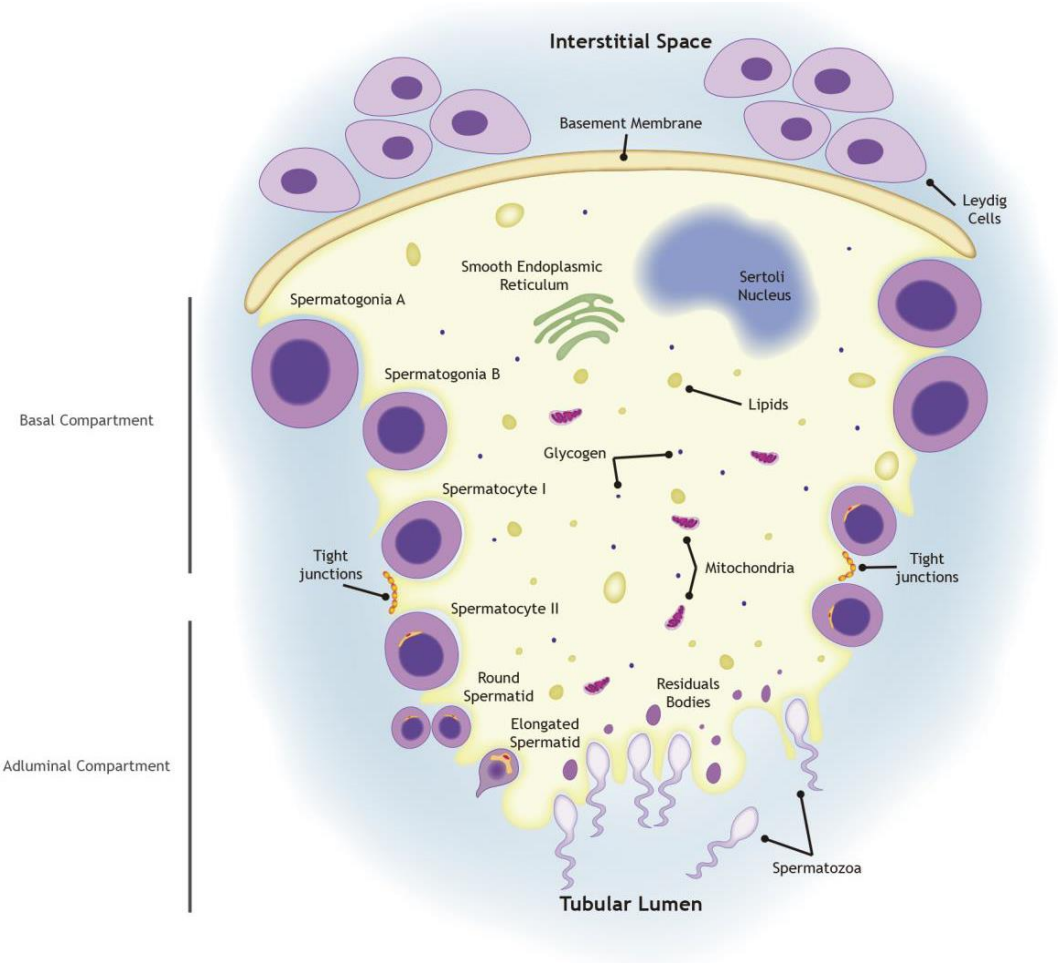

Figure 2: Schematic illustration of the seminiferous tubule, blood-testis-barrier (BTB), spermatogenesis and interstitial tissue. The seminiferous epithelium is composed by Sertoli cells (SCs) and different subtypes of developing germ cells. SCs reside on the basement membrane, under which are the lymphatic endothelium and the peritubular myoid cells. At the interstitial space are located the Leydig cells (LCs), which produce testosterone (T) in the presence of luteinizing hormone (LH). Between adjacent SCs, tight junctions are established, forming the BTB that divides the seminiferous epithelium into basal compartment, where spermatogonia and spermatocytes are found, and into adluminal compartment, containing different stages of meiotic spermatocytes, round spermatids, elongated spermatids and spermatozoa. Adapted from [19].

The Sertoli cell: morphology, function and metabolism

Differentiated SCs are the structural elements of the seminiferous epithelium, playing a main role in the regulation of spermatogenesis. Besides physically supporting spermatogenesis development, SCs also regulate the flow of nutrients, growth factors and other substances to male germ cells, being responsible for determining the spermatozoa produced rates [20].

Within the undifferentiated fetal gonads, SCs are the first cells to differentiate, resulting in the seminiferous cord formation [11]. The germ cells are sequestered inside of these newly formed seminiferous tubules, becoming protected from undergoing meiosis [8]. SCs also influence testis formation in the embryo, since they ensure the regression of the Müllerian ducts *via* secretion of the anti-Müllerian hormone [21]. Such process requires the expression of specific genes on the Y-chromosome [8], namely the *Sry*, the male sex-determining gene expressed by SCs [16].

In mammals, at the time of puberty, SCs suffer a profound alteration on their morphology and function, becoming biochemical and morphologically distinct from the undifferentiated cells. SCs are columnar shaped with a large dimensions volume (from 2000 to 7000 μm^3 in mammals [22]) that allows them to support a vast number of developing germ cells [10]. SCs exhibit prolonged cytoplasmic extensions surrounding germ cells. In most of species, the nucleus of SCs is located at the basal portion of the cytoplasm, presenting large dimensions (up to 850 μm^3) and an irregular shape [23]. Another characteristic of their nucleus is the large nucleolus with a three-partite structure [23]. Ultrastructure images from electron microscopy show that smooth endoplasmic reticulum is abundant on SCs, being associated with the junctional complexes established between SCs and germ cells. This smooth endoplasmic reticulum is organized into reservoirs of lipid droplets, which are involved in the metabolism of lipids or steroids [24].

In addition to creating the adequate microenvironment essential for a suitable development of germ cells into spermatozoa, the BTB also allows the formation of specific intratubular fluid, which is dependent on the function of SCs. In fact, functional BTB consists of three components: (1) an anatomical/physical barrier that prevents the entry of molecules and substances into the adluminal compartment of the seminiferous tubules; (2) an immunological barrier that limits the movement of immune cells of the immune system and regulates the levels of cytokines in the seminiferous epithelium; (3) a physiological barrier, since it contains transporters and channels in the apical and basolateral membranes that are highly dynamic and responsible to the needs of germ cells [25]. Nevertheless, this barrier is “permeable” enough to allow the migration of developing germ cells throughout the seminiferous epithelium, a crucial step for a functional spermatogenesis.

Mammalian spermatogenesis is characterized by continuous cellular differentiations with three main stages: (1) mitotic spermatogonial proliferation and differentiation, (2) meiotic phase and (3) spermiogenesis [26]. This process is highly dependent on SCs, being regulated by the HPT axis. Spermatogonial stem cells, which adhere to the basement membrane where the supporting SCs are also adherent, replicate mitotically not only to guarantee the germ cell line (spermatogonia type A), but also to give rise to spermatogonia type B [26]. Spermatogonia type B will enter meiotic prophase and differentiate into primary spermatocytes (Spermatocyte I). After crossing the BTB, these cells undergo the first division

of meiosis and form the haploid secondary spermatocytes (Spermatocyte II). The second meiotic division differentiates one Spermatocyte II into two equalized round spermatids. Thereafter, cell division stops and spermiogenesis starts to form elongated spermatids, which are finally released into the lumen of the tubule as immature spermatozoa, in a process called spermiation (Figure 2) [26].

Several modifications on SCs structure and function may affect BTB, unbalancing the metabolic cooperation established between these cells and the developing germ cells. Indeed, SCs metabolism and particularly glucose metabolism is pivotal for spermatogenesis and thus for male fertility. Robinson and Fritz [27] showed that cultured SCs are the main source of lactate in the testes, converting the majority of glucose into this metabolite. The rate-limiting step of lactate production is the membrane passage of glucose from the extracellular space, *via* specific glucose transporters (GLUTs) to SCs innerspace [28]. Four GLUTs (GLUT1, GLUT2, GLUT3 and GLUT8) have been identified in SCs to date [29-32]. However, GLUT8 has not been identified in SCs plasmatic membrane, which makes it unexpectable to be involved in the glucose uptake through plasma membrane [33]. Otherwise, GLUT1, GLUT2 and GLUT3 have been identified in the plasmatic membrane of SCs, allowing to assume their role as the primary responsible for glucose import in these cells.

Once glucose enters in SCs cytoplasm, it suffers a series of multi-step reactions catalyzed by several enzymes. The first rate-limiting step in glycolytic metabolism is mediated by phosphofructokinase (PFK) that catalyzes the irreversible conversion of fructose-6-phosphate into fructose-1,6-bis-phosphate [34]. Glucose is then converted to pyruvate and the glycolytic process is completed. The cytosolic pyruvate originated from glycolysis can follow three main distinct paths: (1) it can be converted to alanine by the action of alanine aminotransferase; (2) it can enter the tricarboxylic acid cycle; (3) or it can be converted to lactate by the action of lactate dehydrogenase (LDH). Indeed, LDH has a crucial role in providing lactate to developing germ cells, exporting it from SCs by monocarboxylate transporter isoform 4 (MCT4) (Figure 3) [35]. Additionally, alanine also has an important role, since it can be converted to pyruvate which may be used as a substrate by SCs for several biochemical pathways [36].

Since normal reproductive function is dependent on adequate nutritional state, it is expectable that metabolic disturbs promoted by environmental compounds may affect reproductive function as a result of increased adiposity and/or increased storage of fat-soluble toxicants [37]. Testicular tissue exhibits high lipid content and it easily retains these compounds Furthermore, the epididymal fat, adjacent to testicular tissue, also arises as another source of retention for fat-soluble toxicants, leading to testicular tissue permanently exposed to its effects. Recently, several data reported that the reproductive process is highly sensitive to subtle changes in hormonal levels, especially when induced by environmental toxicants [38]. This is particularly important taking in consideration that these toxicants

easily reach the testicular *milieu* and disrupt whole testicular physiology, compromising male reproductive potential. SCs are particularly susceptible to numerous toxic substances, which are capable of alter the structure and metabolism of these cells [39].

