UNIVERSIDADE DA BEIRA INTERIOR
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Effects of Wi-fi Radiation in *Ex Vivo* Immature Rat Seminiferous Tubules Oxidative Stress

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Resumo

Aproximadamente 15% dos casais mundialmente em idade reprodutiva não engravidam num período de um ano com relações sexuais não protegidas, sendo o fator masculino responsável por aproximadamente 50% dos casos. Ao longo dos anos, a fertilidade masculina tem diminuído sendo associada a vários fatores, como o estilo de vida, o tabaco, o álcool e os produtos químicos. No entanto, com a evolução tecnológica, outro fator foi apontado como uma importante causa para a infertilidade: a radiação eletromagnética (EMR) proveniente de telemóveis, tablets e computadores. Embora existam vários estudos que relacionam a EMR e os efeitos negativos na fertilidade masculina, incluindo o aumento dos níveis de stress oxidativo, nenhum destes estudos utilizou um modelo realístico de exposição à EMR. Desta forma, o nosso objetivo foi construir um modelo realístico de exposição a EMR com o intuito de estudar as influências destas radiações na fertilidade masculina com destaque para o stress oxidativo e a apoptose. Em primeiro lugar foi desenvolvido o modelo de exposição às EMR através da utilização de 3 instrumentos: um módulo wi-fi (rede de transmissão de informação), um mbed (modulo de programação dos pacotes de informação) e um wifly (antena de receção e emissão de pacotes de informação). Depois de desenvolvido o modelo, procedemos à sua validação através da exposição de espermatozóides de ratos *Wistar* com 3 meses ao nosso modelo durante 1h tendo sido avaliados parâmetros espermáticos (motilidade, morfologia e viabilidade). Esta exposição a radiações da gama dos 2.4GHz levou a diferenças estatisticamente significativas da motilidade com um aumento de 2.8 vezes do grupo exposto em relação ao grupo controlo, não se verificando diferenças significativas nos restantes parâmetros espermáticos. Para além disso, procedemos também à seleção da idade dos ratos *Wistar* para estudar a influência das EMR avaliando-se histologicamente a população celular de testículos de ratos com 19, 20, 21 e 22 dias. Tendo em consideração que para o objetivo deste trabalho pretendíamos estudar animais no final do desenvolvimento e maturação das células de Sertoli (SCs), mas com reduzido desenvolvimento das células germinativas foi selecionada a idade de 20 dias para o presente estudo. Sendo assim, dois grupos (controle e exposto) de cultura de túbulos seminíferos (Set) de ratos *ex vivo* imaturos (20 dias) foram expostos a radiações na gama dos 2.4GHz através do nosso modelo durante 72h, avaliando-se a concentração de Total Oxidative Status (TOS) e atividade de Caspase 3. Na exposição de Set de ratos imaturos ao nosso modelo verificou-se diferenças estatisticamente significativas tanto na concentração de TOS como na atividade da Caspase 3, ocorrendo um aumento 2 vezes do grupo exposto em relação ao grupo controlo nos níveis de concentração do TOS e de 1.6 vezes na concentração da Caspase 3. Concluímos que foi possível desenvolver um modelo eficaz na transmissão de EMR, simulando o uso diário de diversos equipamentos existentes na atualidade. Conjuntamente deduzimos que essa mesma utilização pode vir a ter efeitos negativos na fertilidade masculina sendo apoiado pelo aumento das concentrações de TOS e

atividade da Caspase 3 contribuindo desta forma como um fator a ter em consideração na avaliação da fertilidade masculina.

Palavras-chave

Túbulos seminíferos; Infertilidade masculina; Radiação Wi-fi; Stress Oxidativo; Apoptose

Resumo Alargado

Nos últimos anos diversos estudos têm demostrado uma diminuição da fertilidade com o aumento da esperança média de vida. Tendo em consideração que cada vez mais tarde os casais iniciam o processo para se tornarem pais, este fator é apontado como um bom contribuinte para a diminuição da fertilidade. Esta questão tem sido alarmante pelo facto de cerca de 15% dos casais em idade reprodutiva não conseguir engravidar ao fim de um ano de relações sexuais não protegidas. No entanto diversos fatores relacionados com o estilo de vida dos casais são apontados como agravantes para este fenómeno como são exemplos a ingestão de bebidas alcoólicas em excesso, tabaco e até mesmo a falta de exercícios físico e maus hábitos alimentares, sendo que a obesidade é um fator a ter em consideração. Para além disso desde a industrialização que diversos fatores externos contribuem também para a diminuição da fertilidade como são exemplo diversos químicos provenientes da industrialização sendo exemplo o bisfenol-A (BPA) proveniente dos plásticos, produto que tanto utilizamos no nosso dia-a-dia. Apesar das diversas ações para reduzir ou eliminar o contacto com esses fatores ambientais um outro fator tem vindo a ser apontado como uma possível causa para este problema. Com o desenvolvimento tecnológico diversas formas de comunicação foram facilitadas tendo substituído o envio de cartas por telefonemas e nos últimos anos por emails, chamadas ou mensagens usando as redes sociais. Todo isto foi possível com o desenvolvimento de telemóveis e computadores permitindo que estejamos contactáveis a todo o momento e através de diferentes plataformas. No entanto, para que esta comunicação sem limites seja possível existe a necessidade da utilização de radiação eletromagnética (EMR), sendo esta radiação apontada como uma causa possível para o aumento da infertilidade. Tendo em consideração que o fator masculino em casais inférteis é apontado como sendo responsável por cerca de 50% dos casos, o nosso objetivo foi estudar as influências destas radiações, mais concretamente na gama dos Wi-fi, nos túbulos seminíferos de ratos *wistar*. Nos mamíferos os testículos são um órgão vital para o funcionamento do sistema reprodutor masculino, sendo os túbulos seminíferos uma unidade fundamental desse mesmo sistema, visto ser o local de divisão e maturação das células germinativas. Embora existam vários estudos que relacionam a EMR e os efeitos negativos na fertilidade masculina, incluindo o aumento dos níveis de stress oxidativo, nenhum destes estudos utilizou um modelo realista de exposição à EMR. Sendo assim iniciamos o nosso trabalho pela construção de um modelo realista de exposição a EMR, como forma de simular a utilização de dois computadores com dados a serem transferidos entre eles através de uma rede wi-fi. Para tal usamos 3 instrumentos, mbed, wifly e um wi-fi router. A função do mbed é permitir a programação dos pacotes de dados assim como o intervalo de tempo a que são transmitidos, o wifly por sua vez permite a transmissão e receção dos dados através de uma antena. Estes dois sistemas foram conjugados, sendo criado um sistema emissor e um recetor de dados. O

wi-fi router permitia que os pacotes fossem enviados e recebidos através das ondas de radiação correspondente aos wi-fi (2.4Gz). Antes de avançar com a experiência o modelo de emissão e exposição às radiações foi validado expondo ao nosso setup espermatozoides de ratos com 3 meses de idade durante 1h tendo sido avaliados os parâmetros espermáticos (motilidade, morfologia e viabilidade). A influência das radiações wi-fi em espermatozoides de ratos adultos levou a uma diferença estatisticamente significativa de 2,8 vezes na motilidade, não se verificando diferença significativa nos restantes parâmetros espermáticos. Procedemos também à seleção da idade ideal dos ratos *Wistar* para estudar a influência das EMR avaliando-se histologicamente a população celular de testículos de ratos com 19, 20, 21 e 22 dias. Tendo em consideração que para o objetivo deste trabalho pretendíamos estudar animais no final do desenvolvimento e maturação das SCs, mas com reduzido desenvolvimento das células germinativas foi selecionada a idade de 20 dias para o presente estudo. Para avaliar a influências do Wi-Fi, dois grupos (controle e exposto) de cultura de túbulos seminíferos de ratos *ex vivo* imaturos (20 dias) foram expostos à frequência de 2,4 GHz durante 72 horas. Níveis de stress oxidativo foram medido pelo Total Oxidative Status (TOS), tendo sido utilizado homogeneizado de tecido para medição da concentração de TOS assim como para a atividade da Caspase 3. O EMR de dispositivos wi-fi aumentou significativamente o TOS no grupo exposto sendo 2 vezes superior ao grupo controle. Para além disso a atividade da caspase 3 apresentou também um aumento estatisticamente significativo 1,6 vezes superior. Desta forma foi possível concluir, que apesar de nas exposições de espermatozoides de ratos adulto o único parâmetros espermático no qual foi possível verificar diferenças estatisticamente significativas foi a motilidade, o nosso modelo de exposição mostrou ser viável para os estímulos pretendidos. Para além disso o facto de as concentrações de TOS e atividade da Caspase 3 de túbulos seminíferos de ratos de 20 dias se encontrar aumentada leva então a deduzir que estas radiações podem afetar negativamente a fertilidade masculina.

Abstract

Approximately 15% of couples globally of reproductive age fail to accomplish pregnancy within a year of unprotected intercourse, accounting the male factor approximately 50% of the cases. Over the years male fertility has been decreasing being associated with numerous factors such as lifestyle, tobacco, alcohol and chemicals. However, with technological evolution, another factor has been pointed as an important contributor to infertility: the electromagnetic radiation (EMR) from mobile devices such as mobile phones, tablets and computers. Although there are several studies correlating the EMR and negative effects on male fertility, including the increase in oxidative stress, none of those studies used a realistic model of EMR exposure. Thus, our goal was to build a realistic model of EMR exposure and use it to shed light in the association between EMR from wi-fi devices and male (in)fertility, highlighting oxidative stress and apoptosis. Therefore, firstly we developed the EMR exposure model through 3 instruments: a Wi-fi module (information transmission network), a mbed (programming module of information packets) and a wifly (reception and emissor antenna for information packets). After the development of the exposure model, we proceeded to the validation through the exposure of 3-month-old Wister rat spermatozoa for the time frame of 1h and evaluated the sperm parameters (motility, morphology and viability). Spermatozoa exposure to 2.4GHz of radiation lead to a statistically significant difference in the motility with a 2.4-fold difference, however, there wasn't a statistically significant difference in the other parameters. Secondly, we proceeded to the selection of the ideal animals age for the present project to study EMR influences through a histological cell population study of the testis of 4 different ages: 19, 20, 21 and 22 days. As the goal of this work was to study animals in the final stages of development and maturation of SCs and in an early development of germ cells, Wistar rats with 20 days were selected. Thus, two groups of ex vivo immature rats (20 days) seminiferous tubules culture (control and exposed) were exposed to 2.4GHz radiation by the created model for 72h, TOS concentrations and Caspase 3 activity were measured. Immature rats Set exposure to our model resulted in statistically significant differences in both TOS concentration and Caspase 3 activity, with a 2-fold difference in the exposed group compared with control group in TOS concentration while Caspase 3 activity had a 1.6-fold difference. Thus, was possible to conclude through motility results that the developed model is effective in the transmission of EMR, simulating the daily use of diverse equipment existing in the present in our homes. Also, the increased in TOS concentration and Caspase 3 activity supports the hypothesis of the use of these technologies being harmful to male fertility thus being a factor to be taken into consideration in the evaluation of male fertility.

