UNIVERSIDADE D
COIMBRA

Carolina Matos Carola

HEAVY METAL EXPOSURE AND ITS EFFECTS IN MALE REPRODUCTIVE HEALTH: AN *IN VIVO* AND *IN VITRO* STUDY

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pelo Professor Doutor João Ramalho-Santos e pela Doutora Renata Tavares e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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List of Abbreviations

% – percentage

µl – microliters

µm – micrometers

µM – micromolar

5-Br-PAPS – 2-(5-bromo-2-pyridylazo)-5-[N-propyl-N-sulfopropyl)amino]-phenol

ADP – adenosine diphosphate

AR – acrosome reaction

As – arsenic

As_2O_3 – arsenic trioxide

As^{3+} – trivalent arsenite

As^{5+} – pentavalent arsenate

ATP – adenosine triphosphate

BMI – body mass index

BSA – bovine serum albumin

CA – California

Ca^{2+} – calcium ion

cAMP – cyclic adenosine monophosphate

Cd – cadmium

Co – cobalt

Cr – chromium

Cu – copper

DAPI – 4',6-diamidino-2-phenylindole

DNA – deoxyribonucleic acid

EDs – endocrine disruptors

ETC – electron transport chain

$FADH_2$ – flavin adenine dinucleotide

Fe – iron

FSH – follicle-stimulating hormone

h – hours

HCl – hydrochloric acid

HCO_3^- – bicarbonate ion

Hg – mercury

$HgCl_2$ – mercuric chloride

HIV – human immunodeficiency virus

IM – immotility

JC-1 – 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide

kg – kilograms

km^2 – square kilometers

LH – luteinizing hormone

M – molar

Mg – magnesium ion

mg – milligrams

mL – milliliter

mM – millimolar

MMP – mitochondrial membrane potential

Mn – manganese

Mo – molybdenum

mRNA – *messenger* ribonucleic acid

mU – glucosidase activity

Na_2CO_3 – sodium carbonate

$NaAsO_2$ – sodium arsenite

NaCl – sodium chloride

NADH – nicotinamide adenine nucleotide in the reduced form

NAG – neutral alpha-glucosidase

NaOH – sodium hydroxide

Ni – nickel

nM – nanomolar

NP – non-progressive motility

ODFs – outer dense fibers

OXPHOS – oxidative phosphorylation

Pb – lead

PBS – phosphate buffered saline

PI – propidium iodide

PKA – protein kinase A

PNP – p-nitrophenol

PNPG – glucopyranoside

PR – progressive motility

PSA-FITC – *Pisum sativum* agglutinin linked to fluorescein isothiocyanate

ROS – reactive oxygen species

SDS – sodium dodecyl sulfate

Se – selenium

SEM – standard error of mean

SPSS – Statistical Package for the Social Sciences Program

t – time

w/v – weight per volume

WHO – World Health Organization

Zn – zinc

$ZnSO_4$ – zinc sulfate

Abstract

Nowadays, as the industrialization continues to grow worldwide, so does the emission of heavy metals to the environment. These heavy metals can act as endocrine disruptors (EDs) and exert a panoply of effects. In fact, an increasing number of reproductive disorders and a reduction in sperm quality in humans has been reported in the last decades, which is disturbing. Therefore, it became imperative to assess the sperm quality and the overall reproductive health of men that are especially vulnerable by living in heavily industrialized areas, such as Estarreja. This Portuguese city has the second largest chemical complex of the country and contamination by heavy metals was already described in the local. In this context, the present study aimed to evaluate the impact of heavy metals exposure on the reproductive health of men from Estarreja by comparing it with a control group corresponding to adult men from the Center region of Portugal. Furthermore, to achieve a more complete study, an *in vitro* approach was also performed to determine the effects of the two most prevalent heavy metals in Estarreja – arsenic (As) and mercury (Hg) – and their mixtures on important sperm function parameters. The *in vivo* study showed that although no differences were observed in what concerns semen volume and pH, sperm concentration, motility, morphology, mitochondrial membrane potential (MMP), chromatin integrity and accessory glands function, the individuals from the exposed group presented a significant decrease in the percentage of capacitated cells and decreased acrosomal integrity. This is a notable finding and the first study designed in an industrialized scenario, in Portugal, that suggests that heavy metal exposure is negatively affecting male reproductive health. Additionally, the *in vitro* study revealed that although, individually, neither As nor Hg exerted deleterious effects on sperm viability, motility, MMP, chromatin integrity, capacitation and acrosome integrity at environmentally-relevant concentrations, when combined, differences on viability, motility and MMP were observed, indicating that As and Hg may act synergistically. In sum, this project demonstrates that exposure to heavy metals may jeopardize male fertility and reproductive health and advertises for the negative effects that a heavily industrialized world may potentially bring to our lives.