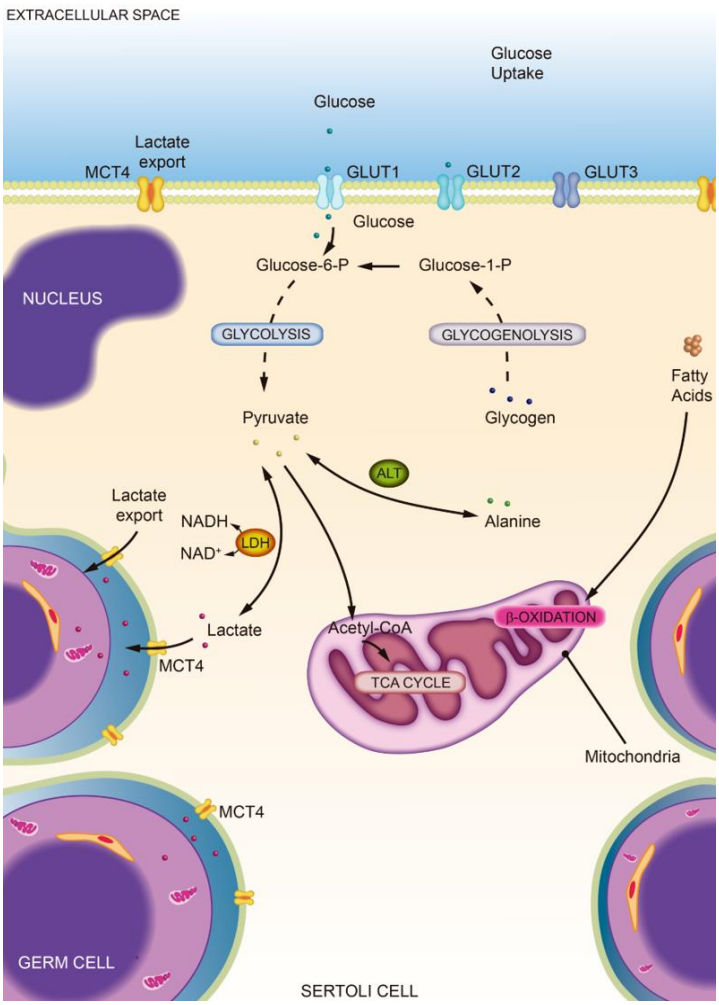

Figure 3: **Schematic illustration of the glycolytic metabolism of Sertoli cells (SCs).** In SCs, glucose from interstitial space is taken through high-affinity glucose transporters (GLUTs), GLUT1, GLUT2 and GLUT3, which are present in the plasmatic membrane. In physiological conditions, the majority of glucose is converted to pyruvate which can follow three distinct paths. It can be converted to alanine by the action of alanine aminotransferase (represented as ALT); it can be converted into acetyl-CoA by the action of pyruvate dehydrogenase; or it can be converted to lactate by the action of lactate dehydrogenase (LDH). Acetyl-CoA enters the mitochondria to be used in the tricarboxylic acid (TCA) cycle, and/or can be converted into acetate. Both acetate and lactate are exported to the intratubular fluid by monocarboxylate transporter isoform 4 (MCT4). These substrates are then taken up by developing germ cells. Adapted from [40].

The obesogen hypothesis

Over the past three decades, obesity epidemics has become a public health concern with the prevalence rates of this disorder reaching 24% of individuals in developed countries [41, 42]. According to the World Health Organization (WHO), in 2014, nearly 2 billion adults were

classified as overweight or obese, conditions characterized by an excessive fat accumulation. Body mass index (BMI), the ratio between weight and the square of the height, is the parameter typically used to evaluate these disorders. Indeed, WHO defines individuals with a BMI greater than or equal to 25 as overweight, and with a BMI greater than or equal to 30 as obese [41]. Obesity is mainly caused by the current lifestyle habits of developed countries, specially the overconsumption of high-energy diets (HED) and decreased physical activity. However, the daily increase on the number of obese people cannot be only explained by lifestyle habits, but also by a permanent exposure of environmental features that may exacerbate their effects, suggesting the environment as "another source" for the development of obesity [43].

The idea that "something" on the environment is able to predispose to weight gain increased the complexity of obesity's etiology, leading to the concept of "obesogens" [44, 45]. The term "obesogen" refers to chemical compounds present either in the environment as in foods, which are able to enhance adipogenesis by increasing the number of fat cells or the storage of fat-soluble toxicants into existing fat cells [37]. Since the increased release of chemical toxicants has been concurrent with the obesity epidemics happening for a few decades ago, cumulative noxious effects resulted from the exposure to these compounds, making the contact with them inevitable [43]. The obesogen hypothesis that has emerged in the recent years proposes that this exposure to obesogens can affect the biochemical pathways that control appetite and/or whole metabolic homeostasis and thus promote the development of obesity. Obesogens can be found virtually everywhere, including in HED or in the surrounding environment. Indeed, there is a wide range of compounds suspected of present obesogenic activity (Table 1). This is a matter of concern because obesity is associated with other comorbidities such as the decline of male reproductive health [46, 47].

Previous evidence had demonstrate a dose-response relationship between BMI and infertility in couples, since even a subtle increase in male weight was already suggested to affect fertility [48]. This may explain, in part, why total fertility rates in developed countries have significantly decreased, reaching the lowest values ever witnessed [49]. Among infertility cases, approximately 30–40% can be attributed to problems with the male partner [50]. Taking into account that today the time interval of men exposed to obesogens is high, this issue deserves special attention from all professionals of the reproductive area to understand how these toxics impact male fertility, especially those that predispose for metabolic disturbances.

One of the first studies demonstrating that obesogens could affect the reproductive capacity through "modulation" of the endocrine system dates from the latest 1960's [51]. Since then, several reports evidenced that the major concern regarding obesogens is based on their capacity to induce endocrine disrupting effects. The storage of obesogens by reproductive

organs results from the high lipid content of these tissues, allowing these compounds to accumulate on testicular lipids and adversely affect whole testicular physiology, specially the formation of germ cells [52]. This happens because most of obesogens are lipophilic and exhibit numerous mechanisms of action, which allow them to disrupt the male reproductive function at either central and/or gonadal levels [53]. Several studies have shown that some obesogens affect testicular metabolism, which is highly dependent on glucose [27, 54-56]. However, it becomes necessary to deepen the knowledge on how these compounds may affect the testicular metabolism, since the disruption of the cooperation between testicular cells can lead to an arrest of spermatogenesis, and therefore compromise male fertility.

Table 1: Summary of the proposed obesogens and their sources of exposure. Legend: 2,4-D - 2,4-dichlorophenoxyacetic; BPA – Bisphenol A; MSG – Monosodium Glutamate; PBDEs - Polybrominated Diphenyl Ethers; PCBs - Polychlorinated biphenyls; PFOA - Perfluorooctanoic acid; PVC - Polyvinyl chloride; TBT – Tributyltin.

Obesogen	Main Sources	Reference
2,4-D	Herbicides	[57]
Benzo[α]pyrene	Residential wood burnings, cigarette smoke, charbroiled food, coal tar and automobile fume emissions	[58]
BPA	Food and drink packaging plastics, medical devices and thermal paper	[59]
Chlorpyrifos	Insecticides	[60]
Diazinon	Insecticides	[61]
Diethylstilbestrol	Cattle feed and medical treatments for breast and prostate cancers	[62]
Fructose	Fruit, vegetables and honey	[63]
Genistein	Soybeans and soy products, fava beans and coffee	[64]
Lead	Water, artificial turf and infant toys	[65]
MSG	Food additives and natural foods such as tomatoes and cheese	[66]
Nicotine	Tobacco, insecticides and nightshade plants	[67]
Parathion	Insecticides and acaricides	[68]
PBDEs	Flame retardant in building materials, electronics, furnishings, plasticizers and textiles	[69]
PCBs	Electric equipment, plasticizers, surface coatings, flame retardants, paints and carbonless copy paper	[70]
PFOA	Crawl and stain repellent on carpets, furniture, waterproof clothing, mattresses and microwavable food items	[71]
Phthalates	Plasticizers, PVC products, infant toys, detergents and personal care products	[72]
TBT	Antifouling paints, wood preservers and stabilizers of plastic products	[73]

Tributyltin as a threat for male fertility

Among the proposed compounds exhibiting obesogenic activity, tributyltin (TBT) is considered the obesogen model, being one of the most toxic substances ever deliberately introduced into the environment [74]. TBT belongs to the organotin family and presents three organic groups covalently bonded to a tin atom (Figure 4) [74]. TBT was discovered to be a particularly potent algaecide and molluscicide, becoming ubiquitous as the active component in marine antifouling ship paints since the mid-1960s [75]. This compound was extremely useful to the shipping industry, since the settlement of aquatic organisms on ships' hulls led to the reduction of their maximum speed and to the upraise of the fuel and maintenance costs [75]. However, the action mechanism of these paints relies on the release of this biocide into the sea, resulting in a widespread environmental contamination of marine ecosystems.

A few years later, studies of adverse biological effects in a wide range of species were associated with TBT contamination of harbors and shipping lanes along European and North American coastlines [76]. As a consequence, severe restrictions have been adopted, including a global ban that entered into force since 2008 [77]. However, present and future restrictions will not immediately remove TBT and its degradation products from the marine environment, since these compounds are retained in the sediments where they persist [78]. In fact, TBT can remain in the ecosystem for tens of decades [79]. Thus, despite its global ban, TBT acts as a long-term source of contamination being its presence in sediments a matter to be aware of.

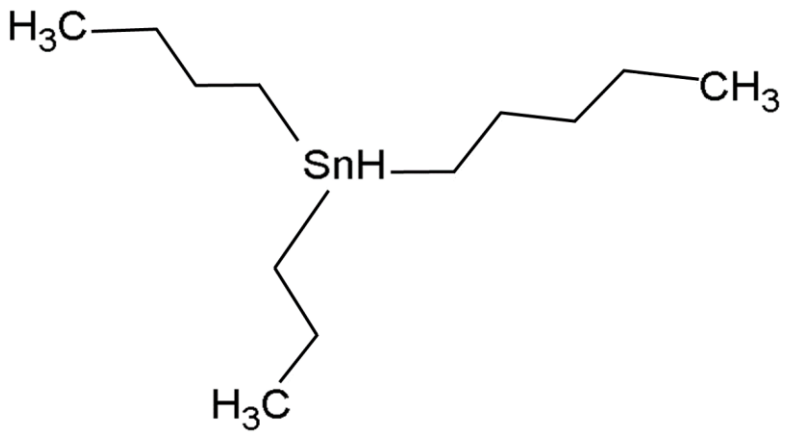

Figure 4: Representative draft of tributyltin (TBT) structure. In the illustration is visible the central tin atom covalently bounded to the three butyl (C_4H_9) radicals. Molecular formula: $C_{12}H_{28}Sn$. Adapted from [80].

TBT enters the human food chain mainly through contaminated marine and freshwater species, but similarly through water consumption, being also present on polyvinyl chloride plastics which are into closer contact to this supplies [74]. Regulatory scientific advisory panels recommend a tolerable daily intake of 270 ng TBT/kg of body weight/day in relation to

human health risk assessments [81]. Moreover, reports from the 1990s evidenced individual dietary sources exposure as high as 375 μg TBT/day in some demographic groups such as Japanese coastal fishing communities [82]. As expected, these levels have decline after the introduction of stricter environmental guidelines. However, TBT can still be found in considerable doses in seafood and vegetable market basket samples [83]. Besides, other "sources" of TBT exposure comes from contaminated household dust (in an estimated range for total organotins between 0.3–28 μg/g) [84] and from wood preservatives [74].

Once in the body, TBT promotes adipogenesis by a covalent bound and posterior activation of the retinoid X receptor (RXR)-peroxisome proliferator-activated receptor gamma (PPARγ) heterodimer, a master regulator of adipogenesis [85]. When activated, this heterodimer stimulates the peroxisome proliferator response element, which promotes the transcription of genes involved in adipogenesis. Thus, RXR-PPARγ controls the differentiation of committed preadipocytes into mature fat cells, maintaining the stability of their differentiated state [86]. Studies using the murine 3T3-L1 preadipocyte cell line model confirm that TBT is an effective promoter of adipocyte differentiation at nanomolar concentrations, which is consistent with its role as RXR–PPARγ ligands agonist [73]. Thus, TBT gives rise to mature adipocytes that are predisposed to acquire lipid droplets [87].