Keywords

Seminiferous tubules; Male fertility; Wi-fi radiation; Oxidative Stress, Apoptosis

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Abbreviations

1G	First Generation
2G	Second Generation
3G	Third Generation
4G	Fourth Generation
8OHdG	8-hydroxy-2'-deoxyguanosine
Apaf-1	Apoptotic Protease Activating Factor 1
BTB	Blood-Testis Barrier
CAT	Catalase
DISC	Death Inducing Signaling Complex
DMEM F12	Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12
E_2	Estradiol
EMR	Electromagnetic Radiation
FSH	Follicle Stimulating Hormone
GHz	Gigahertz
GnRH	Gonadotropin Releasing Hormone
GPX	Glutathione Peroxidase
GSH	Reduce Glutathione
HPT-axis	Hypothalamic-Pituitary-Testicular- axis
LANs	Local area network
LCs	Leydig Cells
LPO	Lipid Peroxidation
LH	Luteinizing Hormone
MW	Microwave Radiation
OS	Oxidative Stress
PBS	Phosphate-Buffered Saline
PDNs	Public Data Network
PSTN	Public Switched Telephone Network
RF	Radiofrequency
ROS	Reactive Oxygen Species
SCs	Sertoli Cells
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SeT	Seminiferous Tubules
WANs	Wide Area Network
WPANs	Wireless personal area Network
T	Testosterone
TOS	Total Oxidative Stress
UMTS	Large-scale cellular network

I-Introduction

1.1-Testicular Structure and physiology

1.1.1- Testicular Anatomy and Function

The mammalian testes are a paired organ suspended by a spermatic cord into the scrotum measuring about 4 cm long and 2.5 cm in diameter with an individual mass of about 10-15 grams[1]. They are the primary gonads as they are responsible for the production of the gametes, being covered by tunica vaginalis in the anterior and lateral surfaces[2]. This organ has a fibrous capsule called *tunica albuginea* and are divided into 250 compartments or lobules which containing seminiferous tubules (SeT) [3] (Figure 1).

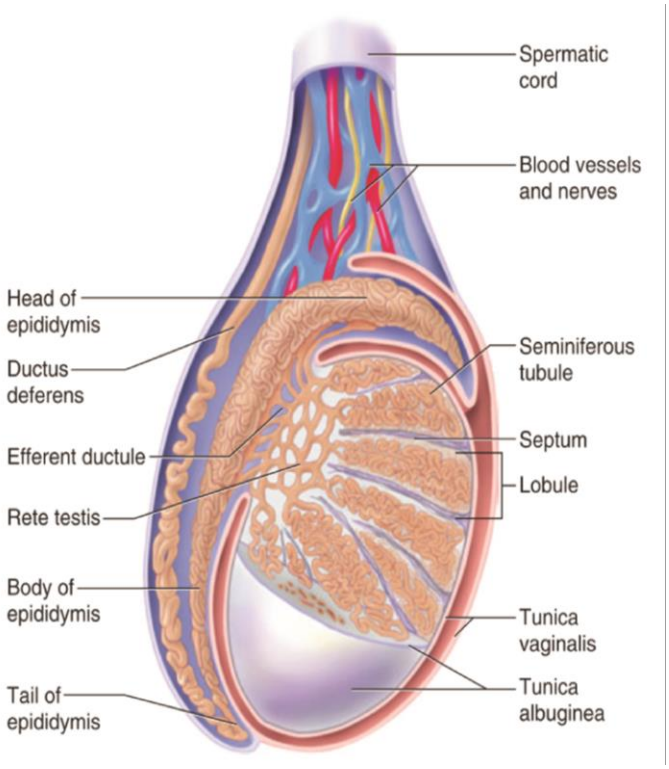

Figure 1.- Representation of mammal testicular anatomy. Testis are suspended by the spermatic cord, being covered by *tunica albuginea* on the inside. They are divided into lobules with a septum dividing which of them. In which septum are the Seminiferous Tubules (SeT) converging to *Rete testis* which in turn congregate to efferent ductule ending in *Epididymis* and progressing to *Ductus Deferens* (Adapted from [2]).

SeT are covered by the seminiferous *epithelium* containing two types of cells: spermatogenic cells and somatic Sertoli cells (SCs) extending from the basement membrane to the lumen of the tubule. In the space between tubules called interstitial space are blood and lymphatic vessels but also Leydig cells (LCs)[1]. SCs are columnar in shape presenting long and thin mitochondria with an oval or pear-shaped nucleus. As far as SCs are concerned they play an important role in the development of the testes and spermatogenesis as in the initial formation of the testis they sequester germ cells inside the recently formed seminiferous

tubules, inhibiting this cells from entering miosis. Also, SCs are responsible for the successful progression of germ cells by producing important factors such as physical support, nutrition and immusupression[4]. The physical support of these cells comes from the blood-testis barrier (BTB) which is a result of the tight junctions between adjacent SCs, creating an adequate microenvironment for germ cells development, dividing the seminiferous *epithelium* into two compartments, the basal and the *adluminal* compartments[5]. Thus the development of meiosis I/II and post-meiotic germ cells take place in the apical compartment being protected from the systemic circulation and immune system[6]. Correspondingly, SCs are also responsible for the secretion of estradiol (E_2) converted from testosterone (T) while LCs are responsible for the secretion of T a hormone essential for male sexual differentiation and expression of male secondary characteristics but also initiation and maintenance of spermatogenesis[7].

Spermatogenesis is the process of the formation of spermatozoom been derived by the proliferation and differentiation of stem cells, starting only in puberty[8]. As SCs and LCs stop proliferating after adulthood germ cells are the only proliferating cells present in the testis[9]. The proliferation of spermatogonial stem cells suffers three phases: the meiotic proliferation of spermatogonia, meiosis of spermatocyte and spermiogenesis being that spermatogenesis results in two types of cells: stem cells and spermatogonia. The stem cells are important for the self-renewal pathway, maintaining germ cells population while spermatogonia are related to mitotic proliferation, meiosis and spermiogenesis (maturation of germ cells)[8]. Spermatogonium (2n) divide through mitosis being present in the basement membrane (Figure 2), moving towards the lumen, they can be divided into 3 stages of differentiation: type A, intermediate and type B[10].

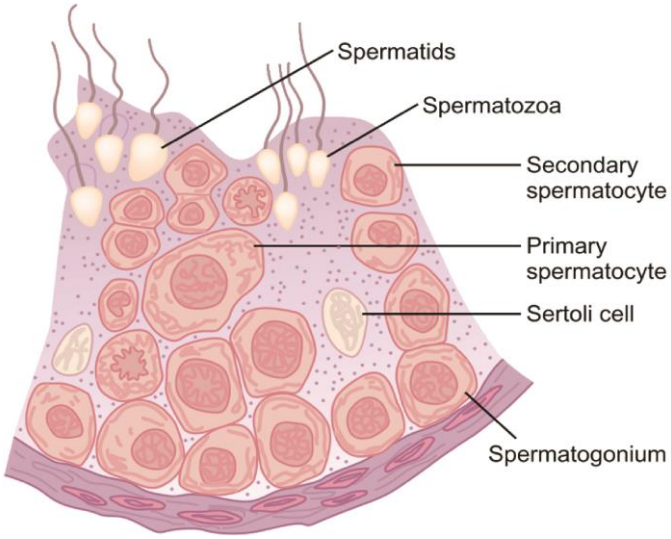

Figure 2.- Germ cell differentiation in Seminiferous tubules (SeT). SeT *epithelium* and germ cells differentiation, spermatogonium and Sertoli cells (SCs) in the basal compartment and near the lumen germ cells start to differentiate into primary spermatocyte, secondary spermatocyte, spermatozoa and finally spermatids (adapted from [7]).

The type A spermatogonium are the supplies of stem cells while type B spermatogonium continues to divide and differentiate into primary spermatocyte[2]. Primary spermatocyte suffers meiosis I reducing the chromosome number to half resulting in the secondary spermatocyte (n) which undertake the meiosis II given rise to 4 daughter cells entitled round spermatids (Figure 3)[2].

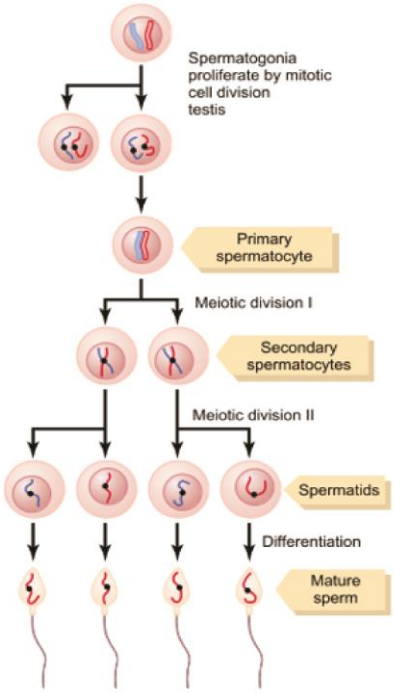

Figure 3.- Germ cell differentiation and different stages of development. In the first step, spermatogonia differentiate into two equals cells by mitotic cell division with one them being a reserve for the maintenance of fertility while the other progresses into differentiation. Spermatogonia then differentiate to primary spermatocyte and thought mitotic division into secondary spermatocyte given rise to spermatids by a second mitotic division. Through a process called spermiogenesis, spermatids differentiate to mature sperm (adapted from [7]).

