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Effects of Wi-Fi Radiation on Metabolism of Rat Immature Seminiferous Tubules *Ex Vivo*

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Resumo

Nas últimas décadas, vários estudos evidenciaram uma diminuição da fertilidade masculina. À medida que as tecnologias sem fios, *Wi-Fi*, e o tempo gasto com a sua utilização estão a aumentar, a relação entre estes dois fatos tem sido um tema de investigação.

Existem vários artigos que demonstram as consequências negativas da radiação eletromagnética de dispositivos sem fios na fertilidade masculina, afetando os parâmetros espermáticos nomeadamente reduzindo a motilidade e viabilidade, aumentando a percentagem de espermatozóides com uma morfologia anormal e diminuindo a concentração espermática. Existem inclusivamente estudos que revelam que a radiação eletromagnética destes aparelhos potencia o stress oxidativo e aumenta as espécies reativas de oxigénio, sendo também capaz de provocar alterações histopatológicas nos órgãos reprodutores masculinos e até mesmo alterar a produção de hormonas importantes para a fertilidade masculina, como a testosterona. No entanto, todos os estudos mostram falhas na concepção de um modelo realista de exposição à radiação e não existem estudos sobre os efeitos da radiação eletromagnética no metabolismo testicular.

Uma função reprodutiva normal é afectada pelo processo de espermatogénese, que por sua vez está dependente do metabolismo testicular, mais especificamente das células de Sertoli que fornecem suporte nutricional às células germinativas em desenvolvimento. As células de Sertoli, localizadas no interior dos túbulos seminíferos, em situações normais, metabolizam a maioria da glucose a lactato, sendo o lactato o substrato preferido para as células germinativas em desenvolvimento obterem energia. Contudo, alguns estudos já demonstraram que em circunstâncias específicas, as células de Sertoli podem usar outros substratos em vez da glucose para obter energia.

Para realizar o estudo, foi construído um *set up* de exposição constituído por diferentes componentes eletrónicos comercialmente disponiveis para expor os túbulos seminíferos em cultura, de uma forma realística à radiação eletromagnética. Para a validação do modelo de exposição construído, espermatozóides de ratos adultos foram expostos durante 1 hora à radiação eletromagnética do *set up* desenvolvido. Desta experiência os resultados revelaram uma diminuição significativa na motilidade dos espermatozóides do grupo exposto em relação aos do grupo controlo, validando o modelo.

Do estudo de metabolismo realizado, expondo túbulos seminíferos de ratos de 20 dias ao equipamento construído, os nossos resultados mostraram que a radiação eletromagnética diminuiu significativamente o consumo de glucose, no entanto contraditoriamente, a produção de lactato aumentou significativamente. A actividade da enzima lactato

desidrogenase foi avaliada e embora não significativo, a radiação electromagnética causou um aumento da mesma. Os resultados obtidos contraditórios entre o consumo de glicose e a produção de lactato sugerem que, quando expostos a radiação eletromagnética de dispositivos *Wi-Fi*, as células responsáveis pelo metabolismo testicular, nomeadamente as células de Sertoli nos túbulos seminiferos, podem usar vias metabólicas alternativas para a produção de lactato e consequentemente obter energia.

Em conclusão, este estudo demonstrou que o modelo de exposição à radiação eletromagnética de aparelhos *Wi-Fi* foi criado e validado com sucesso e que a radiação eletromagnética proveniente destes equipamentos, para além de causar alterações negativas nos parâmetros espermáticos das células germinativas, promove alterações no metabolismo glicolítico normal, sugerindo a utilização de uma via alternativa de obtenção de energia que pode ter efeitos na espermatogénese e afectar a fertilidade masculina.

Palavras-chave

Wi-Fi; Radiação Eletromagnética; Fertilidade Masculina; Túbulos Seminíferos; Metabolismo Glicolítico.

Resumo Alargado

Actualmente, a infertilidade, definida pela impossibilidade de alcançar uma gravidez desejada após um ano de relações sem o uso de qualquer método contracetivo, é um problema de saúde que incide cada vez mais em casais que pretendem ter filhos, sendo afectada pelos mais diversos factores. Sendo que cerca de 30% dos casos de infertilidade são atribuídos a factores masculinos, é de extrema importância o desenvolvimento de um trabalho contíguo entre a investigação e a medicina de maneira a perceber as perspectivas clinicas e os tratamentos a adoptar para minimizar o problema da infertilidade masculina.

Vários factores sendo eles psicológicos, bioquímicos ou ambientais têm vindo a ser apontados por terem um papel no aumento do número de casos de infertilidade. De facto, existem evidências de que problemas de infertilidade masculina são mais comuns em países desenvolvidos, o que nos leva a supor que o estilo de vida nestes países possa contribuir para o número crescente de casos de homens inférteis. Sendo os países desenvolvidos também caracterizados por um maior avanço tecnológico, é plausível questionar se as novas tecnologias podem também ser um factor capaz de influenciar a função reprodutiva masculina. De facto, a exposição a radiação eletromagnética tem sido associada a vários desfechos adversos à saúde. Alguns desses resultados relatados são tumores cerebrais, aumento do risco de cancro de mama, função imunológica alterada, dano de células nervosas, doenças cardiovasculares, abortos espontâneos, problemas de sono e até efeitos de curto prazo na cognição e no comportamento. Desta maneira e uma vez que a internet e os aparelhos *wireless* que utilizam redes *Wi-Fi* são cada vez mais um instrumento de lazer e trabalho presente no nosso dia-a-dia, têm surgido estudos acerca dos efeitos da radiação emitida por este tipo de aparelhos no aparelho reprodutor masculino. Vários estudos demostraram que a radiação eletromagnética proveniente de equipamentos *wireless* têm efeitos negativos na fertilidade masculina, sendo capaz de afectar os parâmetros espermáticos reduzindo a motilidade e viabilidade, aumentando a percentagem de espermatozóides com uma morfologia anormal e diminuindo a concentração espermática. Existem outros estudos que revelam que este tipo de radiação é também capaz de provocar alterações histopatológicas nos órgãos reprodutores masculinos e alterar a produção de hormonas importantes como a testosterona, essencial para uma função reprodutiva normal. No entanto, os estudos acerca deste tema apresentam falhas na concepção de um modelo realista de exposição à radiação e os efeitos desta radiação no metabolismo testicular ainda não foram objecto de estudo. Assim, para colmatar a principal falha encontrada foi criado um *set up* de exposição à radiação eleromagnética de 1.4GHz, passível de ser utilizado noutras experiências onde se pretenda fazer uma exposição à radiação electromagnética mimetizando uma situação real de utilização de internet através da transmissão de pacotes de informação. Para a validação do *set up* construído, espermatozóides de ratos adultos foram expostos

durante 1 hora à radiação eletromagnética do aparelho. Desta experiência os resultados revelaram uma diminuição significativa na motilidade dos espermatozóides do grupo exposto em relação aos do grupo controlo.

Tendo em conta que neste trabalho utilizámos cultura de túbulos seminíferos, verificou-se através de análise histológica se o tempo de 72h em cultura não provocava alterações histológicas nos túbulos, concluindo-se que não houveram alterações. Além disso, uma vez que também era pretendido criar um novo modelo para o estudo do metabolismo em ratos, de acordo com os dados encontrados na literatura, procurou-se determinar a melhor idade para estudar o metabolismo, sendo analisadas secções histológicas de ratos de 19, 20, 21 e 22 dias de maneira a confirmar qual a idade em que o epitélio dos túbulos seminíferos tinha uma população de células de Sertoli bem estabelecida, de extrema importância sendo estas as responsáveis pelo metabolismo testicular, e células germinativas apenas numa fase inicial da espermatogénese. Concluiu-se que ratos com 20 dias possuem um epitélio dos túbulos seminíferos que reúne as condições pré-determinadas, constituindo a idade ideal para estudar o metabolismo testicular em ratos.

O metabolismo testicular, onde as células de Sertoli têm um papel fundamental, é de extrema importância para a espermatogénese, processo fisiológico no qual se produzem os espermatozóides a partir de células germinativas. Estas células, localizadas no interior dos túbulos seminíferos, em situações normais, metabolizam a maioria da glucose a lactato, sendo o lactato o substrato preferido para as células germinativas em desenvolvimento obterem energia. Sendo através do metabolismo glicolítico testicular que as células germinativas obtêm suporte nutricional, um metabolismo testicular alterado tem consequências no processo de espermatogénese e consequentemente na função reprodutiva do homem. Não obstante, alguns estudos já demonstraram sob circunstâncias específicas, as células de Sertoli podem usar outros substratos em vez da glicose para obter energia. Efectivamente, existem estudos que sugerem que que alguns mecanismos metabólicos como o efeito de Warburg podem ocorrer não só em situações de cancro como também nas células de Sertoli. O ciclo de Krebs e a glutaminólise podem ser uma alternativa sendo a glutamina, a leucina e a alanina apontadas como possíveis substratos para obtenção de energia

Para o estudo do metabolismo testicular, túbulos seminíferos de ratos de 20 dias em cultura foram expostos ao equipamento construído. Os nossos resultados revelaram uma diminuição significativa da concentração de glucose extracelular, no entanto contraditoriamente existiu um aumento significativamente da produção de lactato. A actividade da enzima lactato desidrogenase, responsável pela conversão de piruvato em lactato, foi avaliada e embora não significativo, a radiação electromagnética causou um aumento da mesma. Tendo em conta que em situações normais a glucose é transformada em piruvato e posteriormente em lactato pela enzima lactato desidrogenase, seria de esperar que o aumento de lactato fosse acompanhado por um aumento do consumo de glucose, o que não se verificou. Os resultados

obtidos contraditórios entre o consumo de glucose e a produção de lactato sugerem que, quando expostas a radiação eletromagnética proveniente de dispositivos *Wi-Fi*, as células responsáveis pelo metabolismo testicular, nomeadamente as células de Sertoli nos túbulos seminíferos podem adoptar outra via metabólica para obtenção de energia como as mencionadas anteriormente.

Em conclusão, o nosso estudo clarificou que as radiações eletromagnéticas provenientes de equipamentos *Wi-Fi* têm efectivamente efeitos negativos nos parâmetros espermáticos, como a diminuição da motilidade e são efetivamente capazes de promover alterações no metabolismo glicolítico normal, que pode ter efeitos na espermatogénese e afectar a fertilidade masculina.

Abstract

In recent decades, several studies have shown a decline in male fertility. As wireless technologies, which use Wi-Fi, and the time spent with them are increasing, the relationship between these two facts has been a subject of investigation. There are several articles that demonstrate the negative consequences of electromagnetic radiation of wireless devices on male fertility, affecting sperm parameters: reducing motility and viability, increasing the percentage of spermatozoa with an abnormal morphology and decreasing sperm concentration. There are also studies that show that the electromagnetic radiation of these devices enhances oxidative stress and increases reactive oxygen species, and is also capable of causing histopathological changes in the male reproductive organs and even alter the production of hormones important for male fertility, such as testosterone. However, all studies show flaws in the design of a realistic model of radiation exposure and there are no studies concerning the effects of electromagnetic radiation on testicular metabolism.

A normal reproductive function is affected by the process of spermatogenesis, which in turn is dependent on the testicular metabolism, more specifically the Sertoli cells that provide nutritional support. These cells, located within the seminiferous tubules, in normal situations, metabolize the majority of glucose into lactate, with lactate being the preferred substrate for developing germ cells to obtain energy. However, some studies have already demonstrated in specific circumstances, Sertoli cells may use other substrates instead of glucose to obtain energy.

To carry out the study, an exposure set up was developed with different electronic components commercially available to expose the seminiferous tubules in culture, in a realistic way to the electromagnetic radiation. For the validation of the developed set up, spermatozoa of adult mice were exposed for 1 hour to the electromagnetic radiation of the apparatus. From this experiment the results showed a significant decrease in sperm motility of the exposed group comparing to the control group, validating the model.

From the metabolism study performed, exposing seminiferous tubules from 20-day-old rats to the built equipment, our results showed that electromagnetic radiation significantly decreased glucose consumption, however, contradictly lactate production increased significantly. The lactate dehydrogenase activity was evaluated and although not significant, the electromagnetic radiation caused an increase. Contradictory results between glucose consumption and lactate production suggest that, when exposed to electromagnetic radiation from Wi-Fi devices, cells responsible for testicular metabolism, namely Sertoli cells in the seminiferous tubules, may use alternative metabolic pathways to produce lactate and consequently obtain energy.

In conclusion, this study showed that the exposure model was successfully created and validated and that electromagnetic radiations from Wi-Fi equipment, in addition to causing negative changes in the sperm parameters of the cells, promote changes in normal glycolytic metabolism, suggesting the use of an alternative way of obtaining energy which may have effects on spermatogenesis and affect male fertility.

Keywords

Wi-Fi; Electromagnetic Radiation; Male Fertility; Seminiferous Tubules; Glicolitic Metabolism.

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Abbreviations

ADP	Adenosine Diphosphate
ATP	Adenosin Triphosphate
BTB	Blood-Testis Barrier
CAT	Catalase
DHT	5α-Dihydrotestosterone
DIP	Dual In-Line Package
DNA	Deoxyribonucleic Acid
DSSS	Direct Sequence Spread Spectrum
E2	17-ß-Estradiol
EMR	Electromagnetic Radiation
FHSS	Frequency Hopping Spread Spectrum
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
GPx	Glutathione Peroxidase
HPG-axis	Hypothalamic-Pituitary-Gonadal-axis
IDE	Integrated Development Environment
IEEE	Institute Of Electrical And Electronics Engineers
ISC	Industrial-Scientific-Medical
LAN	Local Area Network
LCs	Leydig Cells
LDH	Lactate Dehydrogenase
LED	Light-Emitting Diode
LH	Luteinizing Hormone
MCT4	Monocarboxylate Transporter 4
NAD+	Nicotinamide Adenine Dinucleotide
OFDM	Orthogonal Frequency Division Multiplexing
PBS	Phosphate-Buffered Saline
PCB	Printed Circuit Board
RAM	Random Access Memory
RF	Radiofrequency
RIPA	Radioimmunoprecipitation Assay Buffer
ROS	Reactive Oxygen Species
SCs	Sertoli Cells
SeT	Seminiferous Tubules
SOD	Superoxide Dismutase
SPI	Serial Peripheral Interface
T	Testosterone
TCP/IP	Transmission Control Protocol/Internet Protocol
UART	Universal Asynchronous Receiver/Transmitter

USB	Universal Serial Bus
Wi-Fi	Wireless Networking

1. Introduction

1.1 Anatomy and physiology of the male reproductive system

The male reproductive system is formed by the testis, a system of spermatic channels – *vasa efferentia*, epididymis, *vas deferens*, ejaculatory duct and part of the male urethra, seminal vesicles, bulbourethral glands, prostate gland and penis [1]. The general location of these structures is shown in figure 1.