Interestingly, animal studies proposed that the increase in adiposity due to a TBT exposure is highest in males [73], suggesting that the effects of this obesogen may be gender-dependent. The first reports of the adverse reproductive effects induced by TBT came from marine gastropods, evidencing a dramatic rise in the incidence of imposex [88]. This phenomenon is one of the best documented examples of endocrine disruption in wildlife, being characterized by the masculinization process of hermaphroditic mollusks and associated with a reduction in fecundity [89]. Indeed, imposex was already reported in several TBT-exposed organisms, including bivalves [90] and fish [91] being in the majority of cases related to shell malformations (when applied) and mainly to sterility. Although TBT has been one of the best examples of imposex-inducer in a wide range of marine species [78], the mechanism underlying its action is yet to be fully elucidated. In this regard, some data points the abnormal modulation of the RXR as the main contributor [92], since a significant increase in the transcription of RXR gene was observed at advanced stages of imposex [92].

Even though the effects of TBT were firstly observed in the marine ecosystem, those findings led also to concerns on human health through the consumption of contaminated seafood. Indeed, food chain accumulation and bioaccumulation of TBT is of prime concern for mammals. In this regard, mammal gonadal depots are appointed as the largest contributors to the increased adipose mass and weight gain in TBT-exposed animals, making the impact of this obesogen on the reproductive potential a subject of great concern. Once stored in these

organs, TBT can affect testicular physiology and metabolism, which are crucial for spermatogenesis.

Several studies concerning toxics effects of TBT at testicular levels are based in the damages that this compound induce in testicular cells. The presence of PPARγ and RXR in mouse SCs was already described [93], suggesting that these cells may be a target for TBT. Indeed, TBT was recently reported to primarily affect SCs, which may lead to germ cell damage [39]. Besides, the cytotoxic effects of TBT on LCs isolated from 28 days-old Wistar rats was also assessed [94]. Data evidenced that an *in vitro* exposure in the range of 300–3000 nM of TBT reduced cell viability and affected both T production and redox balance on these cells. TBT also induces oxidative damage and cell death on Sertoli-germ cells co-culture from male Wistar rats [39]. The deleterious effects of TBT on male fertility were already observed in germ cells. Indeed, Si and collaborators [95] reported recently that Chinese Kun Ming mice exposed to 1, 10 and 100 μg of TBT/kg during postnatal period present a dose-dependent decrease on sperm count and motility, suggesting that even a perinatal TBT-exposure may cause long lasting alterations in male reproductive system. In addition, TBT has also been associated with testicular germ cell apoptosis, since apoptotic cells were found in the seminiferous tubules of 21-days old ICR mice after a 3-days oral administration of 25, 50 or 100 mg TBT/kg/day, when compared with the control [96].

The most recent evidences have suggested that cytotoxicity of TBT goes far beyond the structural damages in testicular cells [97]. Zuo and collaborators [98] observed that mice orally administrated with different concentrations of TBT (0.5, 5 and 50 μg/Kg), once every 3 days and during 60 days, elevated blood glucose levels. Despite those authors did not scrutinize the molecular mechanisms that lead to disruption of glucose metabolism, it is suggested that it may happen via GLUTs. According to Yamada and collaborators [97], TBT, at nanomolar levels, was enough to inhibit glucose uptake in human pluripotent embryonic carcinoma cell line. Those authors proposed that TBT reduced the cell surface-bound GLUT1. This decrease may be associated with reduced 5' adenosine monophosphate-activated protein kinase (AMPK), which is involved in the translocation of GLUT1 for membrane surface and thus in an enhanced glucose uptake. Similarly to glucose metabolism, it was also reported that TBT elevated insulin levels, which generally indicates the occurrence of insulin resistance. These facts are of great physiologic relevance, since unbalanced glucose homeostasis and insulin resistance are two conditions that affect the metabolism of SCs [99, 100]. Thus it is possible that TBT may arise as a "candidate" to affect Sertoli cell metabolism (particularly glucose metabolism) with subsequent consequences for male reproductive health.

Aims of the project

The metabolic cooperation established between testicular cells is a complex event and depends on the correct functioning of several metabolic pathways. All these events are affected by a myriad of elements that include environmental factors, such as obesogens. Testicular metabolism and particularly Sertoli cell metabolism, plays a crucial role on the normal occurrence of spermatogenesis. Recent advances have emphasized that the exposure to obesogens is an important contributor to the decline of male reproductive health. Thus, the increased evidences highlighting the dependence of germ cells on the appropriate metabolism of SCs and also that this metabolism arises as a target for obesogens, led us to develop this project.

The general aim of the research described in the present work was to disclose the association between TBT and male infertility, dissecting the toxic effects of this obesogen on Sertoli cell metabolism (particularly glucose metabolism) and the subsequent consequences for male reproductive health.

To achieve it, we firstly aimed to evaluate TBT effects on SCs survival and proliferation through a proliferation test. Then, we measured the messenger ribonucleic acid (mRNA) expression of the SCs markers androgen receptor (AR) and inhibin B, on the rat SCs exposed to non-cytotoxic concentrations of TBT. Besides, we also aimed to evaluate the consumption of specific substrates (glucose and pyruvate) by rat SCs, under the same conditions. We also analyzed how the exposure to this obesogen affected the expression of GLUTS 1, 2 and 3 and enzyme phosphofructokinase 1 (PFK1). To further disclose its effects, the lactate and alanine production was measured, as well as the respective expression and enzymatic activity of LDH and the expression of MCT4.

Materials and methods

Chemicals

Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM:F12) and Gentamicin were obtained from Biochrom GmbH (Berlin, Germany), while Insulin-Transferrin-Sodium Selenite (ITS) supplement, SuperSinal West Pico Chemiluminescent Substrate, Tween 20 and Maxima™ SYBR Green/Fluorescein Polymerase Chain Reaction (qPCR) Master Mix were obtained from Thermo Fisher Scientific (Waltham, USA). Bradford Reagent was obtained from Bio-Rad (Hercules, USA), TripleXtractor reagent was obtained from GRiSP (Portugal) and First-Strand cDNA Synthesis Kit plus Taq 2× Green Master Mix were obtained from NZYtech (Portugal). Polyclonal antibodies (GLUT1, GLUT2, GLUT3, MCT4 and PFK1) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany), while monoclonal antibodies (LDH) were obtained from Abcam Plc (Cambridge, UK). All other chemicals were purchased from Sigma-Aldrich (Roedermark, Germany).

Animals

Twenty-four male Wistar rats (*Rattus norvegicus*) 20-days old from Charles River Laboratories (Barcelona, Spain) were used in the present study. Animals were housed in accredited animal colony (Health Sciences Research Center, University of Beira Interior) and maintained with food and water *ad libitum* in a constant room temperature (20 ± 2ºC) on a 12-hours cycle of artificial lighting. All animal experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European directives for the care and handling of laboratory animals (Directive 2010/63/EU). In accordance with the Portuguese law (Ordinance no. 1005/92 of 23 October), the research team requested a permission to perform this animal experimentation study to the Portuguese "Direcção Geral de Veterinária" (Portuguese Veterinarian and Food Department).

Rat Sertoli Cell Primary Culture

Animals were anesthetized and sacrificed by cervical dislocation. Testes were immediately excised in aseptic conditions and washed in ice cold Hank's Balanced Salts Solution (HBSS) Ca^{2+}/Mg^{2+} free, containing 10000 U/mL of penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B (pH 7.4). Testes were decapsulated and the loosen tissue was washed in the same solution.

SCs were isolated by a method previously described by Oliveira and collaborators [35] with slight modifications. Briefly, the tissue from decapsulated testes was dispersed in a Petri dish containing glycine solution (HBSS plus 1 M glycine and 2 mM etilene diamine tetra acetic acid (EDTA); 0,002% (w/v) Soybean Trypsin Inhibitor; pH 7.2). The tubules were dispersed again in a conical tube containing the same solution plus DNAse (250 U/mL) during 10 minutes at room

temperature. The dispersed tubules were forced through a large-pore Pasteur pipette to uncoil them and further release the interstitial tissue/cells. The tubular pellet was then digested for 15-20 minutes at room temperature in HBSS containing collagenase type I and DNAse (250 U/mL). After digestion, the disaggregated seminiferous tubules were washed three times in HBSS by centrifuging the tubules suspension for 3 minutes at 300x*g*. The suspension was collected and resuspended in culture medium (1:1 mixture of DMEM:F12 supplemented with 10% fetal bovine serum, 15 mM HEPES, 50 U/mL penicillin, 50 µg/mL streptomycin, 0.5 µg/mL fungizone and 50 µg/mL gentamicin; pH 7.4). In order to disaggregate large SCs clusters, the cellular suspension was forced through a 20G needle. For cell culture, the concentration of the clusters on the cellular suspension obtained was adjusted to 5000 clusters/mL, plated on 100 mm^2 culture plates (Thermo Fisher Scientific, Waltham, USA), and incubated at 37ºC in a 5% CO_2, 95% O_2 atmosphere. The day of plating was considered day 0 of culture and the cultures were left undisturbed until day 3.

Experimental Design

After exhibiting 80-90% of confluence, culture medium was replaced by serum-free medium (DMEM:F12 supplemented with insulin (10 mg), transferrin (5.5 mg) and selenium (5 µg) (ITS, Sigma) supplement; pH 7.4). In order to evaluate the effects of TBT on glycolytic metabolism, SCs were cultured in five groups: three in the presence of TBT and two in the absence of TBT (Vehicle and control groups). For the rat SCs exposed to the compound, we selected three concentrations of TBT: 0.1 nM, a subtoxic level; 10 nM, a physiological level; and 1000 nM, a level from which cytotoxic effects have already been reported. All concentrations were diluted in 0.025% ethanol (EtOH). On the other hand, in the vehicle group, SCs were cultured in the serum-free medium supplemented with ITS plus EtOH (0.025%), which allowed us to understand if the concentration of EtOH (0.025%) in which TBT was diluted has some effects on SCs. Besides, in the control group, SCs were cultured only in the serum-free medium supplemented with ITS. Treatments were performed at 37ºC during 6 hours in a 5% CO_2, 95% O_2 atmosphere. 1 mL of culture media was collected at 0 hours and 6 hours of treatment and stored at -80ºC until use. At the end of the treatment, cells were detached from the flask using a trypsin-EDTA solution and collected for protein and ribonucleic acid (RNA) extraction. Total number of cells per culture plate was determined with a Neubauer chamber through a 1:1 dilution of the cell suspension in Trypan Blue.

Cell Proliferation Assay

The effect of TBT on rat SCs proliferation was determined by the colorimetric sulforhodamine B (SRB) assay as previously described by Vichai & Kirtikara [101]. In brief, 2×10^5 cells were plated in each well of 24-well culture plates. Cells were left to grow until reaching 70–80% of confluence. Then, the seeding medium was replaced by ITS or ITS plus TBT (0.1 nM, 10 nM and 1000 nM) and cells were cultured for 6 hours. After treatment, cells were washed twice

in phosphate-buffered saline (PBS) solution and fixed overnight at -20ºC in a mixture of 1% acetic acid and 99% methanol. Afterwards, this mixture was discarded and the plate was left to dry at 37-40ºC. The fixed cells were then incubated with 0.05% (wt/vol) SRB in 1% acetic acid for 1 hour at 37ºC. The unbound dye was removed by washing with 1% acetic acid solution. Finally, bound dye was extracted with 10 mM Tris solution (pH 10). The absorbances were measured at 540 nm, in order to evaluate the effects of different TBT concentrations in the proliferation of SCs comparatively to control group.