At this stage occurs a process called spermiogenesis where round spermatids differentiate into spermatozoon losing the excess of cytoplasm and growing the flagellum[11]. As the spermatozoon end its differentiation it enters the lumen of the tubule beginning a new process, spermiation in which this cells disconnected from the *epithelium* and become free, being then called spermatozoa[12]. However, even though the spermatozoa are free from seminiferous epithelium they still don't have the capacity to fertilize an oocyte as this ability is gained as spermatozoa pass though epidymal regions[13].

1.1.2- Endocrine Regulation

There are two important functions in the adult male testis, the synthesis of T and the production of sperm. Control of this function is led by hypothalamic-pituitary-testicular axis(HPT-axis) in a feedback loop between two crucial hormones the follicle stimulating hormone (FSH) and luteinizing hormone (LH)[14]. These hormones are of major importance because their action affects primarily the LCs and SCs, more precisely the LH acts mainly on

LCs in the testicular *interstitium* promoting the synthesis of T while FSH facilitates spermatogenesis by predominantly acting on SCs and germ cells[14]. This regulation begins with the secretion of Gonadotropin-releasing hormone (GnRH) from the hypothalamus in pulses that can occur at different frequencies through the day (Figure 4)[15]. In turn, GnRH acts on the anterior pituitary given rise to the synthesis and secretion of LH and FSH being that GnRH control is related to aromatization of testosterone to E_2[15].One important factor of FSH action is modulating the release of growth factors by SCs as they are important for the progression of spermatogonia into spermatozoa[14]. One of the influential factors being the secretion of glycoproteins making the transport of ions and hormones easier providing bioprotective function but also the tight junction, making it possible of germ cells to receive a supply of ferritin by the Sc[16].

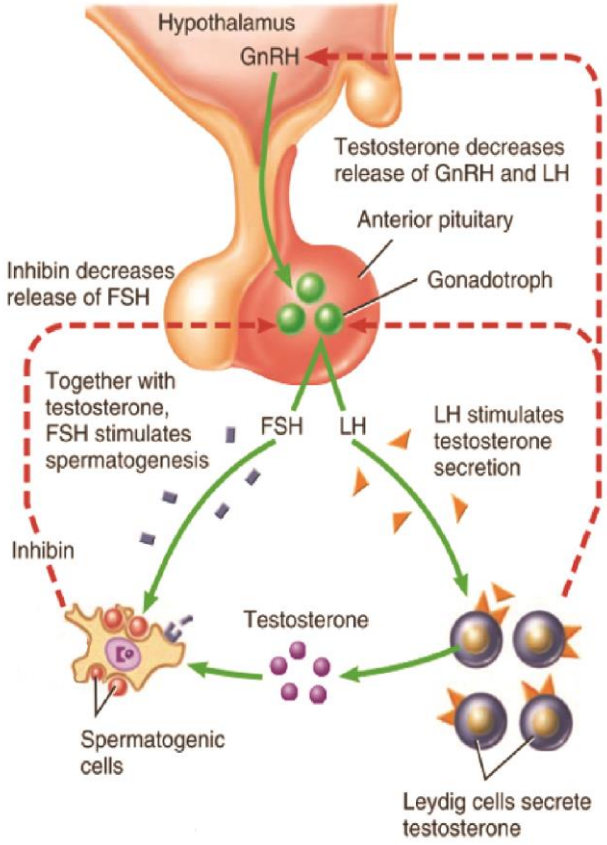

Figure 4.- Endocrine regulation between hypothalamus, pituitary and testis. Gonadotropin-releasing hormone is released from hypothalamus stimulating anterior pituitary to produce follicle stimulating hormone (FSH) and luteinizing hormone (LH). LH, in turn, stimulates Leydig cells (LCs) to release Testosterone (T) while FSH stimulates Sertoli cells (SCs) releasing inhibin. With the rising levels of testosterone and inhibin hypothalamus and pituitary are inhibited from producing more FSH and LH inhibiting, in turn, hypothalamus activity. (adapted from [1])

Inhibin B, produced by SCs, is also important due to its' function as endocrine signal for the integrity and activity of the stem cell system providing a signal to the pituitary to the capacity of sperm production exerting a negative feedback with FSH production[17]. Together the FSH and inhibin axis control the production of sperm and the LH-testosterone axis the androgenization of the organism[18]. With T secreted by LCs entering the bloodstream and

diffusing into the testicular interstitial space, it also interacts with SeT, as a result with SCs being converted into E2 with the remaining T being responsible for the stimulation of specific steps of spermatogenesis [19]. It's important to note that androgen and estrogen balance is fundamental for normal sexual development and reproduction in mammals. In fact, in the testis, the maintenance of this balance is a result of paracrine and endocrine factors.

1.2- Oxidative Stress and Apoptosis

Oxygen is an essential element in the aerobic metabolism from cells, being as a result essential for normal cell functions. However, those functions especially when correlated with metabolism leads to by-products such as reactive oxygen species (ROS). Cellular metabolism depends on the supply of ATP from mitochondria, leading to the production of ROS through mitochondrial respiratory chain [20].

Oxidative stress (OS) is the result of increased cellular damage induced by oxygen and oxygen-derived oxidants known as ROS associated with the class of free radicals. This means that a compound has one or more unpaired electrons being the most implicated when it comes to changes in cell biology[21]. The generation of excessive ROS is called positive oxidative stress occurring when the shift in ROS balance towards pro-oxidants occurs, either by the decreased of antioxidants or excess production of ROS[22]. There are three major types of ROS, hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical (OH)[23]. These ROS in higher concentrations are related with diverse male fertility issues as is an example the decreased in sperm motility and viability [24-26].

The apoptotic process is a genetically encoded suicide program that leads to morphological and biochemical changes[27]. Included in the biochemical changes, responsible for this process are ROS, caspase activation and others. Studies have associated the ability of OS to promote apoptosis by provoking mass cellular damage as a result of lipid peroxidation [28]. However, apoptosis to occur there is a need for several protease families to be implicated being the most prominent the caspases[29]. They are characterized by being cysteine-containing aspartic-specific proteases which exist in the form of zymogens in the soluble cytoplasm, mitochondrial intermembrane space and nuclear matrix of virtually all types of cells [30]. Caspase can be divided into two groups, the initiating caspase composed of caspase 2,8,9 and 10 and the effector's caspase with 3,6 and caspase 7[31]. Initiators caspase is activate through dimerization initiating the apoptotic signal caspase[31]. Two caspases stand out in the extrinsic pathway, caspase 8 and 10 playing an important role in the elimination of defective cells during development and immune system responses while in intrinsic pathways caspase 9 and 2 play the major role being responsible for cells elimination after ionizing radiation, chemotherapeutic drugs and mitochondrial damage [32]. One of the main intervenient in apoptosis is caspase 3 as their pathway activation may be dependent or independent from

mitochondrial cytochrome C release and caspase 9 functions, this caspase is also known as executioner caspase, existing within the cytosol as inactive dimers [33]. However, three different pathways lead to apoptosis:(1) cytotoxic cells, (2) Cell surface receptors, (3) mitochondrial dysfunction (Figure 5) [30].

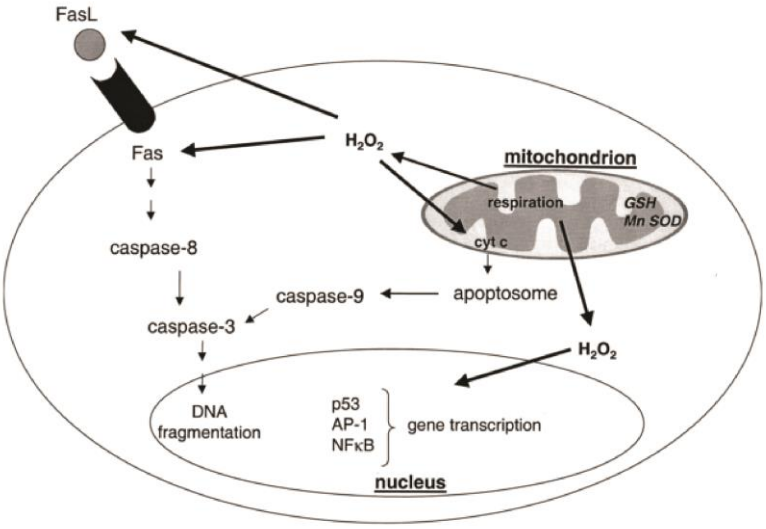

Figure 5.- Pathways for caspase activation and apoptosis. One of the hypotheses behind apoptosis induced by reactive oxygen species (ROS) is by upregulation of the Fas-FasL systems activating caspase 8 and downstream caspase. Also, the release of Cytochrome c and binding to apoptotic protease activating factor 1 (Apaf-1) forms the apoptosome complex which in turn activates caspase 9 activating as a result caspase 3. (adapted from[30])

The first process is important for the protection against intracellular pathogens and tumor cells through lymphocytes which induces apoptosis from perforin/granzyme interaction[34]. The second process occurs when a ligand connects with a cell surface receptor (Fas or TNF-R) leading to the assembly of death-inducing signaling complex (DISC) inducing the activation of caspase 8 which in turn activates caspase 3 activating the caspase cascade leading to cleaves of different subtracts such as caspase dependent endonuclease entering the nucleus and proceeding to cut DNA [35]. The final process occurs when mitochondria dysfunction leads to the release of cytochrome c to the cytosol biding to apoptotic protease activating factor 1 (Apaf-1), forming the apoptosome complex which recruits pro-caspase 9 activating in turn caspase 3 and 7 [32-33].