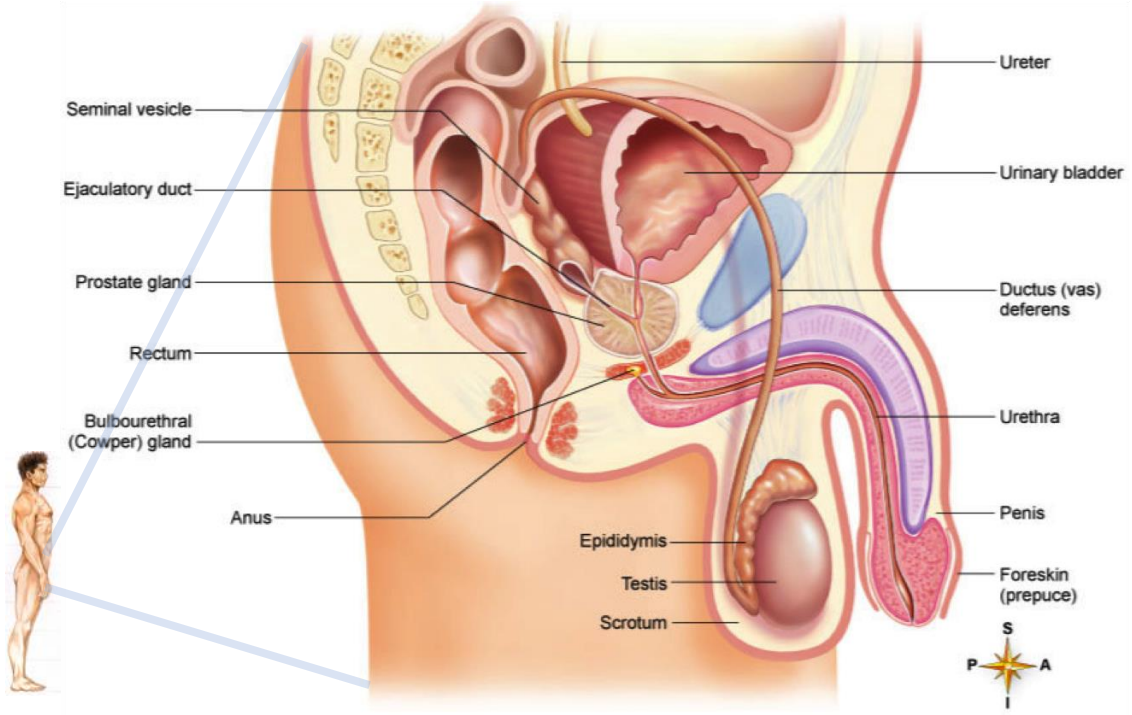

Figure 1: Organization of the male reproductive organs. Sagital section of pelvis showing placement of male reproductive organs. Adapted from [2].

The testis are responsible for the production, nurturing and storage of the male sex cells or gametes called spermatozoa, and the production of androgens, the male sexual hormones [3]. The testicle is a oval shaped organ located outside the body cavity suspended by the body wall by a spermatic cord which contains the vas deferens, a testicular nerve and three coiled blood vessels- the testicular artery and two testicular veins. Each testicle is covered by the *tunica vaginalis* and below this covering is located the *tunica albuginea*. The testicle is divided into approximately 250 testicular lobules wedge shaped separated by septa of connective tissue, each containing one to three seminiferous tubules (SeT; figure 2A), where the sperm is produced in a process called spermatogenesis [4].

The SeT have a lumen lined by a dense seminiferous epithelium containing two types of cells, Sertoli cells (SC, figure 2B) and male germ cells and are covered with a collagenous basement membrane with contractile myoid cells that promote the movement of mature sperm and testicular fluids through the tubules [4].

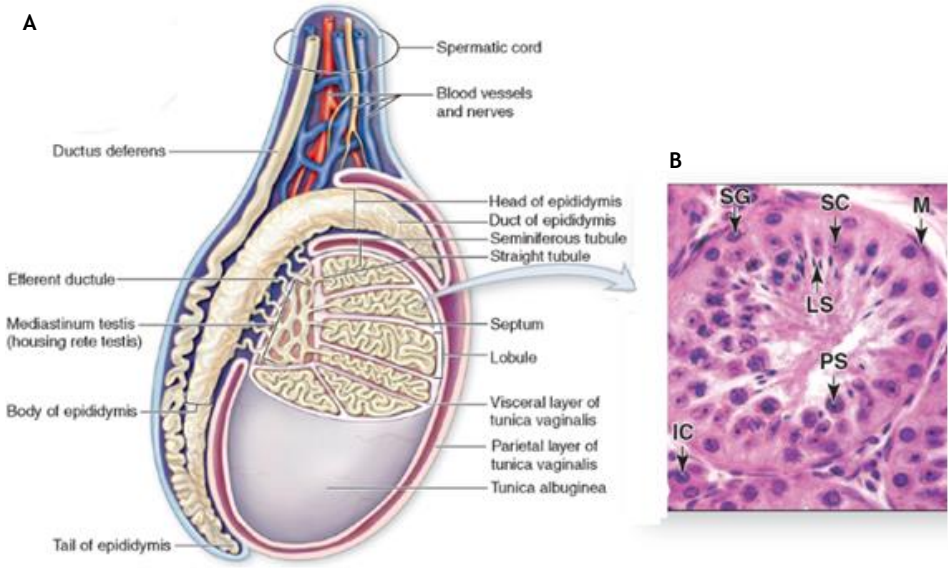

Figure 2: Diagram of the testis. A) The diagram shows a partially sagittal section of the testis. B) A seminiferous tubule cross section shows spermatogonia (**SG**) near the periphery, nuclei of Sertoli cells (**Sc**), primary spermatocytes (**PS**), and late spermatids (**LS**) near the lumen, with interstitial cells (IC) also called Leydig cells in the surrounding connective tissue. The seminiferous tubules are covered with a collagenous basement membrane with contractile myoid cells (M) X400. H&E. Adapted from [5].

SCs are pyramid shaped cells with irregular nucleus whose base lines against the basement membrane of each SeT and the tip is orientated towards the middle of the tube [6]. This cells, also called nurse cells have the function of nurturing, supporting the sperm cells during their differentiation, secretion of testicular fluid into the tubular cavity, proteins, like androgen binding protein and hormones like inhibin and Mullerian-inhibiting substances, production of enzymes that convert testosterone (T) to estrogen and 5α-dihydrotestosterone (DHT) and phagocyte degenerated sperm cells [7]. The adjacent SCs have tight junctions that provide a blood-testis barrier (BTB) which controls the chemical composition of testicular fluid in the SeT and protects the spermatocytes from an attack from the immune system as these are haploid cells and the immune system would recognize them as foreign [8 9]. The number of SCs is determined at puberty (except in some cases discussed later) and the number of sperm production is related with the number of SCs [10].

The interstitial space in the testicle contains small arteries capillaries and veins and in this space is where products like oxygen and glucose diffuse to the SeT from the blood [11]. This is the only way for the sperm cells to get glucose and oxygen because there are no blood vessels inside the SeT [12]. Hormones also pass from the interstitial space to the SeT through the basement membrane and waste products produced in the SeT move from the SeT to the interstitial space and leave this space by small veins [13]. Also in the interstitial space are Leydig cells (LC; Figure 2B). These cells are round and polygonal and have vesicular round nucleus with prominent nuclear membranes and one or two nucleolus. Their function is to synthesize and secrete androgenic steroid hormones [12]

1.2 Spermatogenesis

Normal male fertility is based on a normal spermatogenesis and this process represented in figure 3, has an extremely importance to men's fertility [13]. Spermatogenesis is a process regulated by the hypothalamic-pituitary-gonadal axis (HPG-axis) that happens in the SeT and is highly dependent on SCs, by which a diploid spermatogonium transforms into four haploid spermatids and is characterized by continuous cellular differentiations [14]. Immature germ cells, called spermatogonial stem cells, lay on the basement membrane where they replicate mitotically to guarantee the germ cell line. In this process, two diploid daughter cells are derived from a diploid parent cell, so each spermatogonium has 46 chromosomes. While the spermatogonial cells continue to proliferate, some of them begin another cell division process, meiosis, and become primary spermatocytes. Subsequently, these cells undergo the first division of meiosis and form the haploid secondary spermatocytes with 23 chromosomes. The secondary spermatocytes then undergo a second meiotic division which differentiates one secondary spermatocyte into four haploid equalized round spermatids [15].

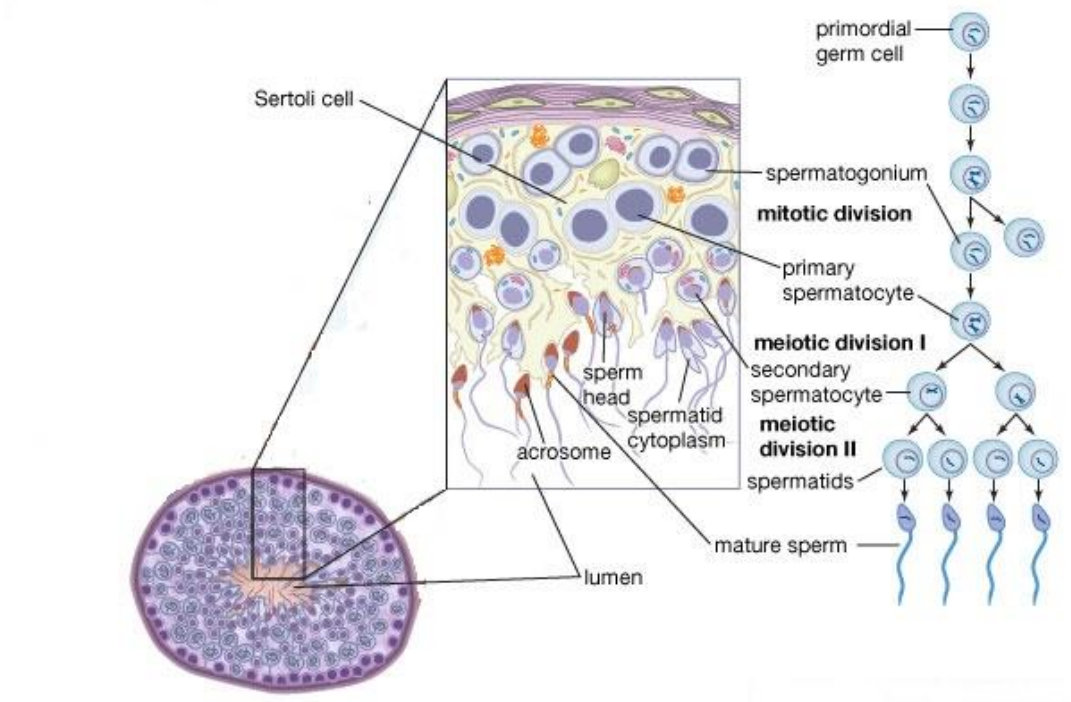

Figure 3: Diagram of spermatogenesis. The initial cells in this pathway are called spermatogonia, which yield primary spermatocytes by mitosis. The primary spermatocyte divides meiotically (Meiosis I) into two secondary spermatocytes and each secondary spermatocyte divides into two spermatids by Meiosis II. These develop into mature spermatozoa, also known as sperm cells. Adapted from [16]

Thereafter, spermiogenesis starts to transform these round spermatids into elongated spermatids, which through a process called spermiation are released into the lumen of the tubule as immature spermatozoa. In this way, in the SeT, the initial stages of spermatogenesis are present in the basal side of the tubule and mature spermatids are nearest to the lumen of the SeT [17].

1.3 Hormonal control of testicular function

The hormonal control of testicular function, represented in figure 4, is in charge of the hypothalamus, which produces pulses of gonadotropin-releasing hormone (GnRH) that leads to the secretion of the gonadotropins from the anterior pituitary gland: the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). LH stimulates LC to secrete T which diffuses into the SeT and enters the SCs which convert it to DHT [12]. Then, T and DHT leave the SCs and enter the testicular fluid to be in contact with the germ cells and contribute to spermatogenesis [18]. FSH acts on SCs, stimulating the synthesis of 17-β-estradiol (E2) from T in the testis. Then, FSH along with T stimulate spermatogenesis [20].

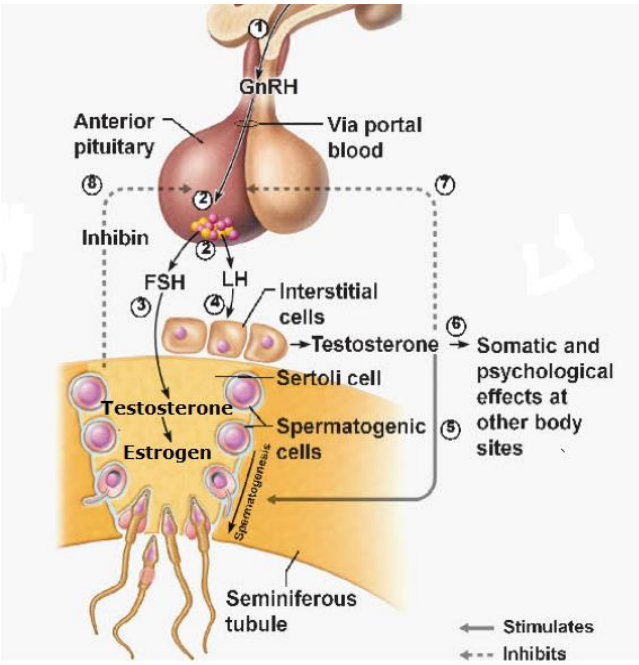

Figure 4: Diagram of the hypothalamic–pituitary–gonadal axis (HPG axis) which includes the hypothalamus, pituitary gland, and gonadal glands. The hypothalamus releases the gonatrophin releasing hormone (GnRH) (1) Which stimulates the anterior pituitary to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) (2). FSH acts on Sertoli cells (SCs) stimulating the synthesis of estrogen from testosterone (T) and spermatogenesis (3) and LH stimulates Leydig cells to produce T (4). T is essential for spermatogenesis (5) and it also has somatic and psychological effects at other body sites (6). A negative feedback mechanism is induced when testosterone reaches high concentrations in the blood, reducing or inhibiting the release of GnRH by the hypothalamus (7), which in turn will decrease LH and FSH production in the pituitary. Inhibin is secreted by SCs and also exhibits a negative feedback effect (inhibitory response) on the production of FSH by the pituitary (8). Adapted from [19]

Estradiol also has stimulating effects on spermatogenesis and it is discussed that the male germ cells are specially stimulated by estradiol and not testosterone as there are not androgen receptors in the germ cells. A negative feedback mechanism is induced when T reaches high concentrations in the blood, reducing or inhibiting the release of GnRH by the hypothalamus, which in turn will decrease LH and FSH production in the pituitary. Inhibin is secreted by SCs and also exhibits a negative feedback effect (inhibitory response) on the production of FSH by the pituitary [21].