Total RNA Extraction

Total RNA was extracted from isolated SCs by TripleXtractor (GRiSP, Portugal) according to the manufacturer's instructions. Briefly, the cellular pellet was initially homogenized in 500 µL of TripleXtractor reagent. To ensure a complete dissociation of nucleoproteins complexes, samples were allowed to stand for 5 minutes at room temperature and then 100 µL of chloroform were added for phase separation. The samples were shaken vigorously for 15 seconds, allowed to stand for 5 minutes at room temperature and then centrifuged at 12000x*g* for 15 minutes at 4ºC. The colorless upper aqueous phase resulting from the centrifugation was transferred to a fresh tube and used to isolate total RNA. 250 µL of propan-2-ol were added to this aqueous phase to allow RNA precipitation. Afterwards, the mixture was centrifuged at 12000x*g* for 10 minutes at 4ºC and the 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, the RNA pellet was air-dried for 5 minutes and then dissolved by repeated pipetting in an appropriate volume of DEPC-H_2O. RNA concentration and absorbance ratio (A260/A280) were determined by spectrophotometry (Nanophotometer™, Implen, Germany).

Reverse transcriptase quantitative polymerase chain reaction

The complementary deoxyribonucleic acid (cDNA) synthesis was performed with the NZY First-Strand cDNA Synthesis Kit MB12501 (NZYtech, Portugal) according to the manufacturer's instructions. Briefly, the reverse transcriptase reaction was performed in a final volume of 20 µL including 10 µL of NZYRT 2× Master Mix containing oligo$(dT)_{18}$, random hexamers, $MgCl_2$ and deoxynucleotide triphosphates (dNTPs), 2 µL of NZYRT Enzyme Mix containing NZY Reverse Transcriptase and NZY Ribonuclease Inhibitor, 1 µg of total RNA and an appropriated volume of DEPC-treated H_2O. Reaction was carried out for 10 min at 25°C followed by 30 min at 50°C, 5 min at 85°C and 20 min at 37°C. The resulting cDNA was stored at -20°C until use.

qPCR was performed to determine AR and inhibin B mRNA expression levels. Specific primers were designed for the amplification of target genes and for β2-microglobulin (ß2MG), which was used as housekeeping gene to normalize gene expression (Table 2). qPCR was carried out in a CFX-Connect system (Bio-Rad, Hercules, CA, USA). The conditions were previously

optimized and the specificity of amplifications was determined by melting curves. qPCR amplifications were performed with 1 µL of synthesized cDNA in a 20 µL reaction containing 10 µL Maxima™ SYBR Green/Fluorescein qPCR Master Mix 2X (Thermo Fisher Scientific), 200 nM of forward and reverse primers specific for each gene (see Table 2 for details) and sterile nuclease-free water. Amplification conditions comprised 3 minutes of denaturation at 95°C, followed by 40 cycles at: 95°C for 30 seconds, a specific annealing temperature for 30 seconds and 72°C for 30 seconds. Samples were run in triplicate in each assay and expression values were normalized relatively to the ß2MG gene using the formula $2^{-\Delta\Delta Ct}$, in accordance with the mathematical model proposed by Pfaffl [102].

Table 2: Oligonucleotides and cycling conditions for quantitative real-time polymerase chain reaction (qPCR) amplification of inhibin B, androgen receptor (AR) and ß2-microglobulin (ß2MG).

Gene	Sequence (5'-3')	Annealing Temperature (°C)	Accession Number
Inhibin B	Forward: CAACATCACGCACGCTGTC Reverse: GACGCCATCTGTCTCTGCAA	60°C	NM_080771.1
AR	Forward: TTTGGACAGTACCAGGGACC Reverse: CTTCTGTTTCCCTTCCGCAG	60°C	NM_012502.1
B2MG	Forward: ATGAGTATGCCTGCCGTGTG Reverse: CAAACCTCCATGATGCTGCTTAC	60°C	NM_012512.2

Total Protein Extraction

SCs were homogenized in lysis buffer supplemented with 1% protease inhibitor cocktail, 1% sodium orthovanadate and 1% sodium fluoride, as described by our group [99]. The homogenate was allowed to stand 30 minutes on ice and the suspension was centrifuged at 14000x*g* for 30 minutes at 4°C. The total protein concentration was quantified using the Bradford Protein Assay Kit II from Bio-Rad (Hercules, USA) according to the manufacturer's instructions and the absorbances were measured by xMark Microplate Spectrophotometer from Bio-Rad (Hercules, USA).

Western Blot

Total protein extracted from rat SCs (25 µg) was mixed with the supplemented lysis buffer plus loading buffer (50% Glycerol (v/v), 20% Tris-HCl (v/v), 10% Sodium dodecyl sulfate (SDS) (w/v), 1.25% β-mercaptoethanol (v/v) and 0.05% bromophenol blue (v/v), pH=6.8). Samples

were separated in 11% polyacrylamide gel (SDS-page). After electrophoresis, proteins were then transferred to previously activated polyvinylidene difluoride (PVDF) membranes. Then, membranes were blocked at room temperature in a 5% skimmed dried milk solution. Afterwards, membranes were incubated overnight at 4ºC with rabbit polyclonal primary antibodies against MCT4 (1:2500, sc-50329), PFK1 (1:500, sc-67028) GLUT1 (1:500, sc-7903) and GLUT2 (1:2000, sc-9117), with rabbit monoclonal antibody against LDH (1:10000, ab52488) or with goat polyclonal antibody against GLUT3 (1:1000, sc-7582). Mouse monoclonal antibody against α-tubulin (1:10000, T9026) was used as the protein loading control for SCs samples. The immune-reactive proteins were detected separately with goat polyclonal anti-rabbit IgG-HRP (1:20000, sc-2004) or with goat polyclonal anti-mouse IgG-HRP (1:20000, sc-2005). Membranes were reacted with SuperSinal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA) and visualized on the Chemidoc MP Imaging System from Bio-Rad (Hercules, USA). Densities from each band were obtained with Image Lab Software 5.1 from Bio-Rad (Hercules, USA). The band density attained was divided by the corresponding α-tubulin band intensity and expressed in fold-variation *versus* the control group.

Enzymatic Assays

The activity of LDH was spectrophotometrically assessed with Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Briefly, samples were diluted in lysis buffer. Then, reaction buffer was added to the plate and the mixture was incubated for 45 minutes at 25ºC. The reaction was stopped by adding stop reaction buffer. LDH activity was measured at 490 nm, using the xMark Microplate Spectrophotometer from Bio-Rad (Hercules, USA), and expressed in percentage *versus* the control group.

Nuclear Magnetic Resonance Spectroscopy

540 µL of the medium culture collected during cell culture were used for proton nuclear magnetic resonance (^{1}H-NMR) analysis. 60 µL of a 10 mM sodium fumarate solution in 99.9% D_2O were added to each sample. ^{1}H-NMR spectroscopy was performed as previously described [99, 103] to determine lactate production, glucose and pyruvate consumption and also variations in alanine production, during the 6 hours of cell incubation in the experimental conditions. Sodium fumarate (final concentration of 1 mM) was used as an internal reference (6.50 ppm) to quantify metabolites in solution: lactate (doublet, 1.33 ppm); alanine (doublet, 1.45 ppm); pyruvate (singlet, 2.36 ppm) and H1-α glucose (doublet, 5.22 ppm) (Figure 5). The relative areas of ^{1}H-NMR resonances were quantified using the curve-fitting routine supplied with the NUTSproTM NMR spectral analysis program (Acorn, NMR Inc, Fremont, CA).

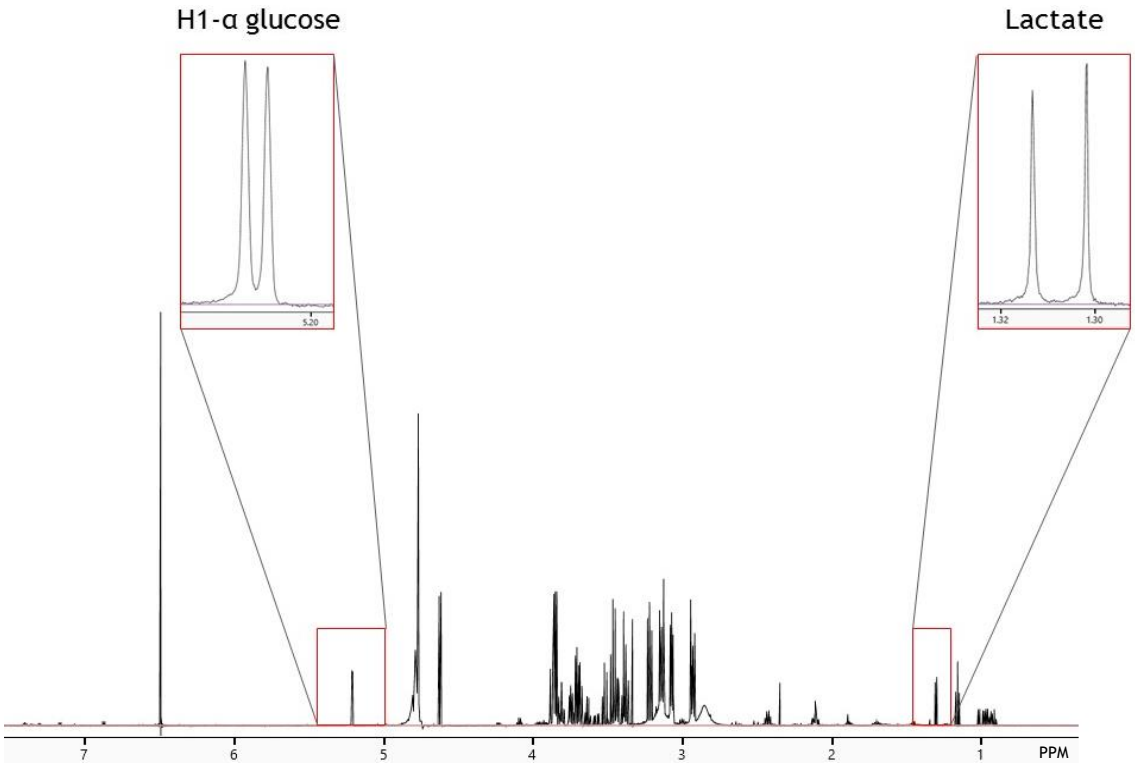

Figure 5: Representative proton nuclear magnetic resonance (^{1}H-NMR) spectrum achieved for the insulin-transferrin-sodium selenite (ITS) supplement Dulbecco's modified eagle medium Ham's nutrient mixture F12 (DMEM:F12) showing the localization of H1-α-glucose and lactate peaks.

Statistical analysis

Statistical significance of differences among experimental groups was assessed by one-way ANOVA, followed by Dunnett's test. All experimental data are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 (GraphPad Prism version 6.00 for Windows, GraphPad Software, California USA). Differences with *p-value* <0.05 were considered statistically significant and were indicated as (*) relative to control and as (#) relative to TBT 10 nM.

Results

Exposure to TBT 1000 nM decreases the proliferation of primary cultures of rat Sertoli cells

To examine the effect of TBT on the proliferation of rat SCs, cells were exposed to different concentrations of TBT for 6 hours and cellular proliferation was determined by the SRB assay. Our results showed that treatment with TBT reduced cell proliferation only when SCs were exposed to the highest dose of TBT (1000 nM) (Figure 6), evidencing a cytotoxic effect. Indeed, this concentration decreased significantly cell proliferation to 27.67 ± 5.78% (*p-value* = 0.0006) when compared with the control group (100%) (Figure 6). Contrastingly, SCs treated with 10 nM or 0.1 nM of TBT, showed a cell proliferation not different from those cells of the control group with values of 95.33 ± 8.41% (*p-value* = 0.6714) and 91.33 ± 12.45% (*p-value* = 0.5621), respectively (Figure 6).