1.2.1- Oxidative Stress, Apoptosis and male (in)fertility

In reproductive biology, the production of ROS happens in a normal physiologic process as reactive oxygen species are important mediators of normal sperm functions an example being the capacitation of a spermatozoa [38].However, it is important to notice that these cells are also highly susceptible to damage induced by this oxidants due to the high content of polyunsaturated fatty acids within the plasma membranes but also due to the low concentration of scavenging enzymes in the cytoplasm[39]. Thus, OS has become a focus of

interest when it comes to male infertility. Evidence indicates that ROS contribute to 30-80% to sperm damage by two different ways, the first being damage to sperm membrane and the second by damage of sperm DNA[40]. As a result, it is believed that ROS is a major contributor to idiopathic infertility as men with this condition generally have a higher seminal ROS levels and lower antioxidant potential than healthy controls [41]. Nonetheless, the principal sources of ROS in semen are leucocytes and abnormal spermatozoa [42-43].

As far as spermatozoa are concern one of the plausible hypothesis recently suggested is that the sperm DNA is attacked by mitochondrial ROS originating from functional defective spermatozoa leading to defects in sperm motility[44]. DNA attacks can occur by different factors such as single and double DNA strand breaks, loss of base to create a basic site, chemical modification of a base, inter or intra strand DNA cross-linkage and DNA proteins cross-links [41]. However, evidence suggests that leucocytes produce more ROS than spermatozoa even up to 1000 times more as a result of the role they play when it comes to infection, inflammation and cellular defenses mechanisms[45]. Consequently in case of male genital tract infection sperm functions are affected by activation of leucocytes to release ROS leading the stimulation of membrane lipid peroxidation (LPO) by OS, resulting in decrease of membrane fluidity of plasma and organelle membranes, damaging membrane function, ion gradients, receptor-mediated signal transduction among other [46-48]. As referred the male reproductive system has contact with ROS in order to maintain its normal function, however, a problem arises when, because of exogenous sources an unbalance is created being one of the reasons the contact with radiations[49](Figure 6).

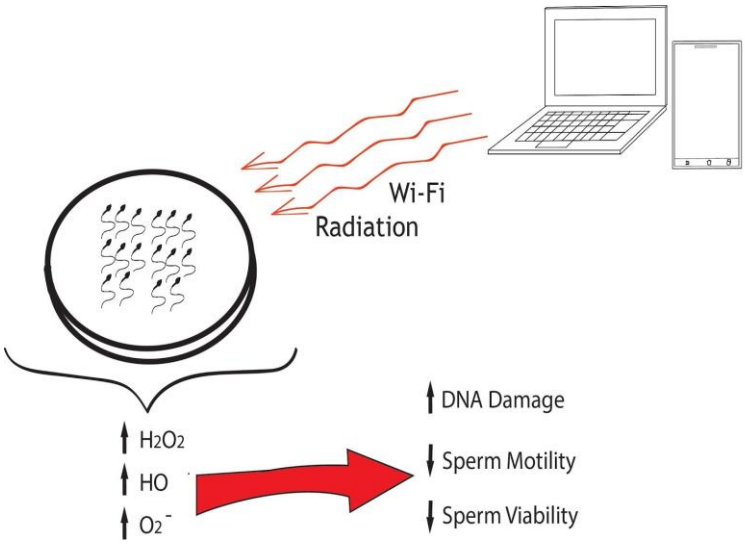

Figure 6.- Effects of Wi-Fi Radiation on Sperm. Spermatozoa exposure to wi-fi radiation from the use of laptops, tablets and mobile phones leads to an increase in hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and super peroxide (O_2^-) leading to a decrease in sperm motility and viability and increasing DNA damage resulting in the decrease of male fertility.

Various studies have been made in order to shed light between oxidative stress and male (in)fertility resulting in the association between oxidative stress and unidentified male

infertility- male sub infertility[50]. Nevertheless, the increased risk of higher oxidative stress levels in male reproductive tissues is also correlated with a higher apoptosis rate [51].

1.3- Wireless technologies

Wireless technology has the beginning of its history in the 1880s as a result of Heinrich Hertz and Guglielmo Marconi experiences [52]. It is important to note that it was Marconi who developed the principle of wireless long-distance communication we still use today by successfully realizing transmission of communication by radio waves between ships at sea [52]. However, only in the second half of the 20th century the key details of wireless mobile systems were developed by Bell Laboratories, being the idea the use of radio frequency (RF) in a group of cells arranged in a specific structure serving unlimited number of users, as a result when calling via RF the calls are systematically handed off from one cell to another being possible to follow a vehicle in movement [53]. Despite this development, it was only possible to provide mobile phones to the mainstream population in the end of the '80s as the innovation in semiconductor technologies made it possible for smaller size phones resulting in the increase of the consumer base. When it comes to computer communication network, the beginning was after the second world war, but the turning point was in the early 1970s as the rapid increase in number of terminals was the force behind local area network (LANs), creating a platform for data communications without the use of Public Switched Telephone Network (PSTN)[54]. In turn, Internet industry was created when LANSs and other Public Data Network (PDNs) were allowed to connect with one another forming Internet with the implementations later on of email and Web browsing the number of users started to increase [54].

There is a consensus that Wireless technology means that transmission of any form of data is conducted through radio waves, infrared waves or microwaves instead of using wires, allowing the support transmission of voice, data and video [55]. Thus, this technology is defined as any wireless technology that uses radio frequency spectrum in any band to facilitate transmission either that been thought text data, voice, video or multimedia services to mobile devices [56]. As a result, people can access network or internet service anytime in any place if there is a network connectivity available. Moreover, the diversity in which wireless technologies are presented led to various ways of use, such as voice-oriented, large scale cellular network (UMTS), to data oriented solutions like wireless LANs (WLANs), wireless personal area networks (WPANs) and wireless sensor network [57]. As a result data oriented market evolved around the Internet and computer communication [54]. It's necessary to note that a wireless system has tree essentials components, a receiver, a transmitter and a radio propagation channel, as for the transmitter and the receiver those are influenced by obstacles around the transition antenna[54]. As far as wireless communication goes there are various ranges of radiation the LANs which are mostly used for laptops and workplaces

because they are designed to have higher data rates than cellular systems[54]. However, in recent years mobile phones undergo a turning point, as the first generation (1G) of mobile phones were only voice-oriented analog cellular and cordless telephones, the second generation (2G) became voice-oriented digital cellular and Personal communication services (PCS) systems, data oriented wireless wide area network (WANs) and LANs but the third (3G) and fourth generation (4G) changed the use of mobile phone[58]. The 3G had higher data transmission speeds, superior network capacity and more sophisticated and enhanced network services while 4G presented the most extensive, widespread, expeditious and high-speed wireless service, being expected to provide data rates from four to ten times higher than conventional 3G[59]. As consequence cellular technology started to shift towards data and Internet services resulting in a wide range of new anytime/anywhere computing and multimedia applications ranging from navigation to mobile video streaming [60].

1.3.1- Wireless technologies and male (in)fertility

It's important to realize that in our daily life we are 24/7 connected via wireless communication in specific wi-fi communication. As a result, different studies reported the consequences of this interaction linking EMR to several health concerns, being brains tumor, increased risk of breast cancer, altered immune function, nerve cell damage, cardiovascular diseases, miscarriages, sleep disruption problems and even short-term effects in cognition and behavior amount them[61-63]. Those observations lead to the questioning of the influence of EMR in male fertility leading to different studies in *in vivo* and *ex vivo* models to improve the understanding of the use of today's technologies, especially wireless technologies, and male infertility. Atasoy *et al.*[64], used a daily exposure of 24h/day for 20 weeks, to 2.437GHz from a Wi-Fi device and observed that the exposed group had a significantly higher level of 8-hydroxy-2'-deoxyguanosine (8OHdG) and a significantly lower activity of Catalase (CAT) and Glutathione peroxidase (GPX). Dasdag and his research group[65], (exposing rats to 2.4 GHz radiation for 24h/day/12months) also found not only a significant decrease in seminal vesicles weight but also in the epididymis. To determine the impact of EMR in sperm parameters Shahin *et al.*[66], exposed mice to nonthermal 2.45-GHz low-level microwave radiation (MW) radiation for 2 h/day for 30 days using a microwave source and pyramidal horn antenna and reported a significant decrease in the number of sperm as well as viability in the experimental group. Also, the number of dead sperm was found to be higher compared to control group. Relating to those results, Yan and colleagues [67], exposed rats to 1.9GHz EMR for two 3-hour periods every day for 18 weeks and the results revealed most of the sperm cells without motion, dead or with straight rigid tails in the exposed rats. Beyond that, they also found numerous clumps of sperm cells where the heads of the cells appeared to be sticking together. Shokri *et al.*,[68] investigated the effects of short- and long-term whole-body exposure to 2.45GHz Wi-Fi radiation exposing one group of rats 1 hour/day/2 months and another group 7 hours/day/2 months and their findings showed a significant reduction in the percentage of motile sperm and a significantly lower concentration and reduction of the

proportion of normal to abnormal sperm in 1-hour and 7-hour groups compared to controlled group. Another research group found that animals exposed for 2 h a day for a total of thirty-five days to 2.45 GHz microwave radiation has an negative effect on testicular organs[69]. Avendaño and colleagues[70], collected human semen samples and incubated them at room temperature under a laptop computer connected to the internet wirelessly for 4 hours with a frequency of 2.4GHz. They found out that laptop Wi-Fi exposure induced a significant decrease in sperm progressive motility.

However, not all research suggests a negative impact of wireless technologies in male fertility, in fact Lee *et al*.[71], used a reverberation chamber to allow whole body rat exposure system in vivo experiments for 45 min/day, 5 days/week with a total of 12 weeks simultaneously to 848,5MHz and 1950MHz radiation to evaluate the effects on sperm parameters, but no significant differences were detected. Also, Oni and research group[72], exposed human semen samples to 2.45GHz from a laptop antenna for 1 hour has not found significant differences. Despite that, Dasdag *et al*. [65], also studied the long-term effects of exposure to radiofrequency radiation (RF) from Wi-Fi equipment by exposing a group of rats to 2.4 GHz radiation for 24h/day/12months and observed that the percentage of head defects was higher in the exposed group compared to the control group. Still, there was no significant statistical difference in sperm concentration, motility and tail defects. Also, studies have been made to evaluate Wireless technologies influence in pregnancy as demonstrated by Gannes, F. P. and group[73] which found none statistically significant differences between the exposed and control groups of pregnant rats and their offspring.