1.4 Testicular metabolism

A normal spermatogenesis and fertility capacity of sperm depends on a correct testicular glucose metabolism. The process by which glucose is transformed into energy is called glycolysis, represented in figure 5. The first step of glycolysis is irreversible and consists of phosphorylation of glucose into glucose-6-phosphate, in the presence of adenosine triphosphate (ATP) and the enzyme hexokinase acting with Mg^{2+} ion as cofactor. Then glucose-6-phosphate is isomerized into fructose-6-phosphate, assisted by the enzyme glucose-phosphate isomerase. In the third step of the glycolysis, a second phosphorylation reaction is observed in which the fructose-6-phosphate is transformed into fructose-1,6-bisphosphate with the intervention of the phosphofructokinase enzyme, which has, as cofactor, the Mg^{2+} ion. Then, due to the action of an aldolase, fructose-1,6-diphosphate is cleaved into two isomeric trioses: phosphoglyceraldehyde and phosphodihydroxyacetone. Next, the only oxidation occurs during the glycolysis process by converting 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid. This oxidation takes place in the presence of inorganic phosphate and is catalyzed by a dehydrogenase which has the nicotinamide adenine dinucleotide (NAD^+) as cofactor. During the step, the energy released by the oxidation is transferred to the formation of a new phosphate bond. As the phosphoglyceraldehyde is oxidized, the phosphodihydroxyacetone will become phosphoglyceraldehyde and oxidized in turn. For each of the glucose molecules that "enters" the glycolysis process, oxidation of two phosphoglyceraldehyde molecules to diphosphoglyceride acid will occur. In the next step hydrolysis of the diphosphoglyceride occurs and the energy released by the hydrolysis is transferred to the synthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate. Finally, 3-phosphoglyceric acid is the subject of several reactions and is transformed into pyruvic acid with the phosphorylation of one more ADP in ATP [22]. Thus, in the course of glycolysis, for each glucose molecule, two molecules of pyruvic acid are produced. At the beginning of the process, energy was invested (2 ATP were consumed). At the end of the process energy was recovered in the form of 4 ATP. The balance is therefore 2ATP per glucose molecule [22]. Pyruvate can then follow three pathways: be converted to alanine by the action of alanine aminotransferase, enter the tricarboxylic acid cycle or, especially under low-oxygen conditions, be converted to lactic acid by lactate dehydrogenase (LDH) action [23].

In the testis, glucose metabolism is carried out by SCs once they support spermatogenesis not only spatially and energetically, but is also required for hyperactivated motility of fully developed germ cells (figure 6) [24] [25]. Although SCs have the ability to metabolize various substrates, they preferentially use glucose [26] [23].

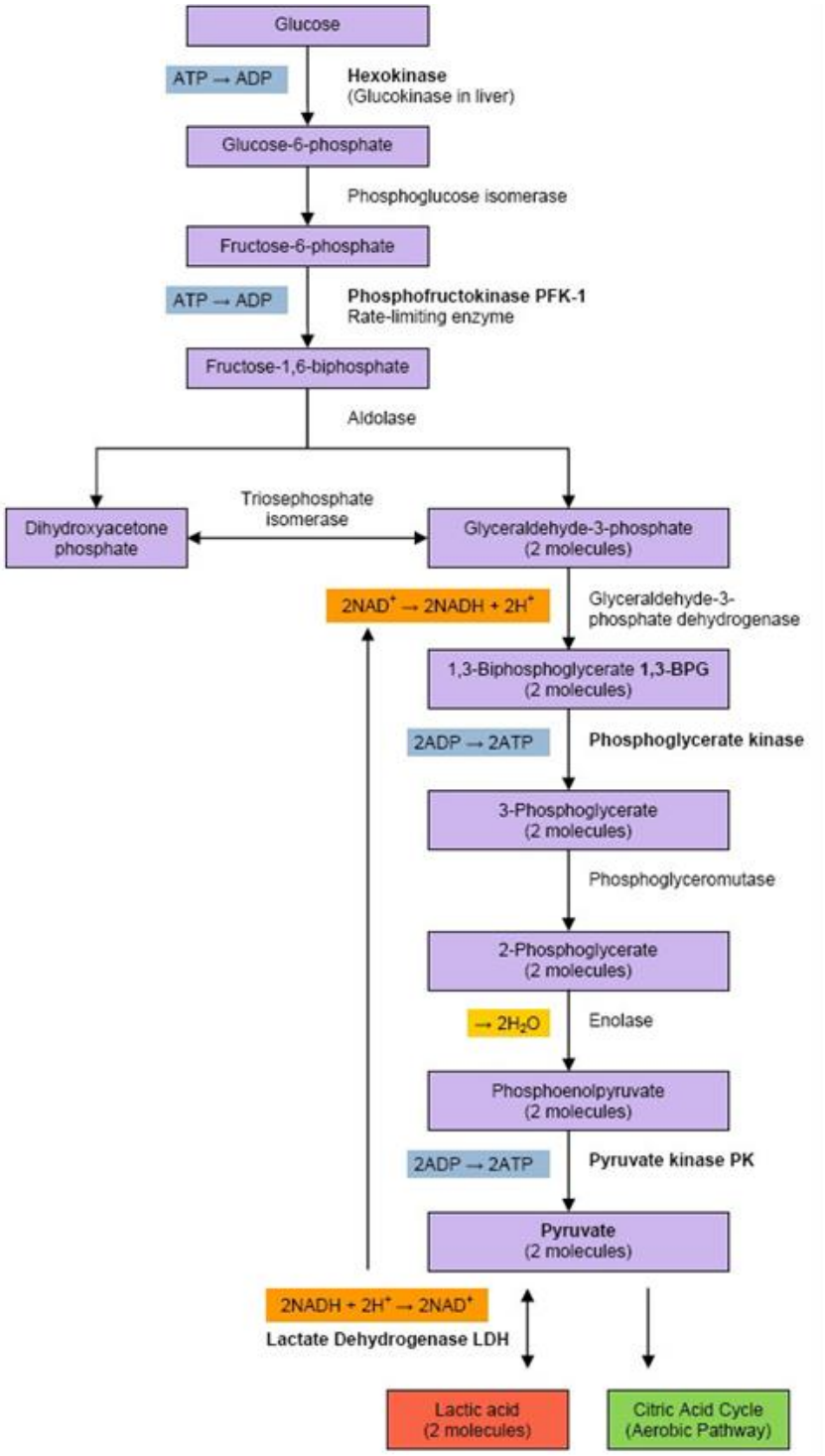

Figure 5: Schematic representation of glycolisys. Adapted from [27].

Lactate is one of the most important products secreted by SCs for maintenance of germinative cells during spermatogenesis because it's their preferred subtract to obtain energy, so SCs convert the most part of glucose into lactate [28]. The control on the production rate of lactate is on charge of specific glucose transporters (GLUTs) that control the membrane passage of glucose from the extracellular space to SCs [29]. So far, there have been identified four GLUTs- GLUT1, GLUT2, GLUT3 and GLUT8 in SCs. GLUT1, GLUT2 and GLUT3 have been identified in the plasmatic membrane of SCs but not GLUT8, so GLUT1, GLUT2 and GLUT3 are assumed to be the primary responsible for import glucose into SCs [31 32 33 34 35]. After being transported to the intracellular compartment of the cell, glucose suffers glycolysis [35]. Since LDH is responsible for the conversion of pyruvate into lactate it has an enhanced importance once lactate is the preferred subtract of germ cells to produce ATP. After produced, lactate is exported from SCs through the active membrane monocarboxylate transporter isoform 4 (MCT4) [23]. Alanine can also be a substrate, because it can be converted to pyruvate and then used by SCs [36].

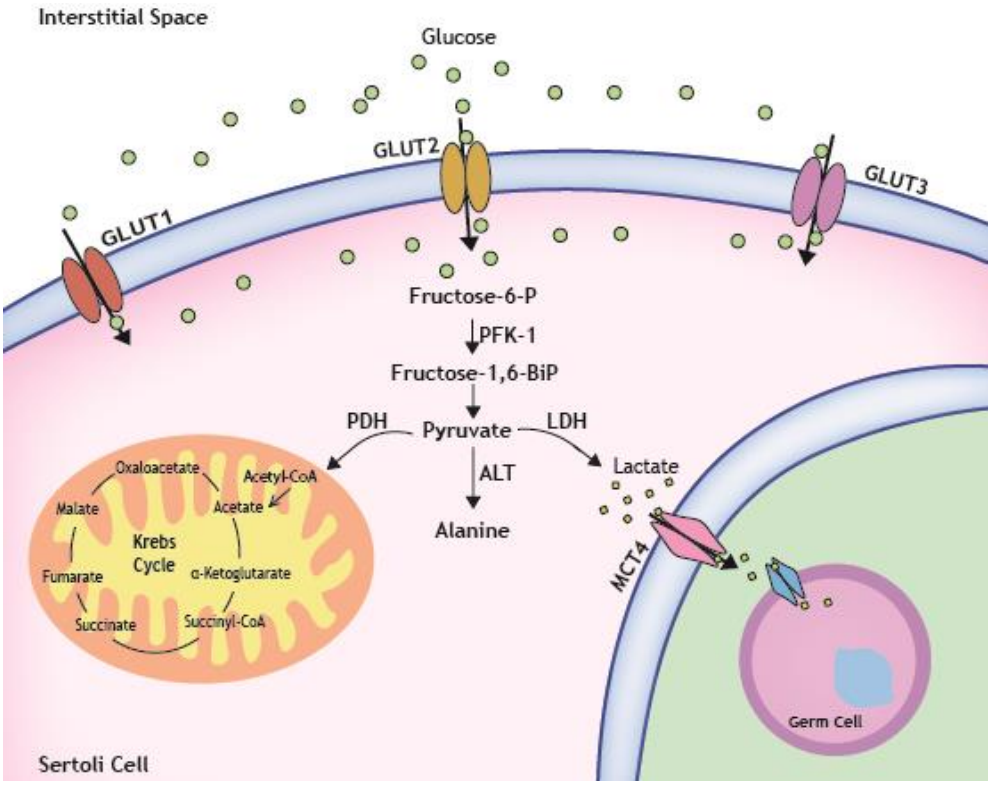

Figure 6: Schematic illustration of the glucose metabolism of Sertoli cells (SCs). In SCs, glucose from interstitial space enters through high-affinity glucose transporters (GLUTs), present in the plasmatic membrane: GLUT1, GLUT2 and GLUT3. Glucose is converted to pyruvate which can follow three distinct 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. Acetate and lactate are exported to the interstitial space by monocarboxylate transporter isoform 4 (MCT4).

It has been shown that SC glucose metabolism is predominantly regulated by the endocrine system especially by sex steroid hormones, follicle-stimulating hormone and insulin [23] [38] [39]. Moreover, glucose may itself regulate its own metabolism and transport. Some studies with cultured mammalian cells showed increased rates of glucose uptake in response to glucose deprivation, suggesting glucose can regulate its metabolism [39].

Since glucose metabolism has such an important role in spermatogenesis, dysfunctions in SC glucose metabolism and transport may lead to male subfertility or infertility.

1.5 Insights on wireless technologies

Wireless devices like mobile phones and computers are increasingly present in our lives and progressively wireless networking (Wi-Fi) interfaces are being incorporated in household devices like audio equipment, bathroom scales, games, running shoes and are present in devices that we use in our daily life like computers or advanced mobile phones [40]. Wi-Fi communication is based on pulse radiofrequency (RF) signals with no signal between the bursts [41]. The first version of communication standards was IEEE (Institute of Electrical and Electronics Engineers) 802.11 family and appeared in 1997. Since then, many other versions of IEEE have emerged such as IEEE 802.11a, b, g and n [40]. Wi-Fi may have different frequency ranges and modulations. The 2.400-2.4835GHz range which belongs to the industrial-scientific-medical (ISC) band (2.4-2.5GHz) is where most of the Wi-Fi devices operate (IEEE 802.11, b, g and n). This band is used for digital communication devices such as cordless phones, wireless interfaces as ZigBee or Bluetooth and for medical purposes. Other Wi-Fi devices operate near 5GHZ, using IEEE 802.11 a or n. The physiological difference of these frequencies is the penetration depth in the body as the higher the frequency, the shorter the penetration [42]. The bandwidth of the channel is 20MHz for all IEEE 802.11 version except IEEE 802.11n which has two 20MHz channels, allowing a higher rate of data transmission. According to the version of IEEE 802.11 that is used, there are different modulations techniques for the pulse such as Direct Sequence Spread Spectrum (DSSS), Frequency Hopping Spread Spectrum (FHSS) and Orthogonal Frequency Division Multiplexing (OFDM). Other parameter that varies according the IEEE 802.11 version is the data rate per stream ranging from 1-2 $Mbits^{-1}$ to 600 $Mbit^{-1}$ for the original and 808.11 n versions respectively. The data rate influences the RF energy necessary, the higher the data rate, the more energy it requires and consequently more pulses [43].

1.6 Physiological effects of EMR from wireless devices on male reproductive system

Electromagnetic radiation (EMR) has been prove to have various physiological effects on male reproductive system (see table 1), and having negative consequences on male fertility.

Several studies have found an association between EMR and oxidative stress showing that Wi-Fi radiation can decrease significantly the activity of reactive oxygen species (ROS) scavenging enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [44] [45]. There are also evidences that EMR from Wi-Fi devices contribute to deoxyribonucleic acid (DNA) damage [46] [47] [48]. EMR can also alter the production of hormones crucial for the proper functioning of the male reproductive system like T [45] [49] [50]. Radiation from wireless devices may even be harmful to male fertility by causing histopathological and ultrastructural changes in the testes such as increase in *membrana propria* thickness and collagen fibers as well as an irregular basal membrane, irregularities in SCs more specifically a nucleus with irregular contour, high number of immature cells in the lumen, tubules with reduced spermatogenic cell lines and also tubules without lumen [51] [52]. Dasdag and his colleagues found observable differences in the tubules diameter and *tunica albuginea* thickness after long term exposure to 2.4GHz radiation (Figure 7) [53].