Keywords: Exposure to heavy metals; male reproductive health; male infertility; human sperm quality and function; *in vivo* exposure and *in vitro* exposure.

Resumo

Atualmente, há medida que a industrialização continua a crescer mundialmente, cresce também a emissão de metais pesados para o meio ambiente. Esses metais pesados podem atuar como disruptores endócrinos (EDs) e exercer uma panóplia de efeitos. De facto, tem sido indicado nas últimas décadas um número crescente de patologias reprodutivas/anomalias urogenitais e uma redução na qualidade espermática em humanos, o que é perturbador. Deste modo, tornou-se imperativo avaliar a qualidade espermática e o estado geral de saúde reprodutiva de homens especialmente vulneráveis por viverem em áreas altamente industrializadas, como Estarreja. Esta cidade portuguesa possui o segundo maior complexo químico do país e já foi descrita no local contaminação por metais pesados. Neste contexto, o presente estudo tem como objetivo avaliar o impacto da exposição a metais pesados na saúde reprodutiva de indivíduos de Estarreja, comparando-a com um grupo de controlo que corresponde a homens adultos da região centro de Portugal. Para além disso, de modo a obter um estudo mais completo, uma abordagem *in vitro* foi também realizada para determinar os efeitos dos dois metais mais prevalentes em Estarreja – arsénio (As) e mercúrio (Hg) – e misturas de ambos em parâmetros importantes de funcionalidade espermática. O estudo *in vivo* mostrou que apesar de não serem observadas diferenças no volume seminal e pH, concentração espermática, mobilidade, morfologia, potencial de membrana mitocondrial (MMP), integridade da cromatina e funcionalidade das glândulas acessórias, os indivíduos do grupo exposto apresentam uma redução significativa na percentagem de células capacitadas e diminuída integridade do acrossoma. Este é o primeiro estudo desenhado num cenário industrializado, em Portugal, que sugere que a exposição a metais pesados está a afetar negativamente a saúde reprodutiva masculina. Adicionalmente, o estudo *in vitro* revelou que apesar de nenhum dos compostos (As nem o Hg), individualmente, terem exercido efeitos deletérios na viabilidade e mobilidade espermática, MMP, integridade da cromatina, capacitação e integridade do acrossoma a concentrações ambientalmente revelantes, quando combinados, obtiveram-se diferenças na viabilidade, mobilidade e MMP, indicando eventual efeito sinergístico dos metais pesados em questão. Em suma, este projeto demonstra que a exposição a metais pesados pode prejudicar a fertilidade e a saúde reprodutiva masculina e adverte para os potenciais efeitos negativos que um mundo altamente industrializado traz para as nossas vidas.

Palavras-chave: exposição a metais pesados; saúde reprodutiva masculina; infertilidade masculina; qualidade e funcionalidade de espermatozoides humanos; exposição *in vivo* e *in vitro*.

CHAPTER I – GENERAL INTRODUCTION

1.1. Male Reproductive System

The male reproductive system is constituted by the testis and several accessory sex organs and ducts that support the process of spermatogenesis, sperm maturation and transport of sperm and other seminal components to the exterior (Figure 1) (Jones, 2015).