II- Aims

Recent studies have emphasized the correlation between the use of wi-fi technologies and the increased of OS as being a possible contributor to the decrease of male reproductive functions. Hence the increased in evidence that highlight the relations between wireless technologies, oxidative stress and a decrease of male fertility parameters leading us to the present project.

The general aim of the research described in this work was to shed light to the association between the use of wireless technologies and male (in)fertility correlating the possible harmful effects of this technology in seminiferous tubules and thus an unbalance in oxidative stress levels and therefore in apoptosis mediated by caspase 3 activity.

To answer the question, we had the following lineup:

- Development and validation of a realistic EMR model.
- Evaluation and selection of the ideal age of development of spermatogenesis by studying the maturation of seminiferous tubules of *Wistar* rats with different ages.
- Evaluation of the effects of EMR in immature rat *ex vivo* SeT culture.

III- Materials and Methods

3.1- Chemicals

Dulbecco's Modified Eagle Medium Ham's Nutrient Mixture F12 (DMEM F12) and Gentamicin were obtained from Biochrom Gmbh (Berlin, Germany). Total Oxidant Status (TOS) was obtained from Rel Assay Diagnostics (Gaziantep, Turkey). Kwik- Diff stain Kit was obtained from Thermo Scientific (Massachusetts, EUA). All other chemicals were purchased from Sigma- Aldrich (St. Louis, Missouri, EUA).

3.2- Instruments

To simulate the everyday life when it comes to the use of wireless communication three instruments were used, a mbed and a wifey, both connected via a wi-fi router. The mbed platform is used to create a cloud environment for the development of fully integrated online software by using C and C++ language [74]. In our case, the platform mbed used was mbed LPC1768 (Figure 7) which is designed for prototyping all sorts of devices, especially those including Ethernet, USB, and the flexibility of a variety of peripheral interfaces and FLASH memory [75].

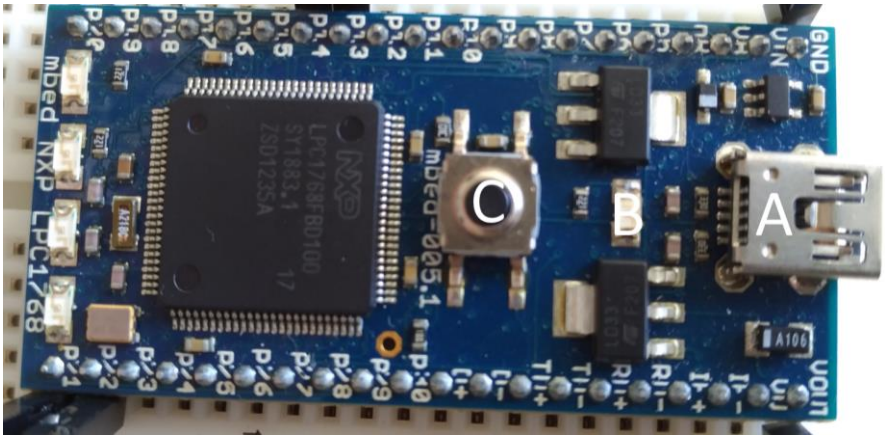

Figure 7.- Example of mbed used in the present work. [A] Represents the micro USB used to power the platform, [B] is a LED light (lights in blue) used to control if the mbed is working and [C] is a reboot bottom used in order to reset the packets been send.

For the connections of interest to be made, pinout, which is a cross-reference between the contacts, or pins, of an electrical connector or electronic component were used (Figure 8).

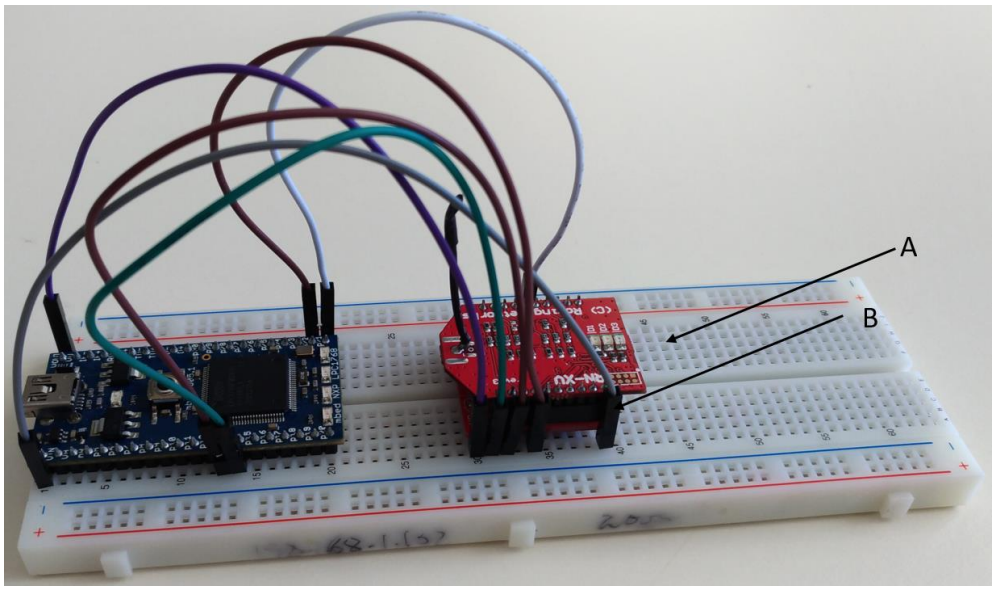

Figure 8.- Platform used for the connections of interest by using pinouts. Pinout is a cross-reference between the contacts or pins of an electrical connector or electronic component used. [A] Pinout; [B) connecter between the wifly and mbed.

The mbed were used to programme the transmission of a setup packet including the type of date and the time interval in which the selected data was being emitted, being in this case a time interval of 0,5 seconds.

The Wifly (RN-XV WiFly Module - Wire Antenna) was the system that worked as the emitter and receiver of the information programmed into the mbed[76]. Therefore, the wifly has an antenna (to transfer and receive data) as well as 3 LED systems (Figure 9) to help identified when the system is turned on (green), no connection is detected (red) and finally when data is being transferred between systems' (yellow).

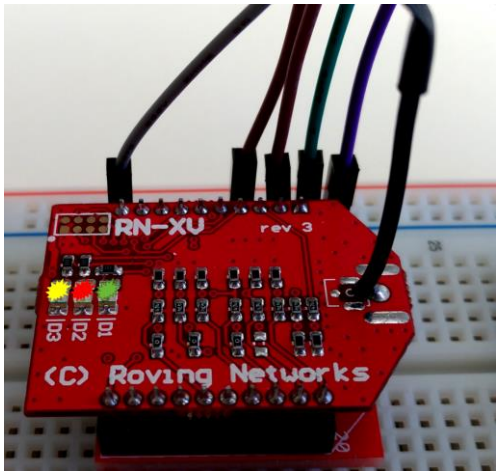

Figure 9.- Example of wifly used in this experimental setup. The LED with the green dot is turn on when the instrument is on, the red is when there is no transmission of data and the yellow dot indicates when the transmission of data is correctly happening. For this System to be corrected used the green and the yellow LED must be on.

For the information to be received and transmitted it was used a wireless system, ergo the need for the wi-fi router (TP-LINK, model number TL-WR740N) (Figure 10).

Figure 10.- Example of wi-fi router used in this experimental setup

3.2.1- Experimental Design

A wi-fi router was placed into a culture incubator alongside a data transmission system as seen in the image below (Figure 11). After the Set were placed in culture plate well they were divided into 2 groups: one placed in the culture incubator with the setup turn on and another group with the set up turned off (control). The plates were taken from the incubator after 72 hours of exposure, the Set were extracted and stored at -80ºC until use. Which incubator was set at 33ºC, 5% CO_2 and 95% O_2.

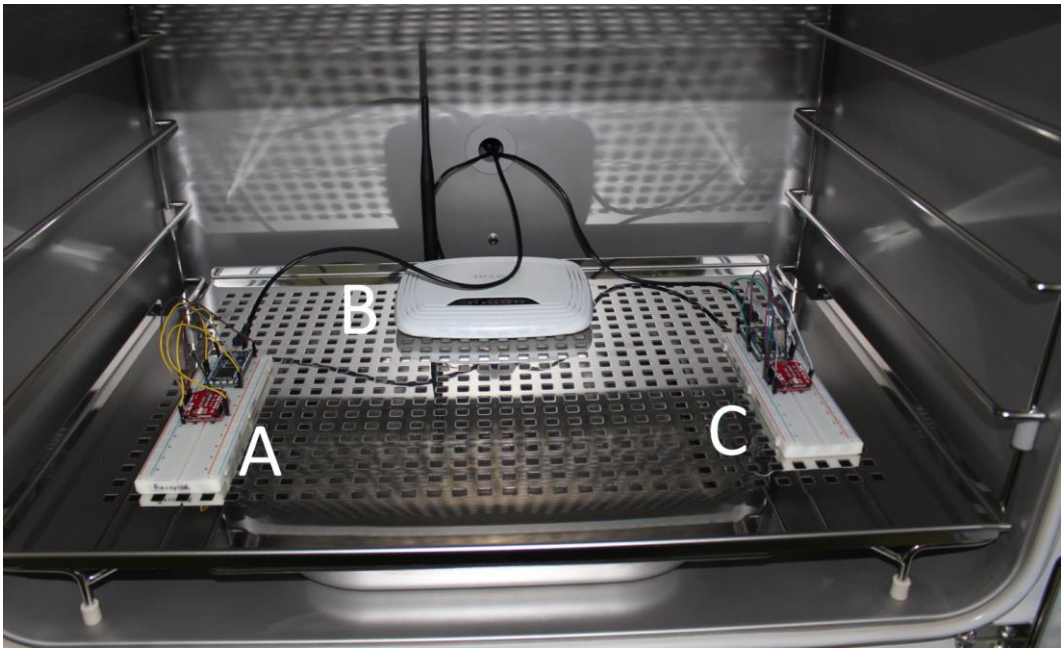

Figure 11.- Setup with wi-fi router, mbed and wifly in culture incubator. Mbed and wifly were power by a USB port connected with a power extension outside of cell culture incubator in which **[A]** is an emissor, **[B]** the wi-fi router and **[C]** the receiver.