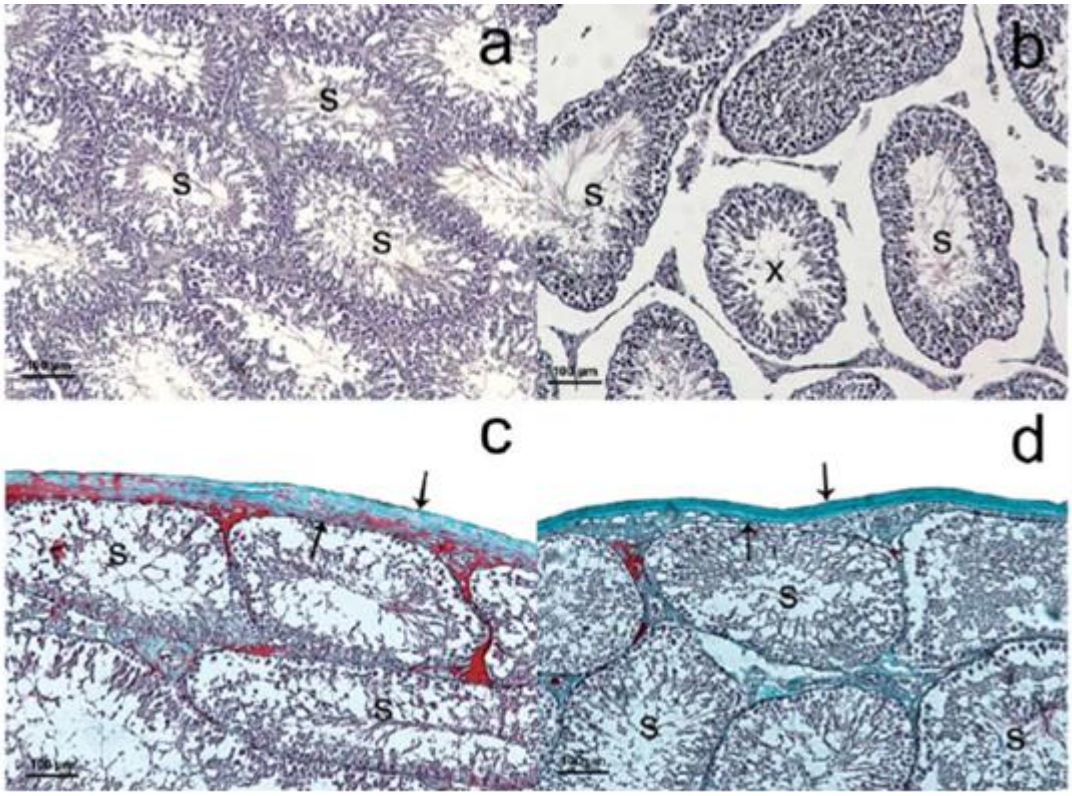

Figure 7: Testes section of a rat after exposure to 2.4 GHz electromagnetic radiation. A and C are from control and B and D are from exposed group. Seminiferous tubules diameter (S) and *tunica albugínea* thickness (arrows) decreased in exposed group. Also a tubule with disorganized view due to loss of germinal epithelium (b) is seen in the figure (arrowhead). H&E (a, b), Masson Trichrome (c, d). [53]

It has also been reported degenerative changes in spermatogenic cells, sharp edge craters, shrinkage on the surface of degenerating cells in seminiferous epithelium, visible debris of degenerating cells and residual cytoplasm and ruptured sperm head and distorted tail [54]. In Shokri *et al.*, [55] study where they exposed rats to 2.45GHz Wi-Fi radiation the 7-hour exposure

group but not the 1-hour group showed a significant decrease in the number of germ cell layers. The exposure for 1 and 7 hours also caused a decline in seminal vesicles weight. As in Shokri *et al.*, [55] other studies also found not only a significant decrease in seminal vesicles weight but also in epididymis. The EMR from wireless devices has been shown to have a negative effect in sperm parameters such as decrease in viability, sperm count and motility [45]. Yan *et al.*, [56] exposed rats to 1.9GHz EMR in a cycle of 3 hours of exposure followed by a 30 minute period without expose and again 3 more hours of exposing for 18 weeks and the results revealed a majority of sperm cells without motion, dead or with straight rigid tails in the exposed rats.

There are also evidences of negative effects of prenatal exposure to EMR radiation on the sperm quality of the descendants. Odaci *et al.*, [57] exposed pregnant rats to 900MHz EMR for 1h/day during days 13-21 of pregnancy and analyzed the epididymis of the rats with 60 days who were born from those exposed female rats. They found a lower sperm motility and viability and also histopathological changes in the epididymis and alterations on spermatogenesis.

However there are also studies that found no effects of EMR on the male reproductive system. Dasdag and his colleagues who exposed rats to long term 2.4GHz EMR found no significant differences in sperm concentration, sperm motility and total morphological defects [53]. Besides, in other studies, Dasdag observed no differences of short (20min/day for 1 month) and long (2h/7days for 10 months) periods of EMR radiation on the apoptotic cell number in the testes [59 60]. Also Saygin *et al.* [59] did not observe any significant effect of Wi-Fi (2.45GHz) on the diameter of seminiferous tubules.

Although there are many studies about the effects of Wi-Fi radiation on several parameters related to male fertility, there are no studies regarding the effects of EMR from wireless devices on testicular metabolism.

Table 1: Recent studies of the negative effects of EMR radiation from wireless devices. Supportive.

Study/Year	n/specie	Exposure	Effects
Fejes *et al.*, 2005[60]	37/human males	4 groups: Cell phone users for less than 15 min/day or over 60 min/day; kept their cell phone within a distance of 50cm for less than 1h/day or for more than 20h/day.	↓ Rapid progressive motile sperm; ↑ Slow progressive motile sperm. (with the duration of possession and the minutes of daily transmission).
Koyu *et al.*, 2005[50]	30/ Sprague-Dawley male rats	EMR 900MHz, 2W/kg, 30min/day for 5days/week, for 4 weeks.	↓ Serum TSH and t3-t4 levels.
Erogul 2006[61]	27/human	EMR 900MHz for 5 min.	↓ Rapid progressive motile sperm; ↑ Slow progressive and nonprogressive, non-motile sperm.

Yan *et al.*, 2007[56]	16/Sprague-Dawley male rats	EMR 1.9Hz, distance of 1 cm for 6h/day for 18 weeks.	↓ Sperm motility; ↑ Sperm cells dead; Clumps of sperm cells.
Wdowiak *et al.*, 2007[62]	304/human males	3 groups: No use of mobile phones, sporadic use for the period of 1-2 years or regular use for more than 2 years.	↓ Sperm motility and vitality; ↑ Abnormal morphology of sperm cells; (with the increase of mobile phone use.)
Agarwal *et al.*, 2008[63]	361/human males	4 groups according to daily active cell phone use: group B <2 h/day, group C 2–4 h/day and group D >4 h/day and a group A as control.	↓Sperm count, viability and normal morphology (with the increase of cell phone use).
Agarwal *et al.*, 2009[64]	32/human males	EMR 850MHz, 1.46W/kg, at a distance of 2.5cm for 60 min.	↓ Sperm motility and viability; ↑ ROS level; No significant differences in DNA integrity.
De Iuliis et al., 2009[46]	22/human males	EMR 1.8GHz, 0,4W/kg-27.5W/kg SAR, incubated for 16h.	↓ Motility and viability.
Kesari *et al.*, 2010[65]	12/Wistar male rats	EMR 0,9W/kg SAR, 2h/day, for 35 days.	↓ Protein kinase C; ↑ Apoptosis.
Al-Chalabi & Al-Wattar 2011[66]	300/human males	4 groups with different hours of active mobile phone use: 4h/day, 3h/day, 2h/day, no active use; 2 groups according the duration of use in years: 1-3 years or 4-6 years; 3 groups according the position of storage: trouser pocket, waist pouch or in the shirt pocket	↓ Sperm count, motility and normal morphology with the increase of active mobile phone use, duration of use in years and proximity of storage position to the testes.
Gutschi *et al.*, 2011[67]	2110/human males	2 groups: men that use cell phones and men that don't use cell phones.	↑ % Of abnormal morphology and teratozoospermia; ↓ Proportion of progressive motile sperm; ↑ Testosterone and luteinizing hormone levels. (in the cell phone users group)
Meo *et al.*, 2011[49]	40/male rats	Mobile phone EMR placed inside the cage and a call was given for 30min/day or 60min/day for 3 months.	↑ Proportion of hypospermatogenesis and maturation arrest; ↓ Serum testosterone level. (in the group exposed to 60min/da).
Esmekaya *et al.*, 2011[68]	30/ Wistar albino male rats	EMR 900MHz, 1.20W/kg, 20min/day for 3 weeks.	Induced oxidative injury in testes by ↑ nitric oxide levels and ↓ antioxidant defense mechanisms.
Kesari *et al.*, 2011[69]	12/Wistar male rats	EMR 10GHz, 0.014W/kg, power density of 0.21mW/cm^2, 2 h/day for 45 days.	↑ ROS levels and apoptosis.
Falzone *et al.*, 2011[70]	12/human males	EMR 900-MHz SAR of 2.0 W/kg, 1 h.	↓ Morphometric parameters, such as the analysis of major and minor axis, area, perimeter and acrosome; ↓ Sperm binding to the hemizona.
Oni *et al.*, 2011[71]	10/human males	Wi-Fi EMR 2.45GHz for 1 hour.	No effects on sperm concentration.

Avendaño *et al.*, 2012[47]	24/human males	Wi-Fi EMR 2.4GHz for 4 hours.	↓ Sperm progressive motility.
Lee *et al.*, 2012[72]	50/Sprague-Dawley male rats	EMR simultaneously 848,5MHz and 1950MHz, 45 min/day, 5 days/week with a total of 12 weeks	Did not observed adverse effects on rat spermatogenesis
Çelik *et al.*, 2012[51]	30/Wistar-Kyoto male rats	2 cell phones, SAR values of 1,58 for 3 months	↑ Membrana propria thickness and collagen fibers; Irregular basal membrane; Nucleus with irregular contour in Sc.
Atasoy *et al.*, 2012[48]	10/Wistar albino rats	2.437GHz from a Wi-Fi device, 24h/day for 20 weeks.	↓ Testicular biopsy score; ↑ Level of 8-hydroxy-2'-deoxyguanosine; ↓ CAT and GPX activity.
Nisbet *el al.*, 2012[73]	33/Wistar albino rats	1800 and 900MHz, 2h/day/90days	↑ Plasma testosterone level ↑ Epididymal sperm motility and normal morphology
Khavanin *et al.*, 2013[74]	28/Wistar male rats	3 exposure groups: EMR 915 MHz,8h/day for 14 and 21 days (group 2 and 3 respectively) and 950MHZ for 8h/day/14days (group 4).	↓ Sperm viability (was more notable in experimental group 3) ↓Sperm motility
Shahin *et al.*, 2014[45]	40/male mice	EMR 2.45GHz, 0.018W/kg, 0.029812mW/cm^2 power density, 2h/day for 30 days.	↓ Sperm count, sperm viability, seminiferous tubule diameter and serum testosterone level. ↑ROS production, total nitrite and nitrate concentration and in mda; ↓ SOD, CAT and GPX activity.
Karaman *et al.*, 2014[52]	21/Wistar albino male rats	2 exposure groups: EMR SAR of 1,52W/Kg, group 1 talk mode for 8 hours and standby for 8 hours for 20 days, group 2 same exposure but after those 20 days the rats were exposed to stand by mode for more 20 days.	↑ Immature cells in the lumen; ↓ Spermatogenic cell lines; Tubules without lumen.
Kumar & Shukla, 2014[54]	24/Swiss male rats	EMR from cell phone during 3h followed by 30 minutes of rest, followed by another 3h exposure/day/5months.	Degenerative changes in spermatogenic cells, sharp edge craters, shrinkage on the surface of degenerating cells in seminiferous epithelium, visible debris of degenerating cells and residual cytoplasm and ruptured sperm head and distorted tail.
Dasdag *et al.*, 2014[53]	16/Wistar albino rats	2.4 GHz radiation for 24h/day/12months.	↑ Percentage of head defects; ↓ Seminal vesicles and epididymis weight; ↓ Seminiferous tubules diameter and tunica albuginea thickness.
Gorpinchenko *et al.*, 2014[75]	31/human males	EMR, frequency range of 900-1800MHz for 5 hours in combined standby/talk mode.	↓Spermatozoa with progressive movement.
Bahaodini *et al.*, 2015[76]	14/male rats	EMR 1 mT,50 Hz low frequency, for 85 days 24 h/day.	↓ Lumen diameter and area of the seminiferous tubules; ↓ Total diameter and cross sectional area; ↑ Number of seminiferous tubules per unit area of testis

Shokri *et al.*, 2015[55]	27/Wistar rats	2 exposure groups: 2.45GHz Wi-Fi radiation, 1 hour/day/2 months and another and 7hours/day/2 months.	↓ Percentage of motile sperm, concentration and proportion of normal to abnormal sperm; ↓ Seminal vesicles weight; ↓ Germ cell layers and ↑apoptotic cells (only in the 7h/day group)

EMR- Electromagnetic radiation; SAR- Specific absorvation rate; ROS- Reactive oxygen species ; CAT- Catalase; GPX- Glutathione Peroxidase; SOD- Superoxide Dismutase; DNA- Deoxyribonucleic acid. ↑- Increased significantly; ↓- Decreased significantly.

2. Aims of the project

A normal spermatogenesis and fertility capacity of sperm depends on a correct testicular metabolism, which in turn depends on the correct functioning of several metabolic pathways. The processes involved on testicular metabolism may be affected by several factors such as environmental factors, a sedentary lifestyle and even the use of devices with Wi-Fi connection. This last factor has been receiving increasing attention from researchers since more and more devices using Wi-Fi connections, such as mobile phones and computers, are present in our daily life. Thus, we are almost constantly emerged in EMR from Wi-Fi networks. Recent advances have highlighted that the exposure to EMR from Wi-Fi networks is an important contributor to the decline of male reproductive health. In fact, there are several papers that evidence the negative consequences of EMR from wireless devices on male fertility affecting the sperm parameters, increasing the oxidative stress and ROS, contributing to histopathological chances in the male reproductive organs and even alter the production of important hormones such as T. However, the possible effects of Wi-Fi networks on testicular metabolism has never been studied which lead us to the development of this project.

The general aim of the research described in this work was to disclose the association between EMR from Wi-Fi devices and male infertility, dissecting its possible effects on testicular metabolism, particularly glucose metabolism, and the subsequent consequences for male reproductive health. It is also intended to develop a new model for the study of the effects of EMR on testicular metabolism using 20-day-old rats.

To achieve this, we first aimed to perform histological analysis to observe the cell populations present in SeT at 19, 20, 21 and 22 days in order to understand what is the most appropriate age for the development of the new model. Secondly we intended to perform an histological analysis to SeT after 72 hours in culture medium to assess if there were any observable histological changes in the tissue after the time of incubation. Then, we wanted to built an Wi-Fi exposure set up, to expose our SeT cultures to EMR on a realistic way. As there are studies showing that EMR has effects on sperm parameters, we decided to confirm these results by exposing sperm cells from an adult rat to EMR from our Wi-Fi network and assess their mobility, viability and morphology after exposure, validating our set up. Lastly, to disclose the effects of Wi-Fi on testicular metabolism, the metabolite production of lactate and glucose consumption were measured as well as LDH activity.

3. Materials and methods

3.1 Chemicals

Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM:F12), Bradford reagent, 3-Isobutyl-1-methylxanthine and phenylmethanesulfonyl fluoride were obtained from Sigma-aldrich (St. Louis, USA); gentamicin was obtained from Alfagene (Carcavelos, Portugal); Protease Inhibitor Cocktail 5 MammCell/Tissue was obtained from PVL (Famões, Portugal); Hematoxilin and aqueous eosin 1% were obtained from Leica Microsystems (Wetzlar, Germany); inclusion agent for histology Histosec was obtained from Merk (Darmstadt, Germany).

3.2 Instruments

The system used to create an internet network was constituted by three components: The WiFly module, the mbed and the internet router.

The wifly module, represented on figure 8, is a standalone device that enables wireless access to a local area network (LAN). It is certified to operate on 2.4GHz IEEE802.11b/g networks and it has a flash memory of 8Mbit and a random access memory (RAM) of 128KB. It also has a slave interface universal asynchronous receiver/transmitter (UART) which is a computer hardware device for asynchronous serial communication in which the data format and transmission speeds are configurable and the electric signaling levels and methods (such as differential signaling, etc.) are handled by a driver circuit external to the UART, and a serial peripheral interface (SPI) which is a synchronous serial communication interface specification used for short distance communication, primarily in embedded systems. The module has internally implemented the transmission control protocol/internet protocol (TCP/IP) stack that is the suite of communications protocols used to connect hosts on the Internet. Once properly set up, the radio trough the wire antenna automatically establishes a connection to the Wi-Fi network. The firmware allows establishing a communication channel between the radio channel and the UART. There were used two wifly modules, one had the function of sending data packets and the other had the function of receiving the data packets. Every time this communication was made the information (data packets) had to always first pass through the internet router. The wifly module also has three light-emitting diodes (LED's) a green one, a red one and a yellow one, each one providing information about the status: If the red LED is blinking rapidly it indicates that the module is not connected to the wireless network. Contrarywise, if the LED is off, the module is connected to the wireless network; If the yellow LED is blinking, each blink means it is either sending or receiving data; if the green LED is on and solid, it indicates the module is connected over TCP, if it is blinking rapidly, it means that no IP address is assigned, if it is blinking slowly, it means that the IP address is assigned but still not conected to TCP. The minimum operating temperature is -40°C and the maximum operating temperature is 85°C. The humidity range operating values are bellow 90% [77].