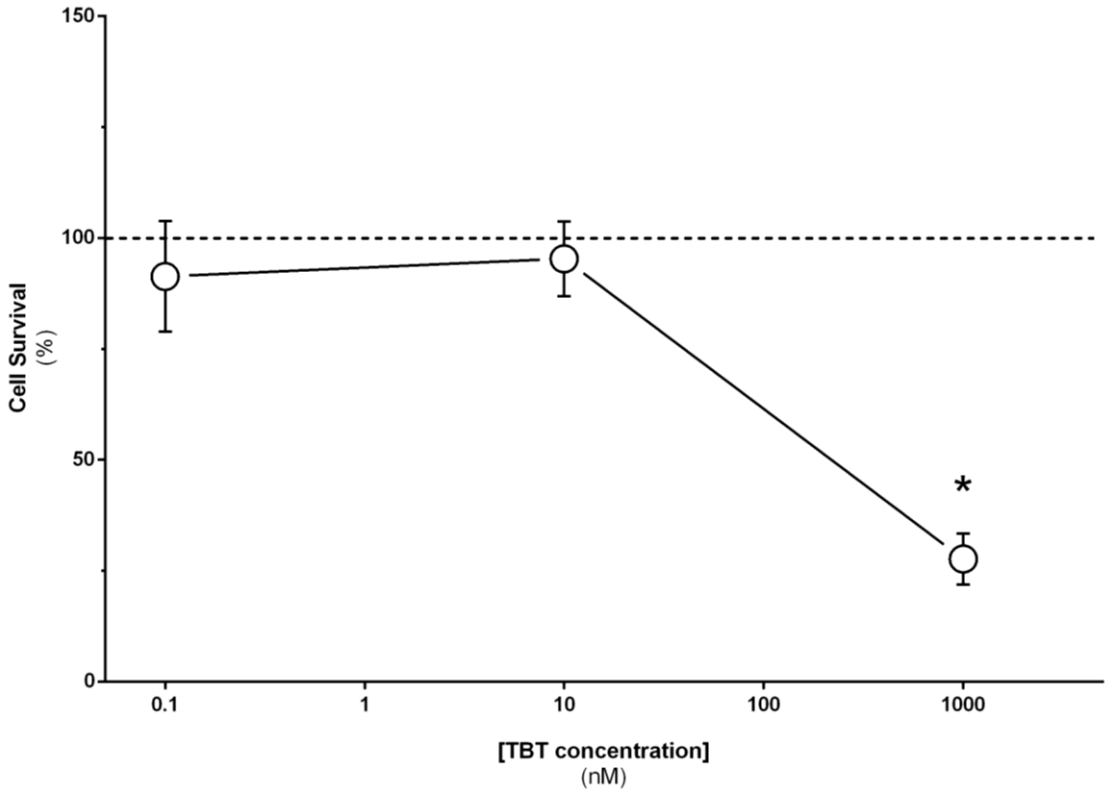

Figure 6: **Effect of tributyltin (TBT) on rat Sertoli cells (SCs) survival after 6-hours treatment.** The figure shows pooled data of independent experiments. Results are shown in percentage and expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Control value was set at 100% (dashed line). Significant results (*p-value* < 0.05) relative to control are indicated as (*).

Inhibin B and AR mRNA expression in rat Sertoli cells is not affected by exposure to TBT 10 nM and 0.1 nM

We evaluated the expression of inhibin B and AR transcripts in SCs exposed to TBT 10 nM and 0.1 nM, since these are often used as markers of SC maturity [11, 104]. Our results showed that mRNA levels of inhibin B were not altered by exposure to TBT 10 nM (0.95 ± 0.17, *p-value* = 0.89) or TBT 0.1 nM (1.03 ± 0.29, *p-value* = 0.94), when compared with the control group (Figure 7, panel A). Identical results were observed for AR, which mRNA levels were not affected by TBT 10 nM (1.04 ± 0.22, *p-value* = 0.89) neither by TBT 0.1 nM (0.99 ± 0.14, *p-value* = 0.99) when compared with the control group (Figure 7, panel B).

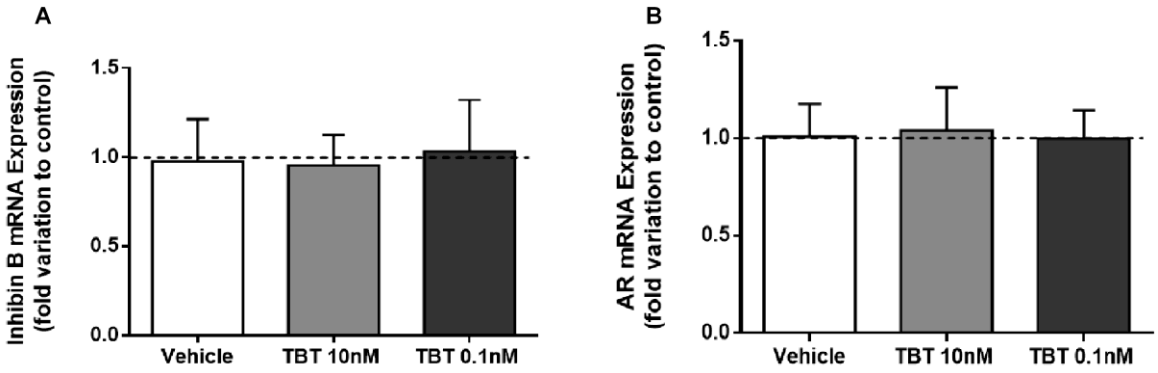

Figure 7: Effect of tributyltin (TBT) on inhibin B and androgen receptor (AR) messenger ribonucleic acid (mRNA) levels in rat Sertoli cells (SCs) after 6-hours treatment. (A) The figure shows pooled data of independent experiments indicating the fold-variation of inhibin B mRNA levels found in SCs of TBT-treated groups when compared with the cells from the control group (dashed line). (B) Figure shows pooled data of independent experiments indicating the fold-variation of AR mRNA levels found in SCs of TBT-treated groups when compared with the cells from the control group (dashed line). Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Significant results (*p-value* < 0.05) relative to control are indicated as (*).

TBT 10 nM increase glucose consumption by cultured rat Sertoli cells

Glucose consumption by rat SCs exposed to different concentrations of TBT was evaluated. After 6 hours of treatment, SCs exposed to 10 nM of TBT showed an increase of 40% on glucose consumption (24.11 ± 2.85 pmol/cell, *p-value* = 0.02) when compared to the cells from the control group (17.11 ± 1.14 pmol/cell) (Figure 8, panel A). In regard to the group of rat SCs cultured with TBT 0.1 nM, a 11.19% non-significant increase in glucose consumption (19.03 ± 1.80 pmol/cell, *p-value* = 0.19) was also exhibited when compared with that of cells from the control group (17.11 ± 1.14 pmol/cell) (Figure 8, panel A).

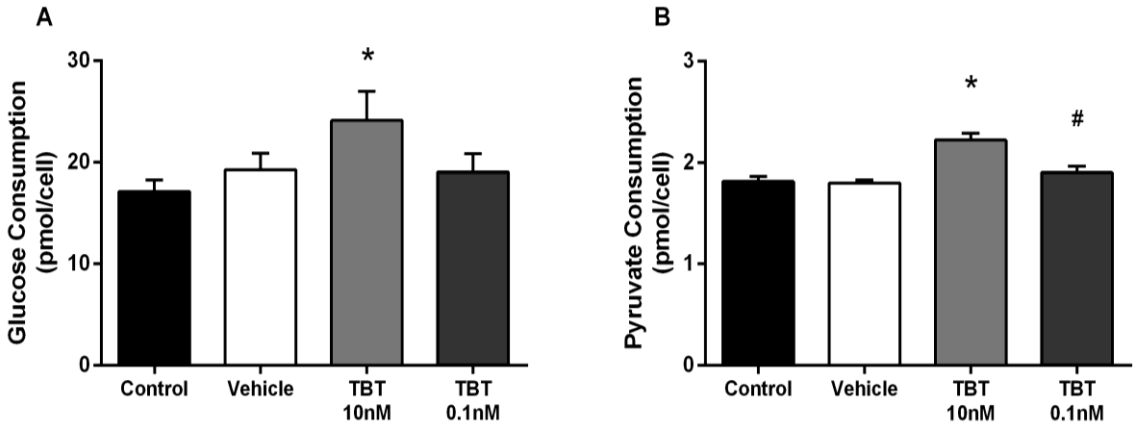

Figure 8: Effect of tributyltin (TBT) on glucose and pyruvate consumption after 6-hours treatment in primary cultures of rat Sertoli cells (SCs). (A) Glucose consumption. (B) Pyruvate consumption. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Significant results (*p-value* < 0.05) relative to control are indicated as (*) and relative to TBT 10 nM as (#).

Exposure to TBT 0.1 nM decreases significantly the protein levels of both GLUT1 and GLUT2, but not GLUT3, in rat Sertoli cells

Glucose is a reliable substrate for cultured SCs and, in normal conditions, these cells uptake extracellular glucose *via* GLUTS located in the plasma membrane [35]. Interestingly, even though glucose consumption was increased in the group of SCs exposed to TBT 10 nM, the protein expression of the analyzed GLUTs was not significantly affected in the cells of this group. Indeed, GLUT1 expression was non-significantly decreased to 0.88 ± 0.25 fold-variation to control (*p-value* = 0.66), when compared with that of the control group (Figure 9, panels A and B). Similarly, the expressions of GLUT2 (0.94 ± 0.13 fold-variation to control, *p-value* = 0.30) (Figure 9, panels C and D) and of GLUT3 (1.24 ± 0.21 fold-variation to control, *p-value* = 0.19) (Figure 9, panels E and F) were not significantly altered, when compared with the control group (1.00).

As concerning SCs exposed to the lowest dose of TBT (0.1 nM), a 44.42% decrease of the expression of GLUT1 (0.56 ± 0.07 fold-variation to control, *p-value* = 0.0006) was observed (Figure 9, panels A and B). Similarly, GLUT2 levels were also decreased by 16.32% (0.84 ± 0.08 fold-variation to control, *p-value* = 0.027) comparatively to those of the cells from the control group (Figure 9, panels C and D). On the other hand, the expression of GLUT3 was not significantly altered (0.91 ± 0.29 fold-variation to control, *p-value* = 0.72) in relation to control group, presenting only a small non-significant decrease of 8.76% (Figure 9, panels E and F).