3.3- Animals

6 Wistar rats 3-month-old 12 *Wistar* rats (*Rattus norvegicus*), four *Wistar* rat which with different ages (19, 20, 21 and 22) and 12 20 days old *Wistar* rats were housed in accredited animal colony (Health Sciences Research Center, University of Beira Interior) and maintained with food and water *ad libitum* at a constant room temperature (20 ± 2ºC) on a 12-hours cycle of artificial lighting. All animal experiments were performed according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European directives for the care and handling of laboratory animals (Directive 2010/63/EU). In accordance with the Portuguese law (Ordinance no. 1005/92 of 23 October), the research team requested a permission to perform this animal experimentation study to the Portuguese "Direcção Geral de Veterinária" (Portuguese Veterinarian and Food Department).

3.4- Model Validation- Sperm Parameters Analysis

After the assembling of the exposure model setup validation was necessary to confirm if Wi-fi radiation were indeed being used and resulted in any alteration. As spermatozoa are one of the most sensible cells to radiation we tested this model by exposing adult rats' spermatozoa to our model for 1h. To do that, each *cauda* was minced in 3 ml PBS solution pre-warmed at 37ºC. Sperm motility was determined by placing 100 ul of sperm suspension on a pre-warmed slide. 10 random fields were accounted for each sample using an optical microscope (1000x magnification) with the diaphragm closed (primo star, Zeiss). For sperm count, an aliquot of sperm suspension was diluted in 490 ul of PBS and 10 ul were placed into a Neubauer's counting chamber (Tiefe Depth Profondeur, Optik labor, Switzerland) for sperm counting using an optical microscope (1000x magnification). Sperm viability was evaluated by using the one-step eosin-nigrosine staining technique. 5ul of sperm suspension was mixed with 10 ul of eosin-nigrosine stain (0.6% eosin and 5% nigrosine) and placed on a pre-warmed slide. Non-viable sperm absorbs eosin thus the head of sperm is colored red while viable head sperm remains white. For sperm morphology, the Kwik- Diff stain Kit (Thermo Scientific) was used. This Kit has 3 sequential solutions: a fixative (methanol), an anionic/acidic dye (eosin) and a cationic dye (methylene blue). The First step was mixing 5ul of sperm solution with 10 ul of PBS and dragged with a coverslip and left to air dry. After dried the slide was immersed in each solution of the staining Kit and left to air dry. The sperm was classified as normal or abnormal (head, neck/midpiece or tail defects).

In sperm viability and morphology, a total of 100 sperm cells in each semen samples were analyzed in random fields under a light microscope (Zeiss, Jena, Germany, Axio Image A1 microscope) with oil immersion (1000x magnification).

3.5- Animals' Age Selection- Histological Assay

Spermatozoa are highly susceptible to radiation and because our focus was not to evaluate the effects of wi-fi radiation on germ cells but in the seminiferous tubules as an all, it was from our interest that our seminiferous tubules were poor on germ cell development. SCs studies prefer rats with less than 21 days old as at that stage of development germ cells are still under development and the final stage of maturation of SCs has been reached [77]. Thus major cells populations are SCs and germ cells population present in SeT are in the early stages of development[78]. Consequently, to confirm the best age of development, 4 *Wistar* rats with different ages (19, 20, 21 and 22 days) were used for histological analysis. As a result, paraffin sections (5 µm) of SeT were deparaffinized in xylene, rehydrated in graded alcohols and stained with hematoxylin (Leica Biosystems, Peterborough, Cambridgeshire, UK) and eosin (Leica Biosystems) (H&E). Each section were analyzed using an optical microscope (630x magnification; Zeiss, Jena, Germany, Axio Image A1 microscope).

3.6- *Ex Vivo* Rat Seminiferous Tubules Culture

12 animals were anesthetized and sacrificed by inhalation of CO2. Testes were immediately excised and transferred to a phosphate-buffered saline (PBS) solution into a petri dish. In aseptic conditions, *tunica albuginea* was cut to exposed the tubules. 6 to 10 mm portions of one testis were placed in culture plate wells containing 5 ml of pre-warmed Dulbecco's modified Eagle's medium/Ham's F-12 culture medium (Sigma-Aldrich, St. Louis, USA) supplemented with 20 mg/L gentamicin 0.1 mM 3-isobutyl-1-methylxanthine, and 1 µg/L of bovine serum albumin (BSA) 10% and incubated at 33ºC, 5% CO_2 and 95% O_2 for 72 hours.[79] At the end of the experimentation Set were recovered from the culture medium and frozen at -80ºC until protein isolation.

3.7- Total Protein Extraction

Set were homogenized in lysis buffer supplemented with 1% protease inhibitor cocktail. The homogenate was placed for 1h on ice and being vortexed with 15 minutes of interval and then the suspension was centrifuged at 14000x*g* for 20 minutes at 4ºC. The total protein concentration was quantified using the Bradford Protein Assay Kit Sigma- Aldrich (St. Louis, Missouri, EUA) according to the manufacturer's instructions and the absorbance's were measured by xMark Microplate Spectrophotometer from Bio-Rad (Hercules, USA). BSA was used to construct a standard curve.

3.8- Total Oxidative Status Assay

After protein extraction 7,5ul were added in a well with 50 ul of reagent 1 prepared according to the manufacturer's instructions and the first absorbance was read at 530 nm after 30s incubation. Secondly, was added 2,5 ul of reagent 2 prepared according to the manufacturer's instructions and incubated for 10 min at room temperature. The absorbance was read at 530 nm. The results were calculated based on manufacturer's instructions.

3.9- Caspase 3 Assay

For the determination of enzymatic activity of caspase 3, 5ul of total protein extracted from rat seminiferous tubules was incubated with the appropriate volume of reaction buffer (20 mM HEPES, pH7.4, 2 mM EDTA, 0.1% CHAPS, mM DTT) and 2 mM of caspase-3 substrate (Ac-DEVD-pNA) in a 96 multi-well plate. The negative control had the same reagents except for the protein sample. The incubation proceeded in a cell incubator at 37ºC overnight. Upon caspase cleavage of Ac-DEVD-pNA, pnitro-aniline (pNA) is release turning the reagents into a yellow color, which is measured spectrophotometrically at 405 nm. The amount of generated product was calculated by extrapolation from a standard curve of free pNA and is directly proportional to the activity of caspase-3.

3.10- Statistical analysis

The statistical significance of differences between experimental groups was assessed by the Student's t-test (GraphPad Software, San Diego, CA, USA). Significant differences were considered when $p < 0.05$. All experimental data are shown as mean ± SEM.

IV- Results

4.1-Model validation -Evaluation of sperm parameters

a) Wi-fi radiation decreases sperm motility

To evaluate the effects of wi-fi radiation in sperm motility we evaluated this parameter at 0h in control and exposed group and after 1h also in control and exposure group. Our results show that there was a statically significant decrease in sperm motility after 1h of exposure to wi-fi radiation (Figure 12). Thus control 0h has an 80.82±1.787 and control 1h has 41.17±7.812 while exposure 0h has 84.17±1.249 and exposure 1h has 14.67±4.417 with a statically significant difference of 2.8-fold between exposed and control 1h.

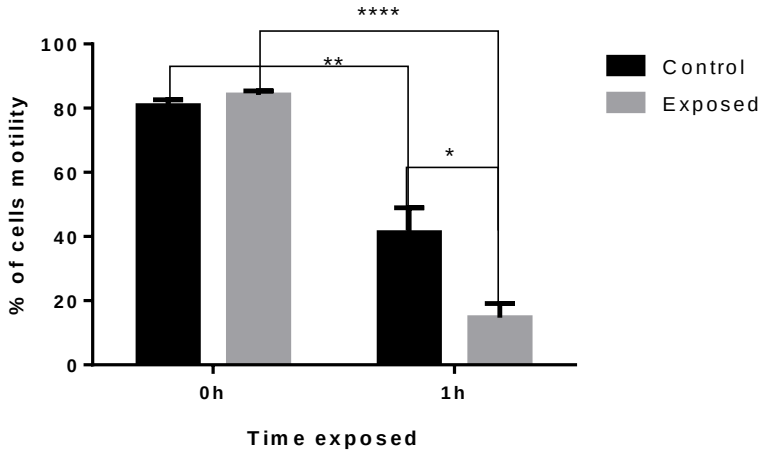

Figure 12.- Evaluation of percentage of sperm motility after 0h and 1h of exposure to Wi-fi radiation. Results are shown in percentage and expressed as mean ± standard error of the mean (SEM) with a n≥5 for each condition. The figure shows the comparison of sperm motility between control 0h and control 1h, exposed 0h and exposed 1h and control 1h and exposed 1h. Significant results are indicated as * (*p-value* < 0.05), ** (*p-value* < 0.01) and **** (*p-value* < 0.0001).

b) Wi-fi radiation had none significant effect on head, neck/midpiece and tail defects of sperm

To evaluate the effects of wi-fi radiation in the head, neck/midpiece and tail defects we evaluated this parameter at 0h in control and exposed group and after 1h also in control and exposure group. Our results show that there was no significant decrease of this parameters after 1h of exposure to wi-fi radiation (Figure 13).