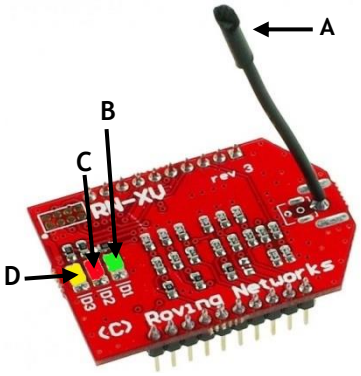

Figure 8: The wifly module. It is a standalone device that enables wireless access to LAN (*local area network). The module is constitued by a* flash memory, a RAM (Random Access Memory), an interface UART (Universal Asynchronous Receiver/Transmitter), a hardware device for asynchronous serial communication in which the data format and transmission speeds are configurable and the electric signaling levels and methods are handled by an external driver circuit; a SPI (*Serial Peripheral Interface),* a synchronous serial communication interface specification used for short distance communication; a TCP/IP (Transmission Control Protocol/Internet Protocol) stack, a suite of communications protocols used to connect hosts on the Internet and a wire antenna (A) that establishes a connection to the Wi-Fi network. The module also has three LED's that provide information about the status of the module. If the green LED (B) is on and solid, the module is connected over TCP, if it is blinking rapidly, no IP address is assigned, if it is blinking slowly, the IP address is correctly assigned; if the red LED (C) is blinking rapidly, the module is not connected to the wireless network, if it is off, the module is correctly connected; If the yellow LED (D) is blinking, it is either sending or receiving data. Adapted from[77]

Mbed, (represented in figure 9, panel A) is a platform and operating system for internet-connected devices based on 32-bit RAM Cortex-M microcontrollers. Such devices are also known as Internet of Things devices. The application for the mbed platform was developed using the mbed online integrated development environment (IDE*)*, which is an online code editor and compiler in which the code was written and compiled within a web browser, and compiled on the cloud using the ARMCC C/C++ compiler. It was used the mbed Microcontroller Board- mbed NXP LPC1768- a demo-board based on an NXP microcontroller, which has an ARM Cortex M3 core, running at 96 MHz, with 512 KB flash, 64 KB RAM. It is packaged as a small dual in-line package (DIP) form-factor which is an electronic component package with a rectangular housing and two parallel rows of electrical connecting pins for prototyping with through-hole printed circuit boards (PCBs), stripboard and breadboard, and includes a built-in universal serial bus (USB) flash programmer. There is also a USB port through which it supplies power to the system and a reboot bottom to restart running the program. There were used 2 mbed boards, each one connected to a wifly module. The Wifly terminals are identified in figure 9, panel B and the RN-XV-171 was connected to the mbed according to the figure. The minimum operating temperature is -65°C and the maximum operating temperature is 150°C. The humidity range operating values are not specified [78].

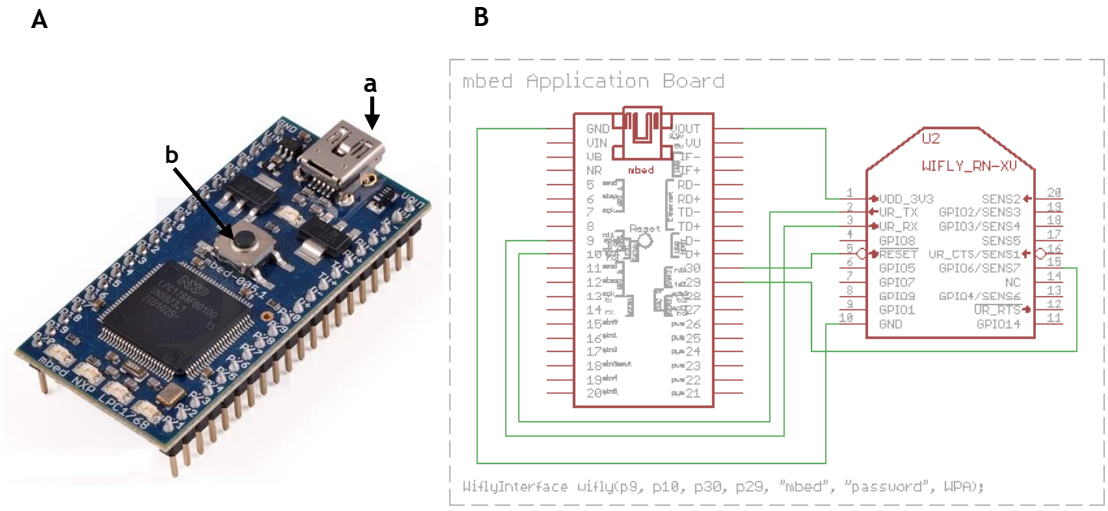

Figure 9: mbed board and its connection to wifly terminals. A) mbed board. The mbed board is a demo-board based on an NXP microcontroller, which has an ARM Cortex M3 core, RAM, a small DIP (dual in-line package) form-factor which is an electronic component package with a rectangular housing and two parallel rows of electrical connecting pins for prototyping with through-hole PCBs (printed circuit boards), a stripboard and breadboard, and a built-in USB (Universal Serial Bus) flash programmer. It has also a USB port (a) through which it supplies power to the system a reboot bottom to restart running the program (b). B) The mbed and Wifly terminals are represented in the figure, through this scheme it is possible to understand how to connect the wifly module to the mbed board. Adapted from[78]

The internet router used was TP-LINK model number TL-WR740N, represented in figure 10, a 150MBps wireless router with 9V power and a frequency band of 2.4 GHz. The minimum operating temperature is 0°C and the maximum operating temperature is 40°C. The humidity range operating is 10 to 90% of humidity [79].

Figure 10: The internet router TP-LINK model number TL-WR740. A 150MBps wireless router with 9V power and a frequency band of 2.4 GHz. a- Wi-Fi horn used to transmit and receive signals; b- Wi-Fi hardware. Adapted from [79]

3.3 Animals

One wistar male rat (*Rattus norvegicus*) 19-day old, 21-day old, 22-day-old and 3-months old as well as twenty-five wistar male rats (*Rattus norvegicus*) 20-days old, were obtained and housed at CICS-UBI animal facilities under a 12-hour light/dark cycle, with food and water available *ad libitum*. Animals were handled in compliance with the guidelines established by the "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European Union rules 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 "Direção Geral de Veterinária" (Portuguese Veterinarian and Food Department). All rats were euthanized with CO_2.

3.4 Development of a new model to study the effects of Wi-Fi on seminiferous tubules metabolism *ex vivo*

3.4.1 Determining the ideal age for the development of the model

There are studies that indicate that mitotic division of SCs ceases after the 15 day of post-natal development in rats, preceding the formation of the hematopoietic barrier created by the inter-sertoli tight junctions between days 16 and 19 [80]. Another study indicates that the mitotic division of the SCs ceases at 18 days [81]. In view of the number of published studies on the metabolism of SCs [82] [83], a model using a rat with an epithelium of seminiferous tubules containing a well established SC population and germ cells exclusively in the early stages of spermatogenesis would constitute an excellent model for the study of metabolism in rats. In order to assess the best age to implement this model, histological sections from SeT from 19 days-old, 20 days-old, 21 days-old and 22 days-old rats were analysed to assess which type of cells were present and to observe the SCs population.

3.4.2 Assessing the possible effects of 72h in culture medium

As the possible effects of the culture medium in SeT with rats at this age have never been assessed, histological analysis was performed comparing t=0 and t=72 of SeT of a 22-day-old rat in culture medium.

3.4.3 Validating the model

Several articles show that radiation from Wi-Fi devices has effects on sperm [60]. In this way, to validate the set up and built Wi-Fi network, spermatozoa from six 3 months-old adult rat were extracted, placed at a Petri dish and exposed 1 hour to the Wi-Fi network in the incubator at 33ºC as represented in figure 11. The sperm parameters were analyzed and compared to the control, constituted by spermatozoa extracted and place at a petri dish in

the incubator for 1 hour at 33ºC but not exposed to the Wi-Fi network. For each rat, one epididymis was used for exposure and the other one as control.

Figure 11: Disposition of the petri dish containg sperm cells for the exposure to the Wi-Fi network. A- Wifly; B- mbed; C- Wi-Fi router; D- The petri dishes are placed between the receptor and sender.

3.5 Effects of Wi-Fi on rat testicular metabolism *ex vivo*

Twenty-four 20-day-old rats were used to study the effects of Wi-Fi on testicular metabolism. The SeT were extracted and cultured, the exposed group (12 rats) received the EMR from the created Wi-Fi network during 72h. The 72 hours were chosen as the exposure time once daily most people are exposed to EMR from Wi-Fi networks 24 hours a day, day after day. Thus, the choice of this exposure time is intended to be as close as possible to the actual situation. The control group (12 rats) was not exposed to the Wi-Fi network.

Every 12 hours it was verified the correct functioning of the system by accessing the network created through the router IP and checking if there was a correct transfer of data packets which indicated that there was continuous communication. Also visually it was possible to check the correct functioning of the system by observing on the mbed a constantly lit green LED which indicates that the mbed has connected correctly to the router and an yellow LED flashing rapidly, indicating that the mbed boards are receiving and sending data packets. A new data packet was transferred every 0.5 seconds. The set up is represented in figure 12.

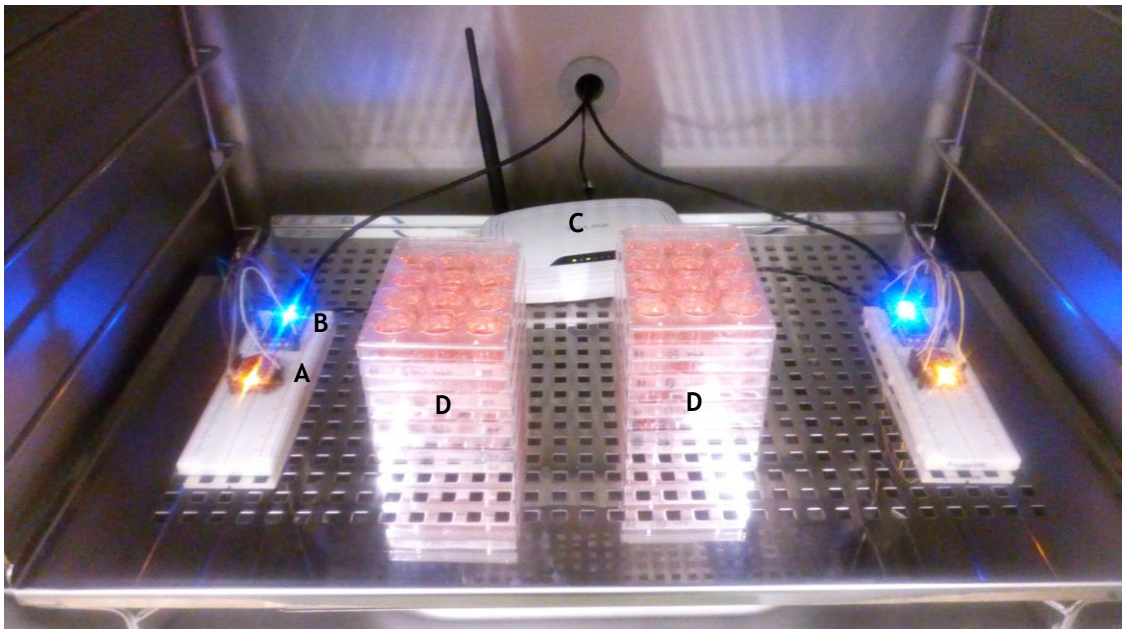

Figure 12: Set up disposition. A- Wifly; B- mbed; C- Wi-Fi router; D- The culture plates are placed between the receptor and sender. At each time 8 plates were expose.

3.6 *Ex vivo* culture of immature rat SeT

Testicles from twenty-four 20-day-old rats and one 22 day-old rat were removed, trimmed free of fat, washed in cold phosphate-buffered saline (PBS) and placed in Dulbecco's modified Eagle's medium/Ham's F12 culture medium (Sigma-Aldrich, St. Louis, USA) supplemented with 20 mg/L gentamicin sulfate, 0.1 mM 3-isobutyl-1-methylxanthine, and 1 µg/L of bovine serum albumin (BSA) 10% at 33°C. *Tunica albuginea* was cut and peeled back to expose tubules. As the immature SeT are very tangled, it is difficult to pick individual fragments, thus, the SeT from one testicle was reparted to the 12 wells of a culture plate (Nunclon D 12 well multidishes; Nunc, Roskilde, Denmark), each well containing 5 ml of pre-warmed culture medium. In this way, 48 plates were used, each one corresponding to a testicle.

3.7 Histological analysis

Testicles from 19 day-old, 20 day-old and 21 day-old animals were extracted and the entire testicles were included side to side in paraffin in order to obtain sections with many cross sections of the SeT which alow us to observe the SeT epithelium. The paraffin sections (5 µm) of SeT were deparaffinized in xylene for 10 minutes and rehydrated in graded alcohols (1 minute in ethanol 100%, 1 minute in ethanol 70% and 1 minute in running water). Then the paraffin sections were stained and differentiated first 7 minutes in hematoxylin, then 1 second in hydrochloric ethanol (differentiator), 4 minutes in running water, 1 minute in eosin 1% and 10 seconds in running water. Subsequently the sections were dehydrated 50 second in ethanol 95%, 1 minute and 30 seconds in ethanol 100% and 2 minutes in ethanol 100%. For the

clarification, the sections were put 2 minutes in xylene. For the assembly, two drops of assembly medium were placed in the section and the lamella was placed on the section exerting some pressure. After dry, the sections were observed on the microscope (primo star, Zeiss, 1000x magnification).

3.8 Sperm extraction

Each epididymis was extracted, trimmed free of fat and the *cauda* was placed on a petri dish with 3 ml of filtered PBS heated at 37°C. With a sterilized scissor the epididymal cauda was minced in order to release the spermatozoa and the solution was homogenized with a Pasteur pipette.

3.9 Evaluation of sperm parameters

3.9.1 Motility

Sperm motility was analysed by placing 100 µl of the previous spermatozoa suspension of the petri dish on a 37ºC pre-warmed microscope slide and covered with a cover slip. 10 fields of the microscope were observed with the diaphragm as closed as possible, always in the center of the microscope slide using na optical microscope (primo star, Zeiss, 1000x magnification) and the motility was classified by assigning a percentage of motility values in intervals of 10%.