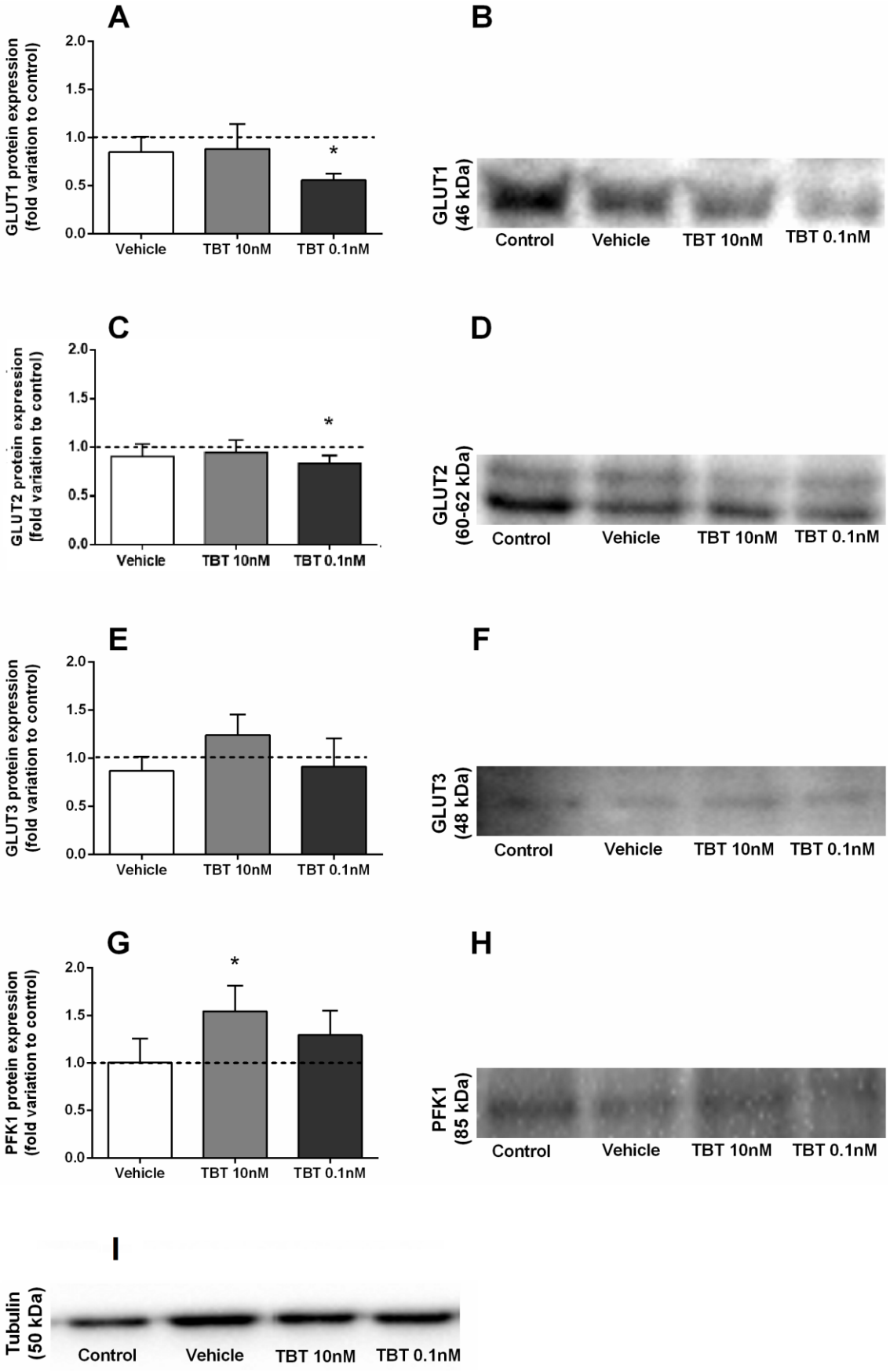

Figure 9: Effect of tributyltin (TBT) on glucose transporters (GLUTs) and phosphofructokinase 1 (PFK1) after 6-hours treatment in primary cultures of rat Sertoli cells (SCs). (A) The

figure shows pooled data of independent experiments, indicating the fold-variation of glucose transporter 1 (GLUT1) levels found in SCs of TBT-treated groups when compared with the cells from the control group (dashed line) with the respective illustrative Western blot experiment (B). (C) Pooled data of independent experiments, indicating the fold-variation of glucose transporter 2 (GLUT2) levels found in SCs of TBT-treated groups when compared with the cells from the control group (dashed line) with the respective illustrative Western blot experiment (D). (E) Pooled data of independent experiments, indicating the fold-variation of glucose transporter 3 (GLUT3) levels found in SCs of TBT-treated groups when compared with the cells from the control group (dashed line) with the respective illustrative Western blot experiment (F). (G) Pooled data of independent experiments, indicating the fold-variation of phosphofructokinase 1 (PFK1) levels found in SCs of TBT-treated groups when compared with the cells of the control group (dashed line) with the respective illustrative Western blot experiment (H). (I) Tubulin illustrative Western blot experiment. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Significant results (*p-value* < 0.05) relative to control are indicated as (*).

Exposure to TBT 10 nM increases PFK1 levels in rat Sertoli cells

After glucose being internalized in the cell, the first rate-limiting step in glycolysis is mediated by PFK1 that catalyzes the irreversible conversion of fructose-6-phosphate into fructose-1,6-bis-phosphate [34]. Hence, the PFK1 expression levels were determined in rat SCs after TBT exposure. Rat SCs exposed to 10 nM of TBT presented an increase of 54.48% on PFK1 levels (1.55 ± 0.26 fold-variation to control, *p-value* = 0.02), when compared to cells from the control group (Figure 9, panels G and H). On the other hand, a non-significant increase of 29.46% was observed in the PFK1 protein levels of rat SCs exposed to 0.1 nM of TBT (1.29 ± 0.26 fold-variation to control, *p-value* = 0.23), when compared to the cells from the control group (Figure 9, panels G and H).

Pyruvate consumption is increased in Sertoli cells exposed to TBT 10 nM

The last step of glycolytic pathway is the conversion of phosphoenolpyruvate to pyruvate, that may be used as a substrate for several biochemical pathways [54]. Thus, we assessed pyruvate consumption by SCs of the different groups, which showed to be sensitive to TBT exposure. Indeed, after the 6 hours treatment, rat SCs exposed to TBT 10 nM evidenced a significant increase in the consumption of this substrate by 22.65% (2.22 ± 0.07 pmol/cell, *p-value* = 0.0008), when compared with the cells from the control group (1.81 ± 0.05 pmol/cell) (Figure 8, panel B). Concerning the rat SCs treated with TBT 0.1 nM, pyruvate consumption was non-significant increased (1.90 ± 0.06 pmol/cell, p-value = 0.15) by 8.72%, when compared with the cells from the control group (Figure 8, panel B).

TBT modulates lactate production in primary cultures of rat Sertoli cells

As one of the key functions of SCs is to produce lactate (from glucose) for the developing germ cells, we evaluated the effect of TBT on lactate production and export. Once pyruvate is produced, it can be converted into lactate in a reversible reaction catalyzed by LDH. Thus, we first measured the amount of lactate produced during the 6 hours treatment. The SCs exposed to TBT 10 nM evidenced a significant increase (by 30.11%) in the production of this metabolite (5.80 ± 0.36 pmol/cell, *p-value* = 0.01) when compared to the cells from the control group (4.46 ± 0.27 pmol/cell) (Figure 10, Panel A). The SCs exposed to TBT 0.1 nM produced similar amounts of lactate in relation to the control group, evidencing a small non-significant increase by 7.88% (4.81 ± 0.32 pmol/cell, *p-value* = 0.42) (Figure 10, panel A).

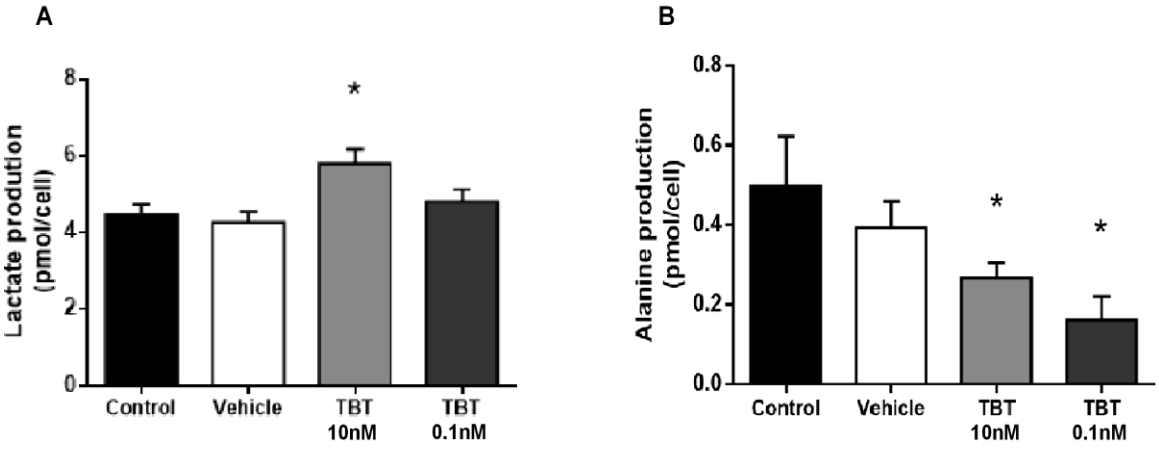

Figure 10: Effect of tributyltin (TBT) on lactate and alanine production after 6-hours treatment in primary cultures of rat Sertoli cells (SCs). (A) Lactate production. (B) Alanine production. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Significant results (*p-value* < 0.05) relative to control are indicated as (*).

Afterward, we analyzed LDH protein levels and our results showed that SCs exposed to TBT 10 nM evidence a decrease of 18.61% (0.81 ± 0.07 fold-variation to control, *p-value* = 0.02), when compared with the cells from the control group (Figure 11, panels A and B). Similarly, in the SCs exposed to TBT 0.1 nM, a decrease of 11.23% (0.89 ± 0.06 fold-variation to control, *p-value* = 0.04) was also observed in the levels of LDH (Figure 11, panels A and B).

We also evaluated LDH activity and observed that SCs exposed to TBT 10 nM had no alteration on the activity of this enzyme (0.99 ± 0.08 fold-variation to control, *p-value* = 0.89), comparatively to that measured in the cells from the control group (Figure 11, panel E). Contrastingly, a significant increase (38.58%) in LDH activity was observed in the SCs exposed to TBT 0.1 nM (1.39 ± 0.15 fold-variation to control) (Figure 11, panel E).

Under normal conditions, the lactate produced in SCs is then exported through MCT4 to adluminal fluid that is in contact with developing germ cells. Thus, we assessed the protein levels of MCT4 after exposure to the different concentrations of TBT. SCs exposed to TBT 10 nM showed a significant decrease in the expression of MCT4 (by 19.55%) (1.20 ± 0.07 fold-variation to control, *p-value* = 0.01) when compared with the cells from the control group (Figure 11, panels C and D). SCs exposed to TBT 0.1 nM also exhibited a significant increase (by 42.86%) in the expression of MCT4 (1.43 ± 0.26 fold-variation to control, *p-value* = 0.03) when compared with those from the control group (Figure 11, panels C and D).