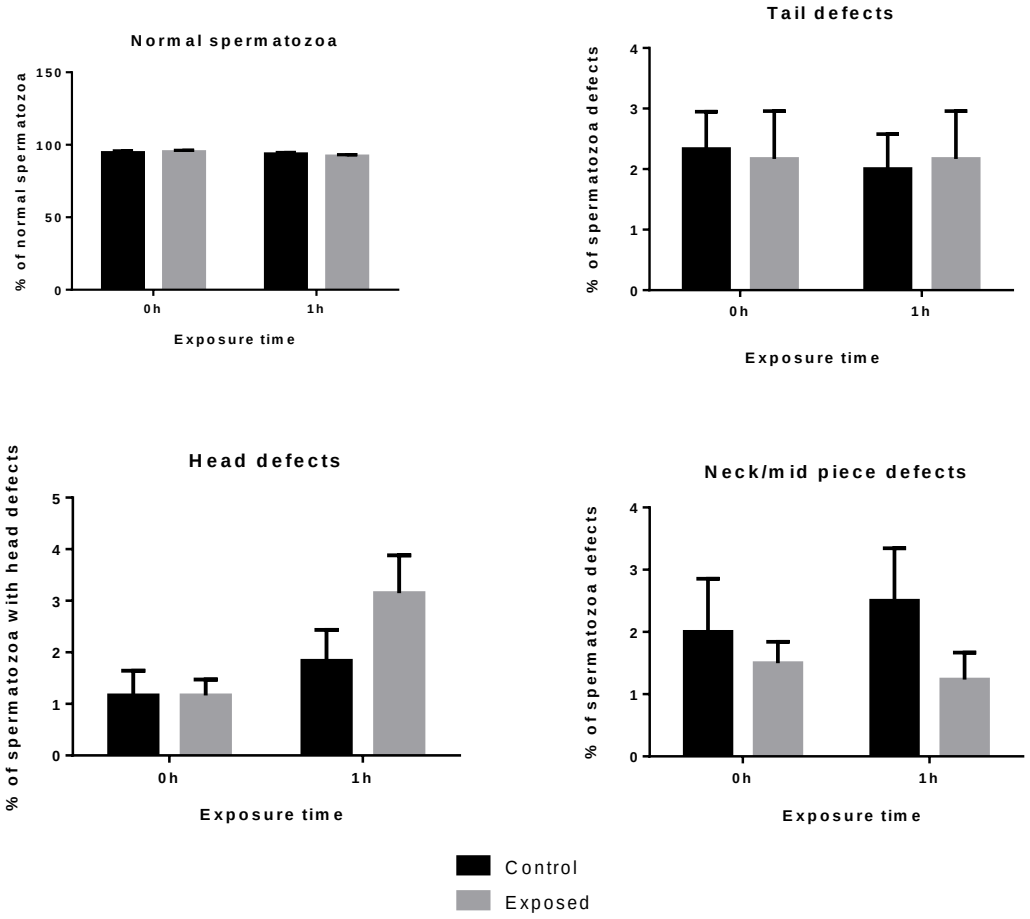

Figure 13.- Evaluation of percentage of sperm morphology defects after 0h and 1h of exposure to Wi-fi radiation. Results are shown in percentage and expressed as mean ± standard error of the mean (SEM) with a n≥5 for each condition. The figure shows the comparison between control 0h and control 1h, exposed 0h and exposed 1h and control 1h and exposed 1h in which **[A]** is the study of normal spermatozoa, **[B]** head defects, **[C]** neck/midpiece defects and **[D]** tail defects.

C) Wi-fi radiation had none significant effect sperm viability

To evaluate the effects of wi-fi radiation in sperm viability we evaluated this parameter at 0h in control and exposed group and after 1h also in control and exposure group. Our results show that there was no significant decrease of this parameters after 1h of exposure to wi-fi radiation (Figure 14).

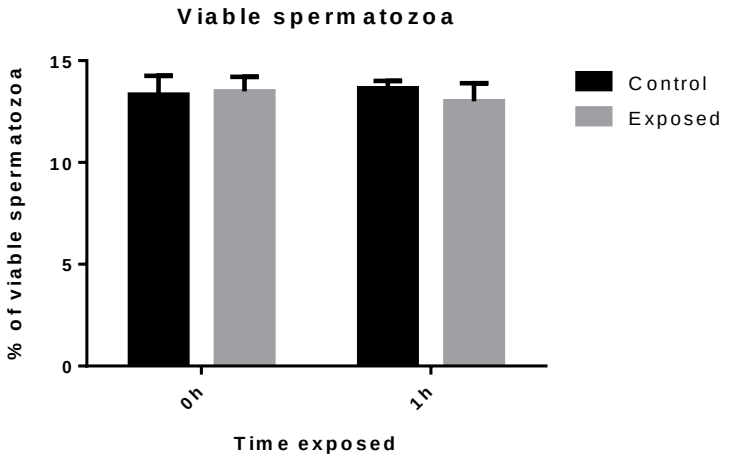

Figure 14.- Evaluation of percentage of viable spermatozoa after 0h and 1h of exposure to Wi-fi radiation. Results are shown in percentage and expressed as mean ± standard error of the mean (SEM) with a n≥5 for each condition. The figure shows the comparison between control 0h and control 1h, exposed 0h and exposed 1h and control 1h and exposed 1h.

4.2- Testis cells population at 19, 20, 21 and 22 days old.

To confirm the best age of development, 4 *Wistar* rats with different ages (19, 20, 21 and 22 days) were used for histological analysis. It was possible to note that at the ages of 22 and 21 days of development rat's SeT are beginning the transition of SCs to the outer layer and entering the period characterized by the maintenance of the first wave of spermatogenesis to round spermatids[78]. However, at the ages 19 and 20 days, germ cell populations were at the same stage of development, as at this stage of development maximum cell density in each tubule occur. As at the age of 20 days, SeT were more developed and different studies with SCs showed a preference to this stage of development we selected rats with 20 days for our study (figure 15).

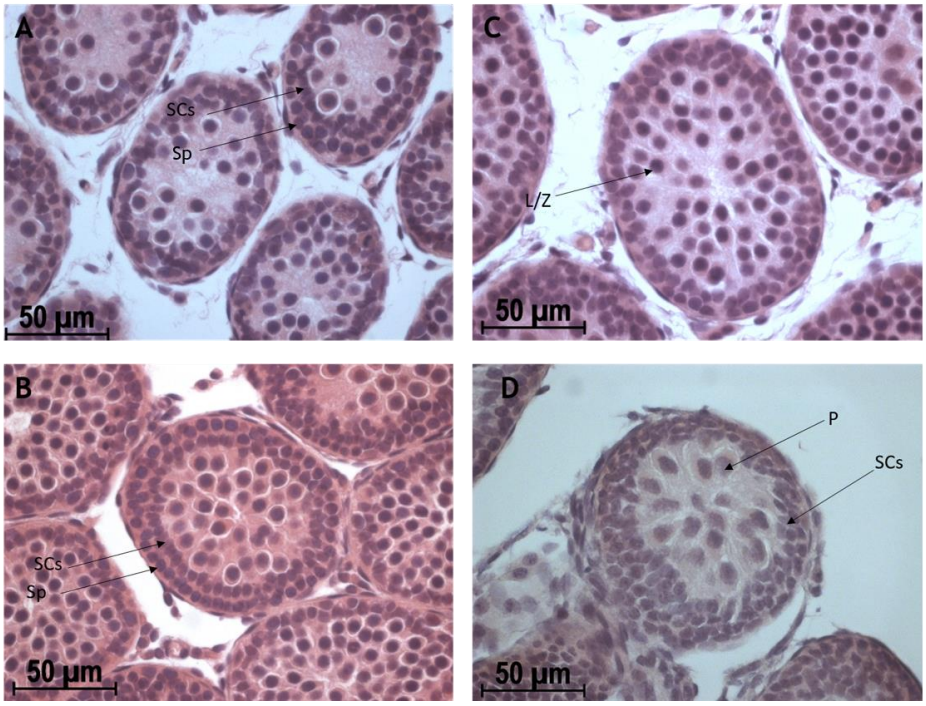

Figure 15.- Seminiferous tubules cells population at different stages of development. [A] At 19 days of development the population of cells in seminiferous tubules consists of Sertoli cells (SC), spermatozoa (Sp) and Pachytene spermatocytes (P) this population is also found at 20 days **[B]**. However, at 21 **[C]** and 22 **[D]** day is possible to note the beginning of the transition of SCs to the outer layer of the Seminiferous Tubules (SeT). At this stages of development Leptotene and Zygotene(L/Z) are also found. Image has an amplification of 630x

4.3- Wi-fi radiation increases Oxidative Status concentration

ROS are produced in the metabolic and physiological process however the balance shift towards oxidative status may be harmful to eukaryotic cells. The oxidants present in the sample oxidize the ferrous ion-chelator complex to ferric ion making a colored complex measured spectrophotometrically.

After 72 hours of culture, TOS concentration was found to significantly increased in seminiferous tubules exposed to Wi-fi radiations compared with control groups with a 2.4-fold increase. Exposed group had a 14.78 ± 0.725 µmol/L compared to 6.094 ±1.209 µmol/L of the control group (figure 16).

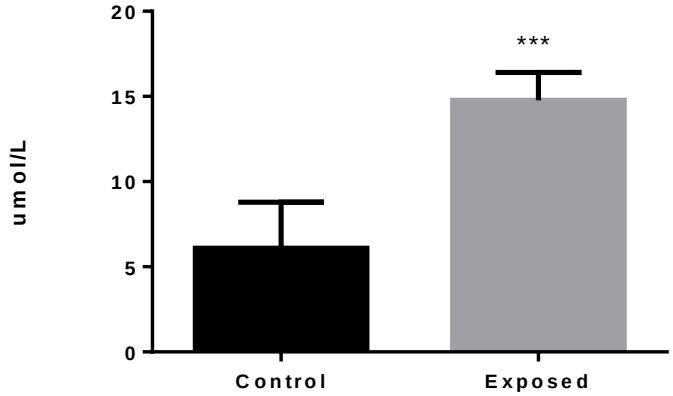

Figure 16.- Evaluation of Total Oxidative Status (TOS) of immature (20 days) rats' seminiferous tubules culture exposure EMR for 72 hours. Results are shown in percentage and expressed as mean ± standard error of the mean (SEM) with a n≥5 for each condition. The figure shows the comparison between control and exposed group. Data are presented as mean ± S.E.M ***($p<0.001$; t-student).