3.9.2 Viability

Using the one step eosin/nigrosin stainig technique sperm viability was assessed. 5 µl of the previous spermatozoa suspension and 10 µl of nigrosin/eosin solution (0,6% eosin; 5% nigrosin; 3% sodium citrate and pH was adjusted to 7 with 1M NaH_2PO_4) were placed on a 37ºC pre-warmed the microscope slide and mixed. A smear was made and the microscope slide was observed on a optical microscope. The head of non-viable sperm cells turn pink as they absorb eosin due to the increased membrane permeability caused by the lost of integrity of the cell membrane while the heads of viable sperm cells remains white. 100 sperm cells were analysed in random fields accordingly to the schematic figure 13, to avoid count the same field more than once, under a microscope (Primo Star, Zeiss, 1000x magnification).

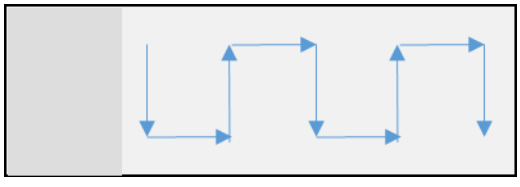

Figure 13: Schematic representation of the orientation of the fields used for the viability analysis in order to prevent the same field from being analyzed more than once.

3.9.3 Morphology

5 µl of the previous spermatozoa suspension and 10 µl of PBS were placed at a microcope slide, a smear was done and the slides were allowed to air dry 37ºC. After dry, the Kwik™-Diff stain kit (Thermo Scientific™) was used to evaluate morphology. First, the slides were immersed for 1 minute in a fixative solution (metanol), secondly, the slides were immersed for 2 minutes on eosin, which is an anionic dye that consequently stains positively charged proteins with red and lastly, the cover slides were immersed for 2 minutes in methylene blue which is an caionic dye that consequently stains negatively charged molecules with blue [84]. After, the slides were rapidly dipped in water, air dried and observed in the microscope. The sperm cells were classified into four categories: normal, head defect, neck/midpiece defect and tail defect. When a sperm cell showed more than one defect, the sperm cell was classified according to the considered most severe defect that was present, being the severity order the following: head, neck/midpiece, tail (from more severe to less severe). 100 sperm cells were analysed in random fields under a microscope (Primo Star, Zeiss, 1000x magnification).

3.10 Total protein extraction

SeT were collected from the wells to falcons and centrifuged at 500G for 5 minutes at 4ºC. Each sample was weighted. Total protein was isolated from rat SeT of all experimental groups using radioimmunoprecipitation assay buffer (RIPA) (150nM NaCl, 1% Nonidet, 5% Na-deoxycholate, 1% SDS, 10% Tris-Base, 1mM EDTA) supplemented with protease inhibitors cocktail and 10% phenylmethylsulfonyl fluoride per mg of tissue and homogenized mechanically with a plastic tool for the effect. The samples were allowed to stand on ice 1 hour and occasionally mixed. Then, samples were centrifuged at 14,000 rpm for 20 minutes at 4ºC, and supernatant containing total proteins was recovered to fresh tubes. Total protein concentration in extracts was determined through the Bradford method (Bradford 1976) with Sigma-Aldrich protein assay dye reagent. BSA was used to construct a standard curve.

3.11 Quantification of glucose and lactate

The concentration of glucose and lactate in the culture medium of rat SeT was determined by means of spectrophotometric analysis using commercial kits (Thermo Fisher Scientific, EUA). Using a 96 well plate, in each well corresponding to samples was placed 1µl of sample medium and 100µl of working reagent, in a well corresponding to standard was placed 1µl of standard provided for the kit and 100µl of working reagent, and in a well corresponding to blank were placed 100µl of working reagent. Each well was made in triplicate. For glucose and lactate kit, incubation time was respectively 5 minutes and 10 minutes at 37ºC. After incubation the plates were read at 590nm. The glucose consumption and lactate production were calculated.

3.12 LDH catalytic concentration

The LDH activity was determined by commercial kit (Spinreact, Spain) based on the fact that LDH catalyzes the reduction of pyruvate by NADH, being the rate of decrease of the NADH concentration determined by means of spectrophotometric analysis proportional to the catalytic concentration of LDH in the sample tested. Using a 96 well plate, in each well corresponding to samples was placed 1µl of sample protein extracted and 150µl of working reagent. After incubation at 37º during 1 minute, the absorbance at 340nm was measured every minute for 3 minutes and the mean absorbance difference per minute was calculated (ΔA/min).

3.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Prism version 6.00 for Windows, GraphPad Software, California USA). The statistical significance of differences between experimental groups was assessed by the Student's t test. Significant differences were considered when $p < 0.05$. All experimental data are shown as mean ± SEM.

4. Results

4.1 Development of a new model to study the effects of Wi-Fi on seminiferous tubules metabolism *ex vivo*

4.1.1 20 day-old is the ideal age for the development of the model

In order to determinate the ideal age for the development of the model histological sections of SeT from 19 day-old, 20 day-old, 21 day-old and 22 day-old were stained with hematoxylin and eosin and observed through the microscope. The age at which the cell population was as intended was 20 days of age (Figure 14, panel B). At this age, spermatogonia and SCs form a double-layered rosette with an outer row constituted by spermatogonial cells and an inter row of SCs. In addition to the population of SCs being well established, at this age there were only germ cells in the early stages of spermatogenesis, in leptotene/zygotene and pachytene stages which are the early stages of prophase 1 of meiosis. At the 19 days of age (figure 14, panel A) there its observable the presence of pré-leptotene spermatocytes and the Sertoli cells and spermatogonia cells are desorganized. Both ages of 21 and 22 days have already spermatocytes at more developed stages of spermatogenesis. The classification made was based on figure 15, representing the different stages of human germ cell development including the different stages of meiosis.

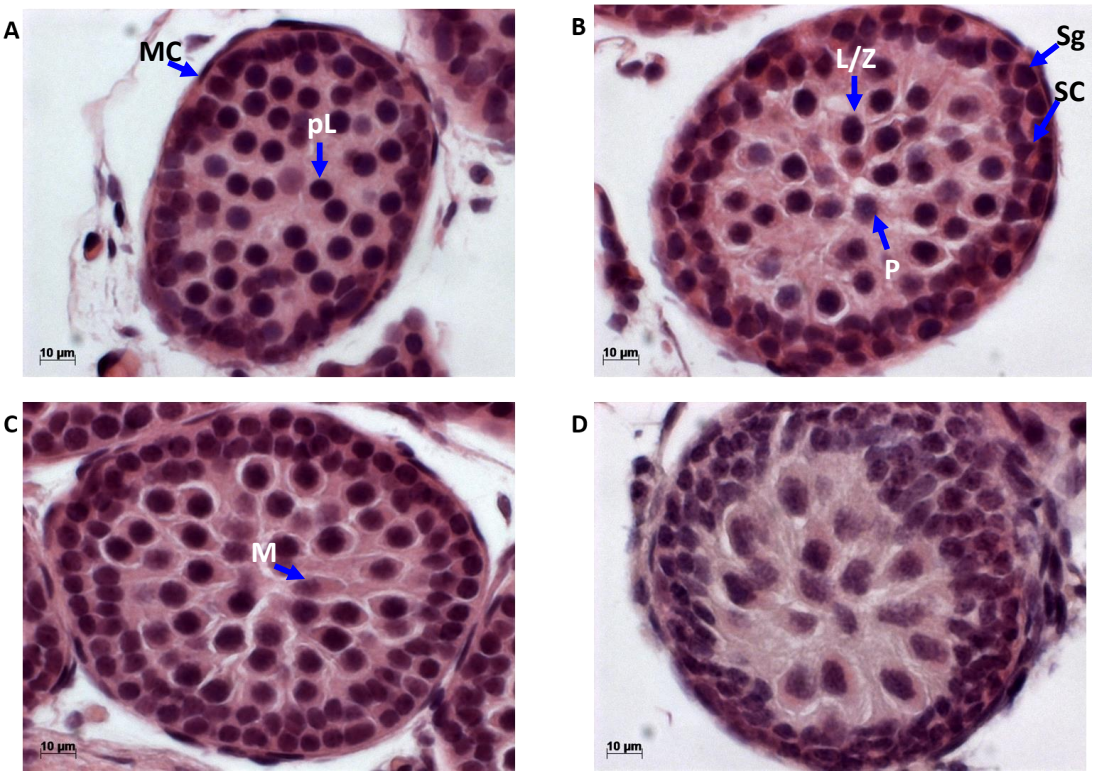

Figure 14: Histological sections from rat seminiferous tubules stained with hematoxylin and eosin. A) Histological section from SeT from a 19 day-old rat¸ B) Histological section from SeT from a 20 day-old rat; C) Histological section from SeT from a 21 day-old rat; D) Histological section from SeT from a 22 day-old rat. 100X. pL- pré-leptotene spermatocyte; MC- myoide cell; Sp- Spermatogonia; SC- Sertoli Cell; leptotene/zygotene spermatocyte; P- pachytene spermatocyte; M-meiotic division.

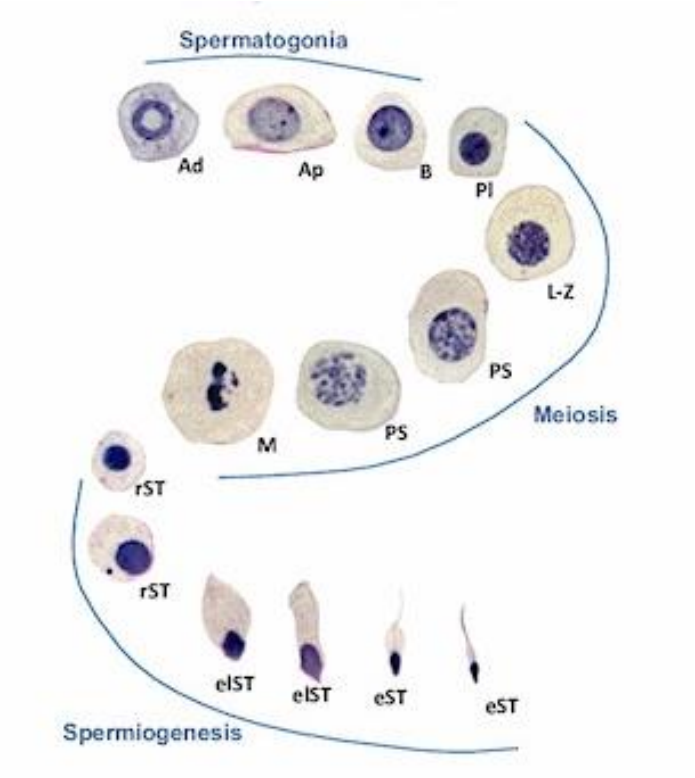

Figure 15: Nuclear morphology of the major cell types found within the human seminiferous epithelium, showing the progress of spermatogenesis. Ad- A dark spermatogonia; Ap- A pale spermatogonia, B: type B spermatogonia, Pl: preleptotene spermatocyte, L-Z: leptotene to zygotene spermatocyte; PS- pachytene spermatocyte; M- meiotic division; rST- round spermatid; elST- elongating spermatid; eST- elongated spermatid. Adapted from [85]

4.1.2 72 hours in culture medium have no effects on seminiferous tubules

To assess if the 72 hours in the culture medium had negative effects medium in the SeT, SeT from a 22 day-old rat were cultured and histological sections were cut after 0, 48 and 72 hours in culture medium and stained with hematoxylin and eosin. As seen in the figure 16, there are no observable differences between t=0h, t=48h and t=72h in culture medium so it is safe to say that the culture medium has no observable effects on the SeT cultured, in this way, any effects that may be present are a consequence of the stimulus.

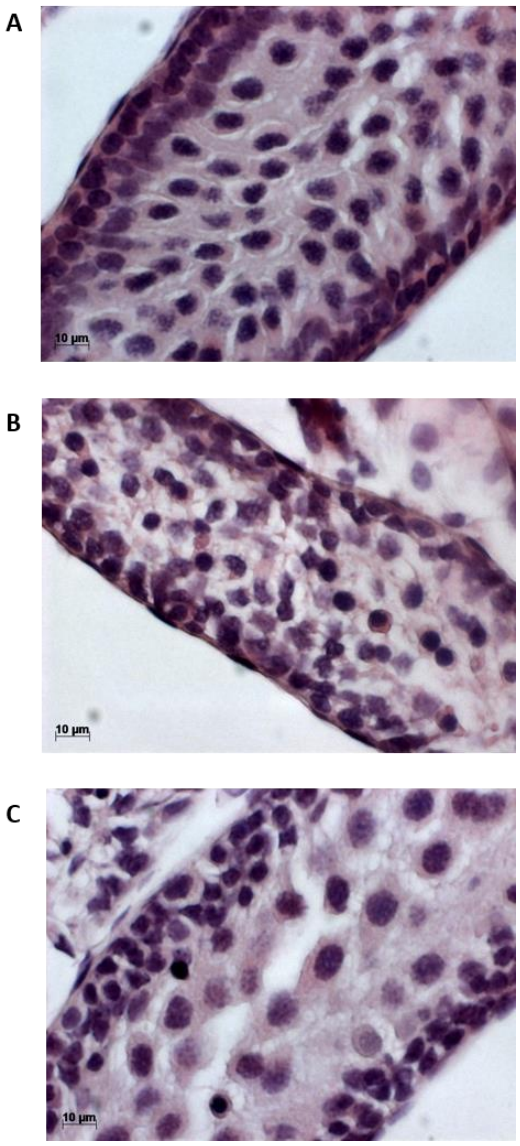

Figure 16: Histological sections from 22 day-old rat seminiferous tubules stained with hematoxylin and eosin. A) Histological section from SeT from a 22 day-old rat¸ after 0 hours in culture medium B) After 48 hours in culture medium; C) After 72 hours in culture medium.

4.1.3 Validating the model

Spermatozoa were divided into two major groups, 0 hours group (parameters analysed right after being extracted from the epididymis), and 1 hour group (spermatozoa who were 1 hour outside the epididymis). For each group were two subgroups, control group, constituted by spermatozoa who were not exposed to EMR, and exposed group, constituted by spermatozoa who were exposed to EMR for 0 hours or 1hour acording to the group they belong. In this way, exposed group from 0 hour group despite being called "exposed" was not exposed to EMR. For the 1 hour group, exposed group was exposed in the incubator with the set up, receiving the EMR for 1 hour, whereas control was placed in another incubator for 1 hour, without the set up.

In what concerns the motility (figure 17, panel A), there were no significative differences for the 0h group comparing the two groups, control (80,82 ± 1,787, N=6, p-value=0,1555) and exposed (84,17 ± 1,249, N=6). However, for the 1 hour group, there was a significant decrease of 26% in sperm mobility for the group exposed for 1 hour to EMR (41,17 ± 7,812, N=6, p-value= 0,0145) comparing to the control, non-exposed group (14,67 ± 4,417, N=6), (with a diference between means of -26,50 ± 8,974).

Regarding the sperm viability, (figure 17, panel B), there were no significative differences for 0 hour group comparing the two groups, control (13,33 ± 0,9189 N=6, p-value=0,7402) and exposed (13,67 ± 0,3333, N=6), neither for 1 hour group comparing the two groups, control (13,50 ± 0,8062, N=6, p-value=0,6867) and exposed (13,00 ± 0,8944, N=6). The percentage of viable sperm in all the groups was very low, approximately 13,37%. Some random fields observed in microscope during viability analysis are represented in figure 18.