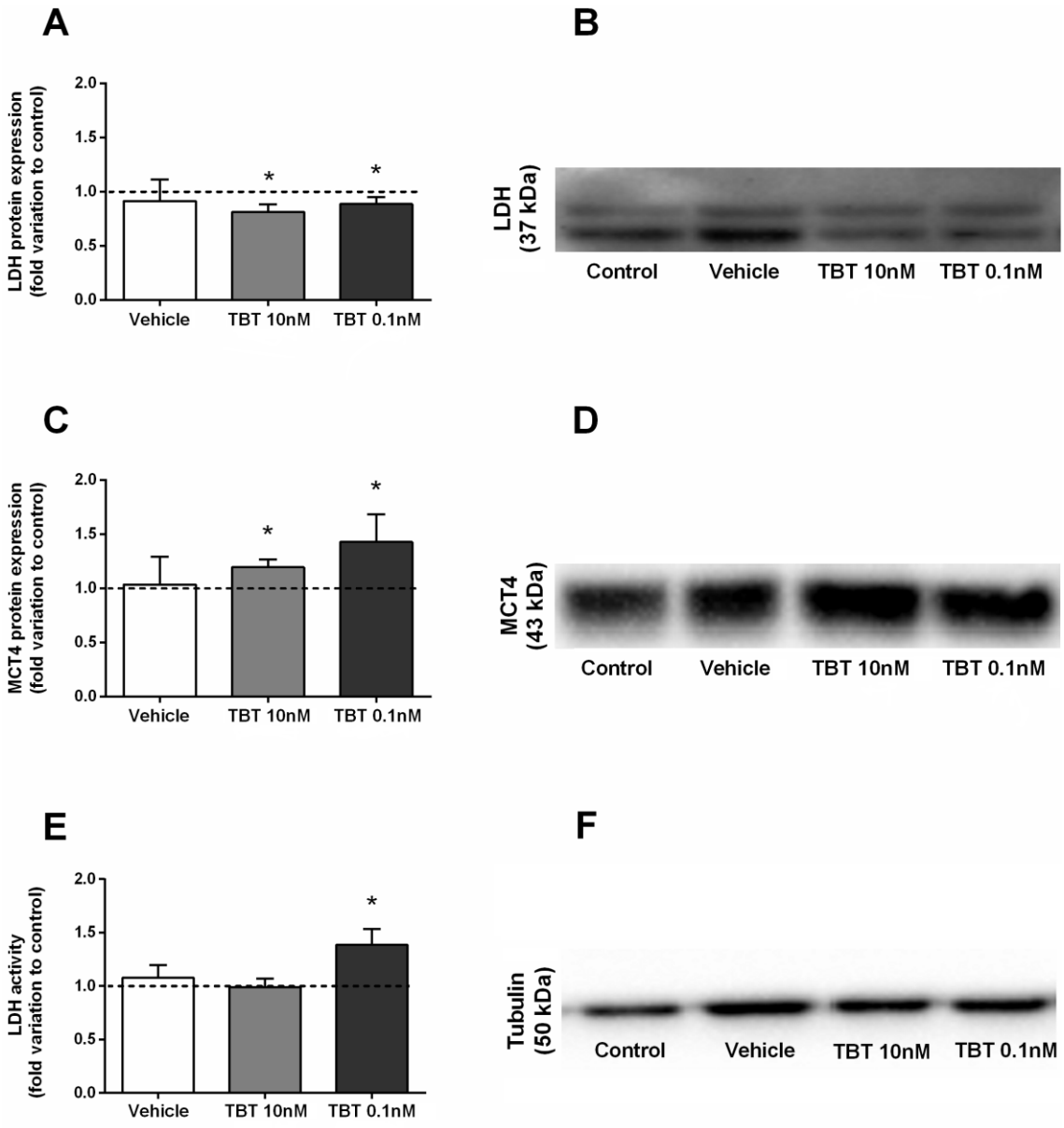

Figure 11: Effect of exposure to tributyltin (TBT) on lactate dehydrogenase (LDH) and monocarboxylate transporter 4 (MCT4) after 6-hours treatment in primary cultures of rat Sertoli cells (SCs). (A) The figure shows pooled data of independent experiments, indicating the fold-variation of LDH protein levels found in SCs of TBT-treated groups when compared with the control (dashed line) with the respective illustrative Western blot experiment (B). (C) Pooled data of independent experiments, indicating the fold-variation of MCT4 levels found in SCs of TBT-treated groups when compared with the control (dashed

line) with the respective illustrative Western blot experiment (D). (E) Panel shows pooled data of independent experiments, indicating the fold-variation of LDH activity in testes of TBT-treated groups when compared with the control (dashed line). (F) Tubulin illustrative Western blot experiment. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Significant results (*p-value* < 0.05) relative to control are indicated as (*).

Sertoli cells exhibit decreased alanine production after exposure to TBT 10 nM and 0.1 nM

Besides lactate, the cytosolic pyruvate may be reversibly converted to alanine by the action of alanine aminotransferase [35]. Thus, we assessed alanine production in these cells after exposure to TBT, which was significantly decreased by 23.16% in SCs exposed to TBT 10 nM (0.27 ± 0.04 pmol/cell, *p-value* = 0.04) when compared with the cells from the control group (0.50 ± 0.12 pmol/cell) (Figure 10, panel B). Similarly, SCs exposed to TBT 0.1 nM also showed a decrease in the production of alanine of 33.80% (0.16 ± 0.06 pmol/cell, *p-value* = 0.03), when compared with cells from the control group (Figure 10, panel B).

Discussion

The incidence of obesity in modern societies has been progressing alarmingly over the few last decades, becoming one of the most prevalent and serious public health concerns [41, 42]. The increased rates are closely related to current lifestyle behaviors, particularly the overconsumption of HED and decreased physical activity. An "external source" for obesity has been recently proposed, based on the permanent exposure to environmental compounds that predispose to weight gain and thus exacerbate some effects of this disease [43]. Such suggestion led to the concept of "obesogens", increasing the complexity of obesity's etiology by arising as important contributors for the deleterious effects observed in human health [44, 73]. Regarding to weight gain, obesogens may act through an increase on the number of adipocytes, through changes in the amount of stored calories or even by affecting the molecular mechanisms that regulate satiety and appetite. However, in addition to this capacity of predispose to weigh gain, obesogens also have a particular feature of acting as potent endocrine disruptors [45]. Thus, it was hypothesized that these compounds are able not only to exacerbate the effects of daily habits, but also to induce endocrine deregulations that therefore may lead to serious metabolic dysfunctions.

TBT, an organotin compound used worldwide until 2008 as algaecide and molluscicide, is considered the obesogen model [74]. The proposed mechanism through which TBT exerts its functions is the activation of the RXR-PPARγ, a heterodimer that promotes adipogenesis by stimulating the differentiation of preadipocytes into mature adipocytes [85]. However, other mechanisms may also be "targeted" by TBT since, as reported by Yamada and collaborators [97], it seems that this toxic compound is able to disrupt glucose metabolism. Indeed, their results evidenced that the exposure of human embryonic carcinoma cells to TBT 100 nM for 24 hours targets the glycolytic system, causing growth cellular arrest. These authors observed an inhibition of glucose uptake *via* a decrease in the level of cell surface-bound GLUT1, reducing also the levels of both glucose-6-phosphate and fructose-6-phosphate as a consequence of this decrease. This inhibitory effect of TBT on glucose uptake was suggested to be mediated by the translocation of GLUT1 to the plasma membrane, a process dependent on AMPK [105]. Since the activity of this key mediator in cellular energy homeostasis was also decreased, the authors proposed a model of TBT toxicity in human embryonic carcinoma cells through AMPK, evidencing an AMPK-dependence of glucose uptake in these cells.

Since TBT is a lipophilic compound, it easily accumulates in tissues with a high lipid content, such as testes and the reproductive accessory organs. The risk of its bioaccumulation is a matter of concern, as TBT is an active compound and able to induce morphological and biochemical alterations directly from where it is stored. Besides, it is also able to activate cytotoxic mechanisms in these organs. Thus, this accumulation has already been reported to impair the reproductive function at multiple levels, with adverse effects ranging from the endocrine control of spermatogenesis to sperm defects [52]. Several deleterious effects in human and animal health have already been associated to the action of this obesogen,

particularly at reproductive level [53]. Si and collaborators [95], for example, reported that Chinese Kun Ming mice exposed to 1, 10 and 100 μg of TBT/kg during postnatal period presented a dose-dependent decrease on sperm count and motility, suggesting that even a perinatal exposure may cause long lasting alterations in male reproductive system. In addition, TBT has also been associated with testicular germ cell apoptosis, since higher levels of apoptotic cells were found in the seminiferous tubules of 21-days old ICR mice after a 3-days oral administration of 25, 50 or 100 mg TBT/kg/day [96]. Likewise, other study also evidenced that caspase-3, which plays a central role on cellular apoptosis, was activated by an increase on stress responsive proteins in germ cells of 28-days old Wistar rats exposed for 6 hours to TBT 600 nM [39]. Still, the exact mechanisms by which this compound acts needs to be clarified.

Within the male reproductive organs, SCs were shown to be highly susceptible to the effects of environmental compounds [106-110]. Indeed, morphological changes were already observed on *in vivo* exposed rat SCs to cadmium [111], as well as biochemical changes, particularly mitochondrial ultrastructural modifications, were evidenced on *in vivo* mice SCs exposed to lead [109]. For SCs cultured *in vitro*, the susceptibly to environmental compounds was already showed for lead exposure [107] but also for TBT, with Mitra and collaborators [39] reporting oxidative damage and SCs death. In fact, these data support that several compounds exhibiting obesogenic properties exert cytotoxic effects on SCs. Such effects may hamper the maturational state of SCs, as observed by the decreased expression of crucial proteins, such as inhibin B [106], but also by alterations in one crucial function of differentiated SCs, the lactate production [106, 108].

In fact, under *in vitro* conditions, the measurement of lactate secretion by rat SCs has been reported to act as an important marker to evaluate the effects exerted by the toxicants on SCs (for review see [112]). This is extremely relevant since the success of spermatogenesis is dictated in part by the metabolic performance of SCs [113]. Particularly, the glycolytic metabolism of SCs is crucial for the normal occurrence of spermatogenesis, since the glucose that is taken up by SCs is the "source" to produce lactate, the preferred energy "fuel" of developing germ cells. In this regard, Hutchison and collaborators [114] reported that an abnormal germ cell development is observed in SCs that fail to proliferate or in which metabolism is perturbed, suggesting that their normal function is required for a successful spermatogenesis. Indeed, formation of competent spermatozoa is an intricate and complex process initiated within the seminiferous epithelium and highly dependent on the correct balance of the whole-body metabolism, which explains why male infertility may be one of the silent health problems that may arise from a permanent exposure to environmental toxicants.

Taking in consideration the aforementioned points, we hypothesized that TBT may impact male reproductive function by altering the metabolism of SCs, the main hormonal target in the seminiferous tubule and a known target for environmental compounds. We proposed to

unveil the mechanisms by which this obesogen affects glucose metabolism of primary cultures obtained from rat SCs. For that, 3 different concentrations of TBT were selected: 0.1 nM, subtoxic levels within the normal range of concentrations to which weight gain and RXR-PPARγ activation were associated [73]; 10 nM, a concentration within the range reported in serum and tissues of male individuals from the general population [115]; and 1000 nM, representing a supraphysiological level with broad cytotoxic effects [116].

Using primary cultures of SCs obtained from 20-days old rats, we were able to clearly determine that distinct patterns of metabolite consumption and key proteins expression involved in the glycolytic pathway were affected in cultured rat SCs exposed to different concentrations of TBT. The results obtained showed that TBT prompts alterations in the mechanisms involved in SC proliferation.