4.4- Wi-Fi radiation increases caspase 3 activity

Caspase are a family of proteases that modulate cells dead, being important in the apoptotic process. Caspase 3 is activated by proteolysis within their interdomain linker being the end-point of multiple pathways that lead to apoptosis [33]. Caspase 3 activity was measured by Ac-DEVD-pNA cleavage by caspase 3 releasing pnitro-aniline (pNA) turning the reagents into a yellow color, which is measured spectrophotometrically.

After 72 hours of culture, caspase 3 activity significantly increased in seminiferous tubules exposed to Wi-fi radiations compared with control groups with a 1.6-fold increase. Exposed group had a 1.616 ± 0.1547 µmol pna/min*µg compared to 1.002 ± 0.1373 µmol/L of the control group (figure 17).

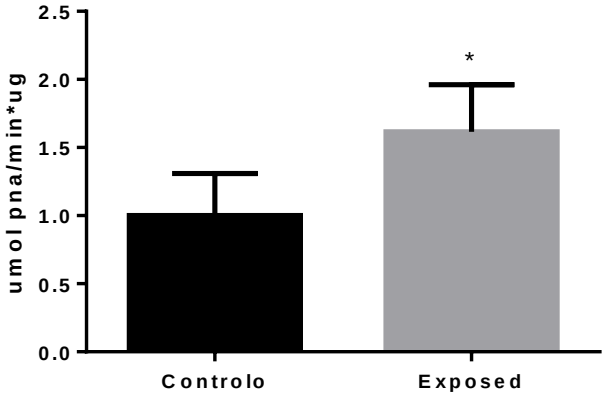

Figure 17.- Evaluation of Caspase 3 activity Assay of immature (20 days) rats' seminiferous tubules culture after EMR exposure for 72 hours. Results are shown in percentage and expressed as mean ± standard error of the mean (SEM) with a n≥5 for each condition. The figure shows the comparison between control and exposed group. Data are presented as mean ± S.E.M *($p<0.05$; t-student).

V- Discussion

Male infertility has been assigned has to be responsible for 45-50% of infertility cases among couples[80]. However, many of them are later diagnosed as having an idiopathic form of male infertility, accounting between 37-58% of the cases being a condition in which fertility occurs spontaneously or as a result of an unknown cause[81]. Thus environmental and lifestyle factors are being taken into consideration as male fertility is known to be sensitive to chemicals present in environment or activities [82]. Those factors are important as male fertility has been reported as declining maybe as a consequence of the rise of the use of chemicals [83]. However, in our modern days, another possible factor has been appointed- the wireless communications systems.

Wireless technologies use has been rising since the turn of the 20th century, as a result, the influence of the radiation, derived from this technology has been a concern especially considering that the average age group of this form of communication has been decreasing[84]. In fact, technology as learning tools has been increasing in the classrooms across the globe highlighting the use of laptops [85]. As a result, the growing use of this technologies both in the professional and personal life have given rise to an increasing concern about their effects on our health, as a result, one aspect that is been talked is their influence on male fertility. Thus, the aim of this project was to create a realistic exposure model to study the influence of wi-fi technologies in seminiferous tubules of *wistar* rats, more precisely by evaluating the influences in oxidative stress and apoptosis.

We started by developing our exposure model and validating its functions by exposing sperm from adults' male *wistar* rats for 1h to our setup and evaluating the sperm parameters. It was found statistically significance in motility, as control 0h has 80.82±1.787 and control 1h has 41.17±7.812 while exposure 0h has 84.17±1.249 and exposure 1h has 14.67±4.417 (Figure 12). However, there were not found statistically significance in morphology and viability between control 1h and exposed 1h (Figure 13 and 14). Although Electromagnetic radiation has been correlated with fertility problems such as a decrease in motility and increase in heads defects, none of the studies used a realistic model to study Wi-fi influences on male fertility. Mahmoudi *et al*. exposed 7 groups of rats to different time exposure and distance from the wi-fi router leading to statistically significant differences between the relative frequency of progressive and rapid progressive sperm in exposed rats compared to the control group[86]. Shokri and colleagues concluded that 2.45 GHz induces a decrease in sperm parameters and an increased in apoptosis-positive cells and caspase 3 activity in the seminiferous tubules in the 7-hour group[68]. Despite the results from these research groups, the fact that Mahmoudi and colleagues' exposure model had a specific time frame in which the rats were exposed to the radiation making this model an unrealistic one, has in our daily life we are in constant contact with these radiations. Also, Shokri and research group used an antenna to simulate the use of this technologies while in our exposure model the simulation came from a wi-fi router, an instrument that is part of our household. Another study evaluated wireless

technologies by using a laptop computer connected to the wireless network and exposing semen sample by placing them beneath the laptop for the duration of 4h resulting in the decrease of spermatozoa motility[70]. Even though our results didn't lead to a significant decrease in viability and morphological changes, the fact that percentage of motility was affected with a statistically significant difference confirms the effects of our model, but also the impact of this radiation on sperm parameters.

One other important step to this project was the selection of the suitable animal age model for this study, thus we used testis from 4 different *wistar* rats with 4 different ages (19, 20, 21 and 22 days) and studied the histological samples from the testis to identify cell populations in the seminiferous tubules. Our selection was based on the fact that at 20 days of age SCs reach their maximum number[87]. Also, at 21 and 22 days is possible to note the beginning of the transition of SCs to the outer layer of the SeT (Image 15. C and D) leading us to discard the use of animals with 21 and 22 days of age. The youngest animals (19 days) weren't considered as their cells population was the same as animals with 20 days but seminiferous tubules weren't as developed (Figure 15 A and B). As a result, we selected for this study animals with 20 days of age.

With exposure model optimized and animals' age selected we exposed SeT culture to wi-fi radiation for 72h and found that SeT exposed to wi-fi radiations had an increased in TOS (figure 16) correlating with an increase in caspase 3 activity (figure 17) when compared with control group. For normal cell behavior, ROS is needed especially when it comes to germ cells as reactive oxygen species has an important physiological role for example in the capacitation and hyperactivation of spermatozoa [49]. However, the excessive production of ROS can be harmful to germs cells and consequently to male fertility as it can induce harmful chemical and structural modifications to sperm nuclear DNA [41]. The fact that SeT culture had a 2-fold increase in total oxidative stress gives rise to the fact that EMR form Wi-fi range is harmful to the tissue being study. It has been described in the literature that high levels of oxidative stress have been related with an increased in apoptotic levels in eukaryotic cells [51]. So, the fact that this culture when in contact with this type of radiation develops a higher concentration of TOS leads to an increase in oxidants and decreased in antioxidants thus shifting the balance towards oxidative status. OS is negativity related with sperm concentration, motility and functions as they are rich in polyunsaturated fatty acids been more susceptible to ROS actions [50]. Also, in mice studies OS has been related to DNA damage in testicular cells[88]. It's important to notice that SCs are subjected to this radiation, along with primordial germ cells and LCs, as they represent the major cellular population in SeT. Therefore, the fact that SCs, the nursing cells to germ cells, are influenced by this radiation may lead to a negative outcome when it concerns to male fertility. In fact, different studies have been made correlating oxidative stress with SCs and the possible impairing of this important nursing cells[89–92]. As far as ERM radiations are concerned, several works have been

developed to study the relation between wireless technologies and oxidative stress in male fertility. In one of those studies Ozoeak and colleagues studied the impact of this radiation on three groups of animals with different ages (4, 5 and 6 weeks) finding that a longer period of exposure leads to an increase in LPO but also that the youngest group of rats had a longer recovering time from the exposure to these radiations[93]. Also, Mailankot and research group[25] found a decrease in GSH and an increased in LPO[25]. Thus, our results are consistent with previous studies.

Apoptosis is a normal occurring process in spermatozoa, being one of the most important reasons the prevention of DNA-damaged cells from participating in the process of fertilization [94]. However, as oxidative stress is related to cells death thought apoptosis we evaluated the caspase 3 activity in the control groups concluding that with wi-fi exposure for 72h there is a statistically significant increase by 1.6-fold in caspase 3 activity (Figure 17). In correlation with our results, Kesari *et al* found that exposed groups to mobile phone range of radiation lead to a significant increase in apoptotic cells[95]. The fact that our exposure model used a more active range of radiation (wi-fi range) leads to an increase in the concern of the effects of these technologies in our physiology Thus, even though our cultures are still in an immature development of germ cells (20 days), therefore not been so susceptible to ROS as they don't have spermatozoa with rich polyunsaturated fatty acids membrane, the high oxidative status leads to cells damages confirmed by the higher activity of caspase 3 in exposed groups.

VI- Conclusions

In the present time, the decrease in human fertility has been among one of the health concerns. The involvement of environmental and lifestyle influences is being pointed out as one of the major causes, however as technology evolves new factors may be placed among those causes, is now the use of wireless technologies among them. Several data evidenced that wi-fi exposure leads to decreased in sperm parameters but also an increased in Oxidative and apoptotic index.

It is important to note that in some parts of the globe, especially in major cities, wi-fi hotspots are increasing in public spaces, leading to an increase in exposure time by decreasing the time outside of our homes and workplace where we weren't exposed to these radiations. With the decrease in fertility and increase in demographic pressure the fact that we are constantly in the presence of this radiation in our daily life increases the concerns of this prolonged use and exposure.

In this dissertation, we have established a relation between prolonging use of wireless technologies, more specifically wi-fi, with the increase of oxidative stress and caspase 3 activity. Also, this was the first study that used a realistic model which demonstrated that EMR affects TOS and caspase 3 activity. As caspase 3 is the end-point of the apoptotic pathway, we haven't the knowledge about what pathways are influenced by this radiation. Thus, further studies will be necessary to elucidate the pathways of apoptosis as well as other effects in the male reproductive system.

VII-References

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