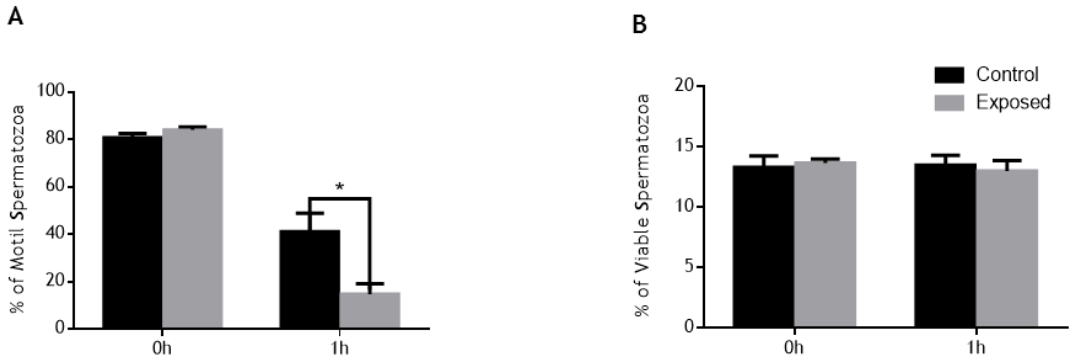

Figure 17: Effect of EMR exposure on epididymmal spermatozoa viability. A) The figure shows the percentage of motil spermatozoa, comparing both groups, control and exposed, from 0 hours and 1 hour group. There were no significative diferences between the 0 hour group, however there was a significative decrease in the 1 hour group comparing the control and exposed groups. B) The figure shows the percentage of viable spermatozoa, comparing both groups, control and exposed. There were no significative diferences between any of the groups. 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 (*).

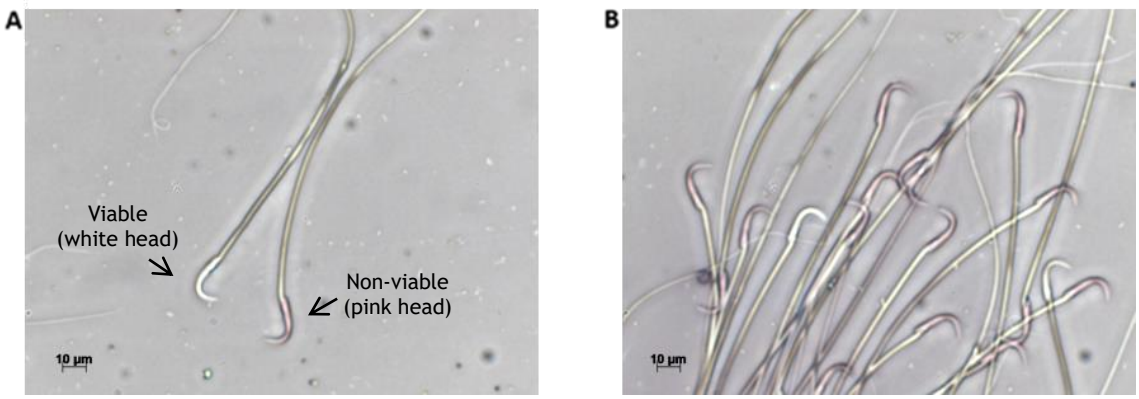

Figure 18: Random fields observed in viability analysis. A) A viable sperm cell and a non-viable sperm cell; B) A few viable and lots of no-viable sperm cells. (Stained with eosin/nigrosin). 100X

For the percentage of normal epididymal spermatozoa (figure 20, panel A), there were no significative differences for 0 hour group comparing the two groups, control (94,50 ± 1,335, N=6, p-value=0,6993) and exposed (95,17 ± 1,014 N=6), neither for 1 hour group comparing the two groups, control (93,67 ± 1,054, N=6, p-value= 0,1526) and exposed (86,50 ± 4,507, N=6). For the percentage of epididymal spermatozoa with head deffects (figure 20, panel B) for 0 hour group there were no significative differences comparing the two groups, control (1,167 ± 0,4773, N=6, p-value= 0,4054) and exposed (1,833 ± 0,6009, N=6). However, exposure for 1 hour to 2.4 GHz EMR caused a significant increase, by 1%, comparing the exposed group (1,167 ± 0,3073, N=6, p-value= 0,0234) to control (2,833 ± 0,5426 N=6), (with a difference between means of 1,667 ± 0,6236).

For the percentage of epididymal spermatozoa with neck/midpiece defects (figure 20, panel C), there were no significative differences for 0 hour group comparing the two groups, control (2,333 ± 0,6146, N=6, p-value=0,8713) and exposed (2,167 ± 0,7923, N=6), neither for 1 hour of exposure comparing the two groups, control (2,000 ± 0,5774, N=6, p-value= 0,2487) and exposed (3,000 ± 0,5774, N=6). For the percentage of epididymal spermatozoa with tail defects (figure 20, panel D), there were no significative differences for 0 hour group comparing the two groups, control (2,000 ± 0,8563, N=6, p-value=0,5995) and exposed (1,500 ± 0,3416, N=6), neither for 1 hour group comparing the two groups, control (2,500 ± 0,8466, N=6, p-value= 0,2456) and exposed (1,333 ± 0,4216, N=6). Some of the defects found in the exposed group 1 hour to the EMR are represented in figure 19.

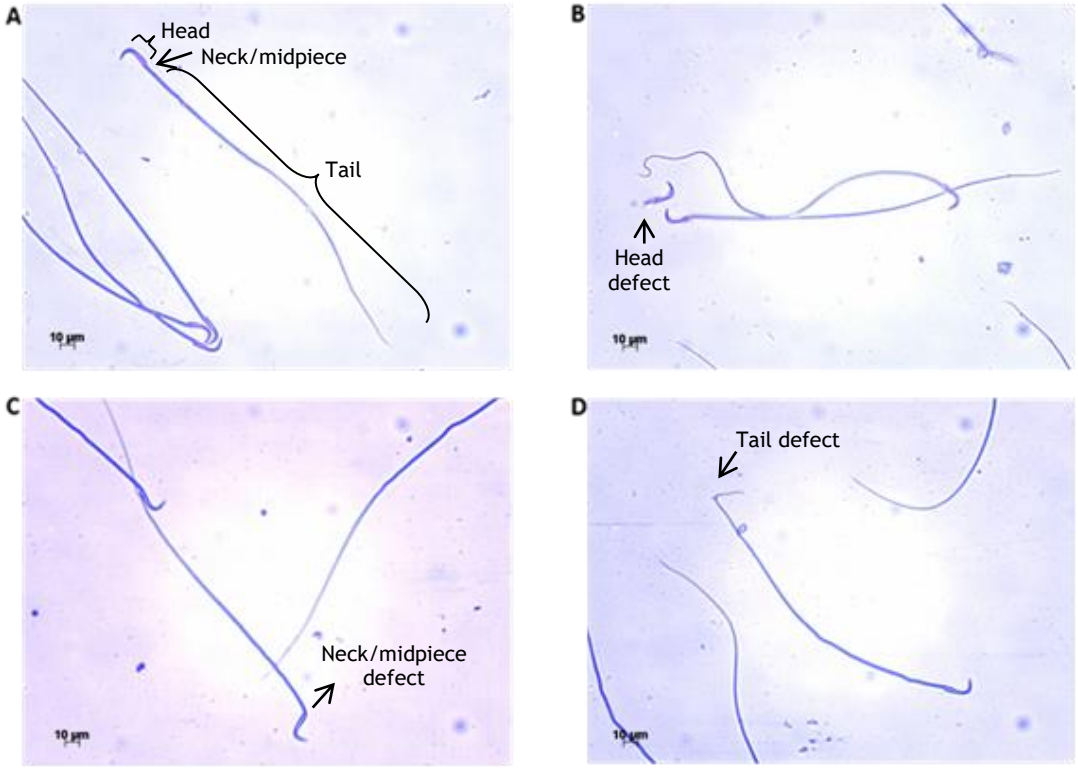

Figure 19: Sperm cells found during sperm morfology analysis from the group exposed 1 hour to EMR. A) Normal sperm cell; B) Head defect; C) Neck/midpiece defect; D) Tail defect. 40X

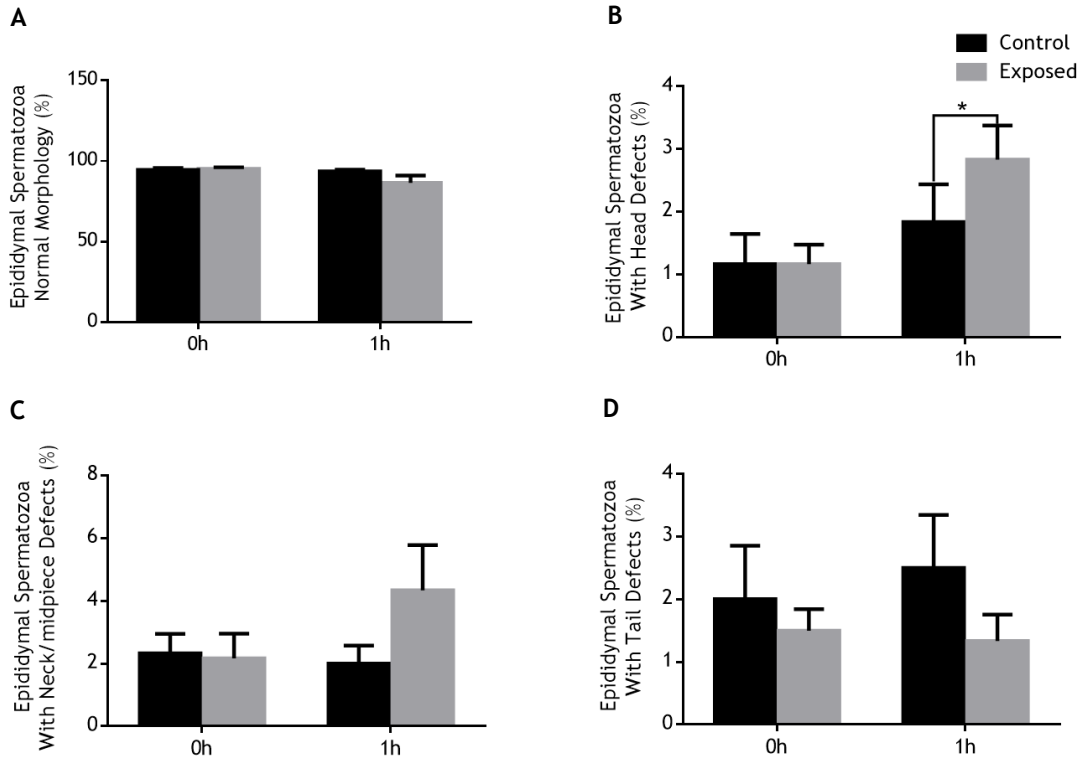

Figure 20: Effect of 1hour EMR exposure on epididymal spermatozoa. A) The figure shows the percentage of normal epididymal spermatozoa, comparing both groups, control and exposed, from 0 hours and 1 hour group. There were no significative diferences between any of the groups. B) The figure shows the percentage of epididymal spermatozoa with head defects, comparing both groups, control and exposed. There was a significative increase on the percentage of epididymal spermatozoa with head defects between control and exposed on the 1 hour group. C) The figure shows the percentage of epididymal spermatozoa with neck/midpiece defects, comparing both groups, control and exposed, for 0 hours and 1 hour group. There were no significative diferences between any of the groups. D) The figure shows the percentage of epididymal spermatozoa with tail defects, comparing both groups, control and exposed, for 0 hours and 1 hour group. There were no significative diferences between any of the groups. 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 (*).

4.2 EMR from Wi-Fi altered SeT glycolytic metabolism

4.2.1 EMR from Wi-Fi device inhibited glucose consumption

Since glucose is the most important fuel of the cells responsible for testicular metabolism, in order to evaluate whether exposure during 72h to EMR of 2.4GHz corresponding to the EMR emmited from Wi-Fi devices alters testicular glycolytic metabolism, metabolic alterations in glucose consumption and lactate production were evaluated. Our results evidenced that the extracellular glucose consumption (figure 21) was significantly decreased on exposed group (2,242 ± 0,4855, N=6, p-value=0,0029) comparing to the control group (0,01042 ± 0,5173, N=6), (with a difference between means of -2,231 ± 0,7095).

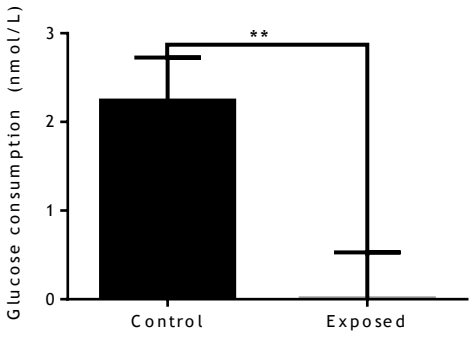

Figure 21: Wi-Fi effects on glucose consumption. 72 hour exposure to 2.4GHz EMR significantly decreased glucose consumption in rat immature SeT. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Significant results (p-value < 0.05) relative to control are indicated as (*).

4.2.2 EMR from Wi-Fi device increases lactate production

Lactate is one of the most important products secreted by SCs for maintenance of germinative cells during spermatogenesis because it's their preferred subtract o obtain energy. In this way, lactate concentration was calculated. The results from the quantification of extracellular lactate concentration (Figure 22) showed that exposure for 72h to 2.4GHz EMR radiation significantly increased lactate production on the exposed group (2,902 ± 0,3875, N=6, p-value= 0,0022) comparing to the control group (1,616 ± 0,08674, N=6), (with a difference between means of 1,286 ± 0,3971).

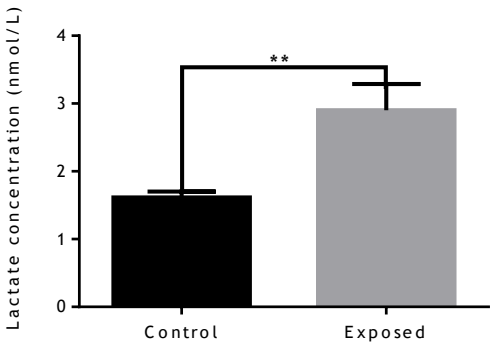

Figure 22: Wi-Fi effects on lactate production. 72 hour exposure to 2.4GHz EMR significantly increased lactate production in rat immature SeT. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Significant results (p-value < 0.05) relative to control are indicated as (*).

4.2.3 EMR from Wi-Fi device increases LDH activity

Since the production of lactate was increased in the exposed group, it was decided to study the LDH activity, the enzyme responsible for reversively converting pyruvate to lactate. The results evidenced an increase in LDH activity (figure 23) in the exposed group (39,30 ± 3,379,

N=6), comparing to control group (27,99 ± 5,511, N=6), although this increase was not significantive (p-value=0,1109).