An evident cytotoxic effect was observed in the rat SCs exposed to the highest dose of TBT (1000 nM). Indeed, a decrease in the proliferation was observed in the cells of this group when compared to the control group. These results are consistent to those reported by Ferreira and collaborators [116] which also evidenced a decreased cellular survival to 20% in cells exposed to this concentration of TBT. Although these evidences became from neuroblastoma cells, they show that TBT, at relative high doses, affects cellular viability. However, it is important to highlight that SRB assay does not evaluate cell death. Indeed, its principle is based on the measurement of cellular protein content, with the colorimetric evaluation allowing us to estimate the total protein mass and to relate it with cell number. Through this assay, we observed that cells from the group incubated with TBT 1000 nM exhibited a decrease in SCs proliferation when compared to that observed in SCs of the control group. Since SCs exposed to this high dose were detached from culture plate at the end of the 6 hours treatment and knowing that the SRB assay allows the toxicity screening of TBT to adherent cells, we pointed that TBT affect cell adhesion molecules (CAMs) in SCs. This is consistent with previous data where a decrease in the expression of genes related to CAMs was observed on primary culture of porcine aortic endothelial cells after a 7 hours exposure to TBT 100 nM and TBT 500 nM [117]. From a functional point of view, such decrease is consistent with the hypothesis that TBT is able to affect cellular structure and thus cellular adhesion, increasing the number of detached cells from the culture medium. Likewise, a dose-dependent decrease on CAMs intracellular adhesion was also reported in prostate cells from rats exposed to 2.5, 10 and 20 mg TBT/kg, being associated with changed prostatic tight junctions and aspects of cell polarity, as well [118].

Since only the highest dose of TBT decreased cellular proliferation on SCs, we assessed if intermediate and low levels of TBT affect SCs maturity and function. In order to achieve it, we evaluated AR and inhibin B mRNA expression. These proteins are produced by mature SCs and are often used as biomarkers of gonadal disorders on clinical studies [119], making them strong predictors of mature SCs function [11, 104]. In the present conditions, SCs treated with

both intermediate levels (10 nM) or sub-nanomolar levels (0.1 nM) of TBT did not show differences in the expression of AR and inhibin B transcripts. In this regard, and albeit the reasoning of these results needs to be clarified, we suggest that it would be reasonable to hypothesize that although TBT decreased cellular proliferation, there was a maintenance of SCs maturity. This is consistent with data from human prostate cancer cells reported by Yamabe and collaborators [120]. In this study, little changes in AR mRNA levels were observed by the treatment with TBT 100 nM, when compared to the control.

Although the maturational state of SCs was not influenced by TBT exposure at intermediate and sub-nanomolar doses, these observations do not invalidate that other features of mature SCs are affected, such as the glycolytic metabolism. Indeed, we already know that glucose consumption is affected in other cellular systems mainly through disruption of translocation mechanism of GLUT1 [97]. In this regard, we observed that SCs exposed to different concentrations of TBT exhibit alterations in the glycolytic profile being those alterations more severe in the group of SCs exposed to intermediate concentrations (10 nM). Glucose uptake by SCs occurs through the action of specific GLUTs present on their plasma membranes [28]. Three glucose transporter isoforms (GLUT1, GLUT2, GLUT3) have been so far identified in the plasma membrane of SCs [29-32]. SCs exposed to 10 nM of TBT exhibited an increased glucose consumption and no alterations on the levels of GLUTs. Indeed, a previous study showed that TBT at nanomolar levels (100 nM) compromises a key step of the glucose metabolism by downregulating GLUTs' expression in human embryonic carcinoma cells [97].

Besides the effects of TBT being dose-dependent [121, 122] it is important to highlight that this compound can also exert different actions under the presence of other substances [123], as happens at physiological level. Indeed, testicular environment is rich in androgens, mainly T, which might condition TBT effects. T, particularly, was already reported to be implicated in the regulation of glucose uptake and metabolism not only in SCs, but also in other systems [124].

Once inside the cells, glucose enters the glycolytic process and PFK1 is responsible for the irreversible conversion of fructose-6-phosphate to fructose-1-6-bis-phosphate, in a rate-limiting step [125]. SCs exposed to intermediate levels of TBT showed an increase of PFK1 protein levels, which may contribute to a higher glycolytic activity as observed by the increased glucose consumption. The end product of glycolysis is pyruvate, a crossroad metabolite that can be reversibly converted into lactate, alanine or acetyl-CoA. Similarly, SCs exposed to 10 nM of TBT exhibited also an increased consumption of pyruvate. In this regard and although this approach did not mimic the physiologic conditions, cultured rat SCs exposed to intermediate levels of TBT responded in order to increase the consumption of both substrates that are mainly used to ensure lactate production to be delivered to germ cells [54]. In fact, the increased substrate consumption was followed by an increased production of lactate in these cells, though LDH protein levels were decreased. The increased production of

lactate may result from the high LDH activity and the increased expression of MCT4, which serve as the main lactate exporter in the "nurse cells". Thus, these effects may compensate the decrease in the protein levels of LDH to sustain lactate supply.

Alanine is another important product of glucose metabolism, which is required as a precursor for several anabolic reactions and specifically for protein synthesis. In our experimental conditions, SCs showed a decrease in the production of this metabolite when exposed to both concentrations of TBT. Our results suggest that cells exposed to 10 nM of TBT redirect their metabolism from alanine production towards pyruvate, to guarantee the adequate levels of lactate. Importantly, the lower levels of alanine together with the high amounts of lactate observed in the group of cells exposed to TBT 10 nM lead to a higher cytosolic redox state usually observed in tissues with glycolytic activity [56, 126]. As the reduction of pyruvate into lactate is related to the re-oxidation of NADH to NAD^+ and the lactate/alanine reflects the NADH/NAD^+ in these conditions, we may assume that these SCs present higher redox state [127].

SCs from the group exposed to a subtoxic concentration (0.1 nM) exhibit a different metabolic response compared to SCs exposed to intermediate levels of TBT and to those of the control group. Indeed, although the decreased protein expression of GLUT1 and GLUT2, we observed no changes in glucose consumption. This may happen due to the different kinetic properties exhibited by the several GLUTs isoforms. GLUT1 and GLUT3 present some of the highest affinities to glucose in GLUTs family ($K_m \approx 3$ mM for GLUT1 [128] and 1.5 mM for GLUT3 [129]), while GLUT2 presents low affinity for glucose ($K_m \approx 17$ mM) but high capacity for its transport [130]. GLUT3, particularly, reveals the highest affinity for glucose, ensuring a maximal and efficient glucose uptake in physiological conditions. This leads to suggest that GLUT3 may arise as the main contributor in a compensatory mechanism for glucose uptake. Besides, even though the decreased expression of GLUT1 and GLUT2, the activity of any of the GLUTs analyzed may be increased and thus contribute also to the maintenance of glucose uptake. In fact, the first steps of glucose metabolism seemed to be compromised and even pyruvate consumption was significantly decreased when compared both with SCs from the control group and those exposed to 10 nM of TBT.

Environmental compounds typically are active in the range of nanomolar to micromolar, with some of them being able to show effects at even lower concentrations. This may be attributed to the nonmonotonic dose-response curves that TBT may exhibit. This raises a question whether TBT at these levels may compete with the high amounts of androgens present in the testicular environment. Indeed, Bettin and collaborators [131] showed that both TBT and T compete for the same metabolic system with the high T levels swamping its molecular target to the exclusion of TBT.

Several data from other groups showed that adult male animals exposed to TBT exhibit an adversely affected reproductive capacity. Yan and collaborators [132] showed that male pubertal mice exposed to TBT exhibited lower sperm counts and increased sperm abnormalities, accompanied with decreased sperm viability, while Kim and collaborators [96] found that the number of apoptotic germ cells inside the tubules was increased in the TBT-exposed animals. However, our results showed that these previous studies reporting loss of germ cells are not due to an inhibition of energy substrate production by SCs, speculating that the stimulation of lactate production may be a useful *in vitro* marker for TBT toxicity. Indeed, TBT seems to exert its effects through an exacerbated glycolytic metabolism and the decreased alanine production corroborated the redirection of glucose metabolism to lactate production. The evidence of higher redox state that we suggest from our metabolic analysis needs confirmation, but is also a clear indicator of the toxicity induced by TBT at concentrations reported in serum and tissues of individuals from the general population.

In our study we have scrutinized the effects of TBT on the metabolic pathways of primary cultures obtained from rat SCs. To the best of our knowledge, few works have explored the impact of TBT on SCs function being these results the first attempt to understand the mechanisms by which TBT can alter SCs metabolic function, with a possible impact on spermatogenesis. This work gave new insights about the effects of TBT on rat SCs metabolism, leading us to suggest that this obesogen contributes for an abnormal function of SCs by impairing its metabolic performance. Besides, our use of these prepubertal rather than adult SCs may also provide relevance to developmental aspects of ongoing spermatogenesis in adults. Although observations in SCs primary cultures may not exactly represent an *in vivo* situation, and must be analyzed under that scope, the results presented here are of great significance, representing a further step to elucidate the regulation of glucose metabolism of SCs by environmental compounds, specially obesogens that may directly influence spermatogenesis and thus male fertility. We believe that further research on the functioning and regulation of possible key mechanisms by which testicular metabolism pathways are compromised after short or long-term exposure to TBT is crucial, not only for the enlightenment of a process that is central to spermatogenesis and male fertility, but also to stimulate the debate on how the metabolic alterations caused by what male individuals are exposed may lead to subfertility or infertility. *In vivo* studies will also be necessary in order to better determine the eventual cumulative effects of this obesogen with the naturally modulation of SCs metabolism by endogenous factors, that may hamper spermatogenesis and contribute to male infertility.

Conclusions

There are growing concerns about the possible severe consequences of the so called "obesogenic hypothesis" on male fertility. There has been a consensual awareness regarding the contribution of lifestyle factors in obesity epidemics, but the role of environmental compounds in this topic is still a matter of intense debate. Several data evidenced that individuals from a very early age (even in prenatal period) are permanently exposed to obesogens, which induce several changes in male reproductive function that may end-up in infertility.

The basis of male fertility relies in fully functional spermatogenesis, an intricate and complex process that depends on the correct balance of the whole-body metabolism. From a clinical point of view, the identification of possible features by which testicular metabolism pathways are compromised after both short- and long-term exposure to obesogens should deserve special attention. SCs, whose glycolytic metabolism is crucial for a metabolic support of germ cells, are very sensitive to environmental toxicants. Therefore, SCs may constitute a reliable model in the field of reproductive toxicology, since they may help to disclose the mechanisms by which obesogens may affect male fertility [112].

Through this project, it was possible to conclude that high concentrations of TBT are clearly cytotoxic to SCs. Moreover, we were able to confirm that the exposure to intermediate/physiological levels of TBT (10 nM) reveal to affect the key events of the SCs glycolytic flux. Indeed, in the group of SCs exposed to this concentration, we observed an increase in glucose and pyruvate consumption, which was reflected in an increased production of lactate. Interestingly, we also observed that although subtoxic levels of TBT were not able to alter glycolytic profile of SCs, the protein expressions of both enzymes and transporters involved in the metabolism of glucose were sensitive to these levels, showing that even sub-nanomolar doses of TBT may affect one of the main functions of SCs. These results are a first attempt to understand the mechanisms by which TBT affect SCs metabolic function and thus spermatogenesis. However, further studies will be necessary to clarify in depth these mechanisms and to determine the eventual cumulative effect of this obesogen with the naturally modulation of SCs metabolism by endogenous factors.

As infertility grows to epidemic proportions, a demographic pressure is already bringing subfertility and infertility topics into the spotlight. Since the exposure to environmental contaminants may be involved in the 20% of the infertility problems with unknown reasons [133] and knowing that the release of environmental compounds continues to increase, the number of obesogenic studies focused on male reproductive health is also expected to grow in the next years. The study of testicular metabolism has arisen as one of the new challenges that reproductive biology faces in the next century, with the enlightenment of key regulatory mechanisms by which spermatogenesis is affected being critical in order to highlight new therapeutic approaches. This subject will certainly become a research hot topic for those who

are interested in environmental toxicology and male infertility, one of the most blatantly visible, yet somewhat overlooked health problems.

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