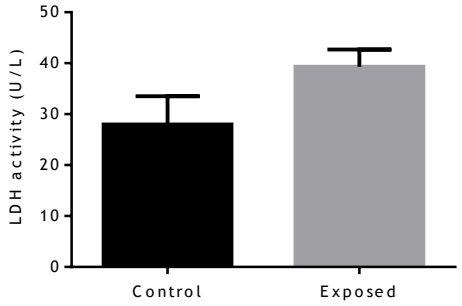

Figure 23: Wi-Fi effects on LDH activity. 72 hour exposure to 2.4GHz EMR caused na increase in LDH activity in immature rat SeT, nevertheless this increase was not significative. Results are expressed as mean ± standard error of the mean (SEM) (n = 6 for each condition). Significant results (p-value < 0.05) relative to control are indicated as (*).

5. Discussion

Wireless devices using W-Fi interfaces are increasingly present in our lives and are an essential part of our daily activities. This type of apparatus uses electromagnetic radiation for the transmission of signals, which in turn has been proved to have various physiological effects in particular on male reproductive system, having negative consequences on male fertility. Several studies have shown that EMR from Wi-Fi devices contributes to DNA damage [46 47 48], causes histopathological and ultrastructural changes in the testes and on SC [51 52], alters the production of hormones crucial for the proper functioning of the male reproductive system like T [45 49 50], causes degenerative changes in spermatogenic cells [54] and on its number[55] and has a negative effect on sperm parameters[45 56]. However, there is a lack of studies regarding the effects of the EMR from these devices on testicular metabolism.

Since processes involved on testicular metabolism may be affected by several factors such as environmental factors and even a sedentary lifestyle, it is plausible to hypothesize that EMR may have effects on testicular metabolism. Since the EMR studies found did not use realistic models of radiation exposure, once some of them only placed sperm under a laptop computer, or placed animals in small cages inducing them stress [47], it was necessary to built a realistic and reproducible model of EMR exposure.

A closed Wi-Fi network was created inside the incubator, being constituted by the wifly module, the router and the mbed. The Wifly module was chosen because it is certified to operate on 2.4GHz networks, the same frequency used in our common internet networks. Also the easier verification of signal transmission and its correct operation was taken in account, once it has three LED's (green, red and yellow) each one providing information about the wifly status as previously described. Mbed was selected to be part of the network construction once it is easy to programme through an online platform using a relatively simple compliler, the ARMCC C/C++ and because of the presence of a USB port through which it supplies power to the system which was ideal for our situation, since we wanted to place the system inside the incubator and pass the cables through a small hole to be able to connect all devices to a power plug on the outside. The capability of easily connect the wifly module to the mbed, following a schematic already provided, was also a plus point of using these two components. The internet router used was a simple, commercial router that satisfied the needs of the network we wanted to build.

The EMR from wireless devices has been shown to have a negative effect in sperm parameters such as decrease in viability, sperm count and motility [45 56], in this way, to validate the exposure set up sperm cells from adult rats were exposed during one hour to the network built. The short time expose (one hour) is justified by the fact that a longer ex vivo exposure could compromise sperm quality and influence the results. The results showed that EMR caused a significant decrease in motility in the exposed compared to control. Also the number of sperm with head deffects was significantly increased in the exposed group. However, all the other sperm parameters did not suffer significative alterations inclusively viability. The

fact that it is an ex vivo study may justify the differences between control and exposed groups not being significant in the other parameters, as in *in vivo* studies EMR caused significant effects on sperm parameters. For exemple Yan *et al*., [56] exposed rats to 1.9GHz EMR in a cycle of 3 hours of exposure followed by a 30 minute period without exposure and again 3 more hours of exposure for 18 weeks and the results revealed a majority of sperm cells without motion, dead or with straight rigid tails in the exposed rats. However, Dasdag and his colleagues who also used an *in vivo* model, found no significant differences neither with long or short term exposure in sperm concentration, sperm motility, total morphological defects and apoptotic cell number in the testes [53 59 60].

Since the ultimate goal was to study the effects of EMR on testicular metabolism, it was considered pertinent to determine the best age to study testicular metabolism using rat SeT. In view of the number of published studies on the metabolism of SCs [82 83], we hypothesized that a model using a mouse with an epithelium of seminiferous tubules containing a well established SC population alongside with germ cells in the early stages of spermatogenesis exclusively, would constitute an excellent model for the study of metabolism in rats. There are studies revealing that mitotic division of SCs ceases after the 15 day of post-natal development in rats, preceding the formation of the hematopoietic barrier created by tight junctions formed by SCs between days 16 and 19 [80]. Another study indicates that the mitotic division of the SCs ceases at 18 days [81].

Although there are studies that show that post-mitotic terminally differentiated SCs from adult animals could, under certain conditions re-enter the cell division cycle, as it happens in horses that presented a greater number of SC during the breeding season, in knock-out mice with DBKO or P27KO removed, in circumstances of testicular transplantation or hypophysectomy, among other situations, since the rats used for this work do not fall into any of the cases above described, and since adult SC numbers are generally stable, we assumed that the population of SC in these case would stay stable [10].

According to previous studies that showed the ages in which there was a well-established SC population and germ cells only in the initial stages of spermatogenesis, we decided to confirm the data present in the literature and perform an histological analysis of SeT from rats aged 19 to 22 days. The ideal age with the ambitioned characteristics was shown to be 20 days of age, since in addition to the population of SCs being well established, there were only germ cells in the early stages of spermatogenesis once the germ cells that were in the most developed state were leptotene/zygotene/pachytene spermatocytes. In the histological section of the SeT of the 19-day-old rat it was still possible to observe a certain disorganization of the cells that was no longer observable in the 20-day rat SeT since a higher level of organization was present. In the 21-day-old rat SeT it was observed na augmented number of germ cells at a more advanced stage, which was not intended since we wanted the study to focus on the contribution of SCs and not germ cells. In the 22-day-old rat SeT an

even higher number of germ cells in an advanced stage of spermatogenesis were observable. The results were consistent with histological images of rat SeT of the same age present in the literature, as espected [86].

We also intended to discard the hypothesis that the time in culture medium could influence the results found. For this purpose, SeT from a 22-day-old rat were placed for 72 h in culture medium without receiving any EMR and a histological analysis was performed at t=0h, t=48h and t=72h given that, if changes were observed in the SeT, the results found later could be a consequence of these histopathological changes and not due to EMR exposure. There were no observable differences between SeT for t=0h, t=48h and t=72 in culture medium so it is safe to say that the culture medium has no effects on the SeT cultured, in this way, the effects found are a consequence of the stimulus.

In the testicles, glucose metabolism is carried out by SCs once they support spermatogenesis not only spatially but also energetically [24 25], however SeT and not SC cultures were used in this study. The choice of SeT culture instead of SC culture is justified by several studies that show that this model is more suitable to mimic the testicular cellular environment *ex vivo* [87 88].

Although SCs have the ability to metabolize various substrates, they preferentially use glucose [26 23]. In this way it was pertinent to study the possible effects of EMR on glucose concentration. Also lactate which is one of the most important products secreted by SCs for maintenance of germ cells during spermatogenesis because it is their preferred subtract o obtain energy is an object of study with interest [28]. To provide germ cells their favorite substract, SCs convert the most part of glucose into lactate so it was expected that if the glucose concentration decreased, the lactate concentration would increase, however the results evidenced that the extracellular glucose concentration was significantly decreased on exposed group but contradictorily lactate production increased. Since LDH is responsible for the reversible conversion of pyruvate into lactate it has an enhanced importance to further disclose the increase in lactate production, thus, the LDH activity was assessed. The results from LDH kit revealed that a 72hour exposure to EMR caused an increase in LDH activity and although the increase was not significant, it is in accordance with the increase in lactate production in the exposed group once if LDH is more active, it is expected that lactate production will be higher since there is more conversion of pyruvate to lactate. These contradictory results between glucose consumption and lactate production suggest that when exposed to EMR from Wi-Fi devices the cells responsible for testicular metabolism, namely SCs in the SeT, may use alternative metabolic pathways for the production of lactate and consequently obtain energy. Some studies have already demonstrated that SCs can use other substrates rather than glucose to obtain energy. Indeed, some studies propose some metabolic mechanisms to occur both in cancer and SCs, such as the Warburg effect.

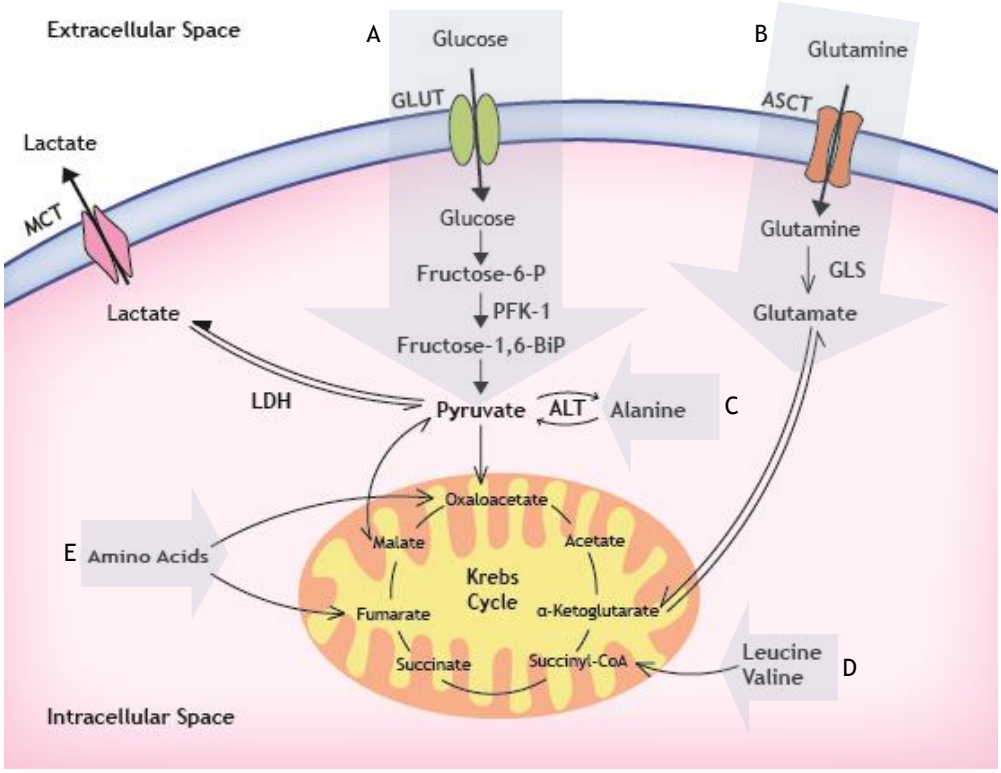

Figure 24: Metabolic pathways in testicular metabolism proposed under Wi-Fi EMR radiation. Usually, Sc metabolize glucose into pyruvate throught the enzyme phosphofructokinase (PFK) (A). Then, pyruvate is converted into lactate by lactate dehydrogenase (LDH) and exported through specific monocarboxylate transportes (MCTs). Since under certain conditions SCs can use alternative fuels to obtain energy, it is proposed that under Wi-Fi EMR radiation may use alternative pathways. Glutamine may have an importante role, entering through alanine–serine–cysteine transporter (ASCT) and being converted to glutamate by glutaminase (GLS) (B). Glutaminolysis derivated α-ketoglutarate is a Krebs-cycle intermediate that in last instance may turn into pyruvate and through LDH be converted in lactate and exported through MCT. Also leucine and valine can do the same path (D). Several amino acids may also replenish Krebs cycle at oxaloacetate and fumarate (E). Also alanine may be converted to pyruvate by alanine aminotransferase (ALT) and through LDH be converted in lactate and exported through MCT (C).

In normal conditions glucose is metabolized into pyruvate through several reactions which is then converted to lactate, but under specific circumstances, Krebs cycle and glutaminolysis may be an alternative [89]. Glutamine, which is known to be involved in anabolic pathways, has been identified as a substrate capable of providing the energy required for testicular metabolism once it can be degraded in the Krebs cycle and in order to produce ATP. Furthermore, Grootegoed et al [35] have shown that the single oxidation of glutamine and leucine can yield much of the required energy by SCs [90]. Glutamine oxidation leads to its conversion to glutamate and then to a-ketoglutarate, which is followed by oxidation of a-ketoglutarate via the citric acid cycle and provide intermediates for fatty acids and amino acids synthesis [35]. Glutamine also decreases the incorporation of alanine into proteins, which in turn may be beneficial since alanine can be converted to pyruvate by ALT and subsequently used by SC as a substrate to produce lactate through the action of LDH [36]. Besides glutamine and alanine, leucine and valine can also alter the normal metabolic course in SC since they

decrease the oxidation of glutamine [36]. In fact, Kaiser et al., showed that SC can adapt its energy metabolism to the oxidative substrates available to fulfill their role in spermatogenic energetic supply.

Once the culture medium used had glutamine, the Wi-Fi radiation may have induced SC present in SeT of the immature rats to use glutamine instead of glucose as the substrate, leading to the use of this alternative metabolic pathway. These alternative pathways are represented in figure 24.

The control on the production rate of lactate is on charge of GLUTs - GLUT1, GLUT2 and GLUT3, that control the membrane passage of glucose from the extracellular space to SCs, and after produced, lactate is exported from SCs trough the active membrane monocarboxylate transporter isoform 4 (MCT4) [29] [23]. In this way, the study of the expression of these transporters may give more answers about the effect of the EMR from Wi-Fi devices in testicular metabolism. The study of ALT and LDH expression may also be usefull to understand which substract is leading to lactate production.

6. Conclusions

In recent years, concern over the possible effects of Wi-Fi equipments on fertility has been increasing as they are increasingly being used around the world.

The uneasiness, about the effects of EMR on male fertility has been reflected in several published studies, however the way radiation exposure was conducted and whether the effects are actually representative of reality remains a topic of debate.

A good reproductive capacity depends on the correct process of spermatogenesis which in turn is affected by testicular metabolism that occurs in the seminiferous tubules by SC. In this way, and since this subject has never been studied, the effect of the EMR emitted by Wi-Fi devices in testicular metabolism deserves particular attention. Since SCs are located within the SeTs and the fact that the SeT epithelium from a 20-day-old rat consists predominantly of SC and germ cells at an early stage of the spermatogenesis, the use of SeT cultures of 20-day-old rats consists of a reliable model for the study of the effects of EMR on testicular metabolism.

Through the work developed, it was possible to built a set up of exposure to EMR emitted by Wi-Fi devices, mimicking a situation of internet use through the transfer of data packets that made it feasible to study the real effects of EMR on metabolism using the new model described above. The study conducted to validate the set up showed that EMR does indeed have effects on sperm parameters, specifically in the motility in which the exposed group had a significant decrease in the percentage of motile sperm compared to control.

Through this project, it was possible to conclude that Wi-Fi devices affect testicular metabolism since EMR decreased significantly extracellular glucose concentration, however increased lactate production and LDH activity. The results found are the first evidence that the EMR of Wi-Fi devices has effects on the testicular metabolism, altering its normal glycolic pathway.

Althouth some alternative pathways have been suggested in the discussion of this dissertation, further studies will be necessary to understand in depth these mechanisms and to determine the actual pathway that is being used.

The study of the effects of EMR and Wi-Fi devices is gaining more and more interest from the scientific community since in recent years evidence of its negative effects on male fertility has arisen trough several published papers.

This topic will certainly continue to be a research topic and this dissertation intends to be a starting point for the study of this subject, which can provide useful answers to the problem of male infertility.

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