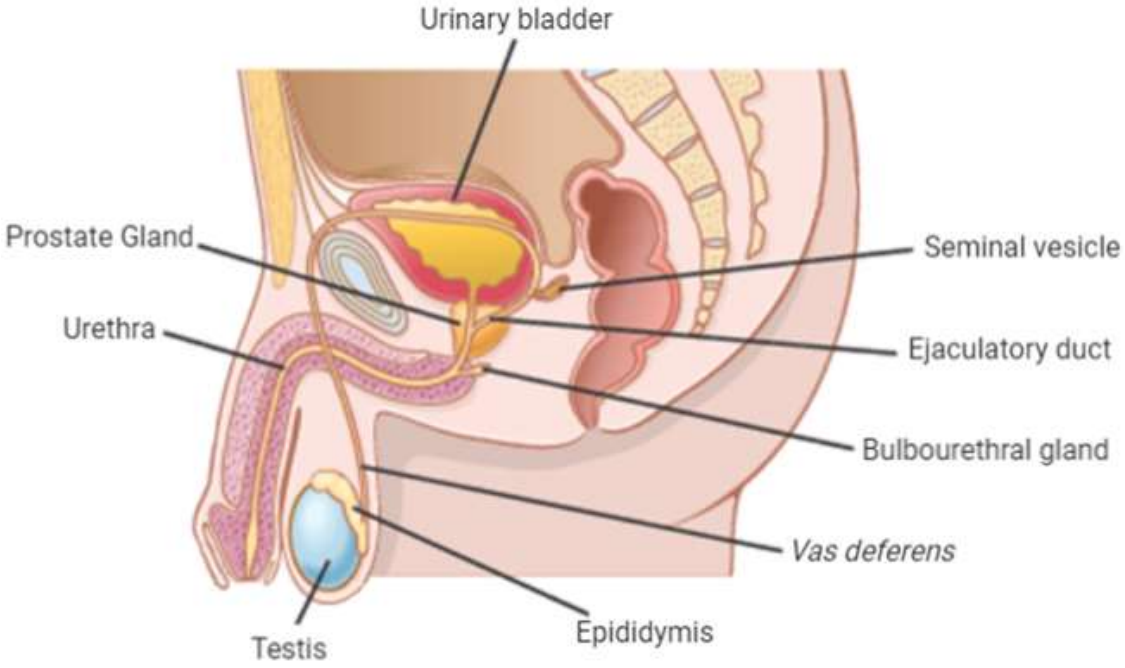

Figure 1: Schematic representation of the male reproductive system. The male reproductive system is constituted by the testis, the accessory sex organs (seminal vesicles, prostate gland, bulbourethral glands and epididymis) the *vas deferens* and the ejaculatory duct (Adapted from Guyton & Hall, 2016).

The testis is composed by the seminiferous tubules where the spermatogenesis – the process by which the sperm are formed – occurs in strict association with the Sertoli cells and the somatic cells of the seminiferous epithelium that provide support and nutrition as well as an immunoprivileged environment for the developing cells (O'Donnell et al., 2001) (Hess & de Franca, 2009). Spermatogenesis comprises three main phases: mitosis, meiosis and spermiogenesis (Hess & de Franca, 2009). In the first phase, the multiplication of the spermatogonial stem cells occurs, originating either other stem cells (which allows its self-renewal) or spermatogonia committed to undergo meiosis and spermiogenesis, leading to the formation of sperm (Schulz & Miura, 2002). In the second phase, primary spermatocytes enter in meiosis which involves the duplication of the deoxyribonucleic acid (DNA) content and genetic recombination that is followed by the reduction of the chromosomes via two cell divisions. While in the first meiotic division secondary spermatocytes are formed, the second one gives rise to haploid round spermatids (Schulz & Miura, 2002) (Hess & de Franca, 2009). In the last phase, the round spermatid endures a series of complex cytological transformations that

transform it into a spermatozoon. Such transformations include nuclear condensation, the formation of the acrosome, phagocytosis of the majority of the cytoplasm by the Sertoli cells and formation of the flagellum (De Kretser et al., 1998). After its formation, the spermatozoon detaches from the Sertoli cell and is released into the lumen of the seminiferous tubule in a process called spermiation (Neto et al., 2016). The spermatogenesis process is highly regulated and, essentially, relies on the action of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH), both secreted by the pituitary gland, and testosterone secreted by the Leydig cells (Tavares, 2014). The secretion of gonadotropin releasing hormone by the hypothalamus triggers the secretion of LH by the pituitary gland that stimulates the Leydig cells to produce testosterone (Holstein et al., 2003). Testosterone together with FSH act on Sertoli cells to provide support and maturation of germ cells (Holstein et al., 2003) (Kim & Kim, 2015).

Nevertheless, the sperm cells recently formed in the testis are not motile, so they have to pass throughout the efferent ducts and reach the epididymis where they undergo maturation and acquire motility in a continuous process since the epididymal head to the tail (Guyton & Hall, 2011). The tail of the epididymis conducts to the *vas deferens*. During this path that ends when spermatozoa reach the urethra, sperm receive secretions from the epididymis, the seminal vesicles, the prostate gland and the bulbourethral glands comprising the seminal plasma (Jones, 2015).

1.2. Spermatozoon

The spermatozoon is the male haploid gamete and it can be divided into two main compartments – head and tail (Figure 2) (Schuff, 2018). The cell is surrounded by a single plasma membrane and each compartment plays a specific and extremely important role for its biological function, which is to reach the oocyte and fertilize it (Amaral et al., 2013).

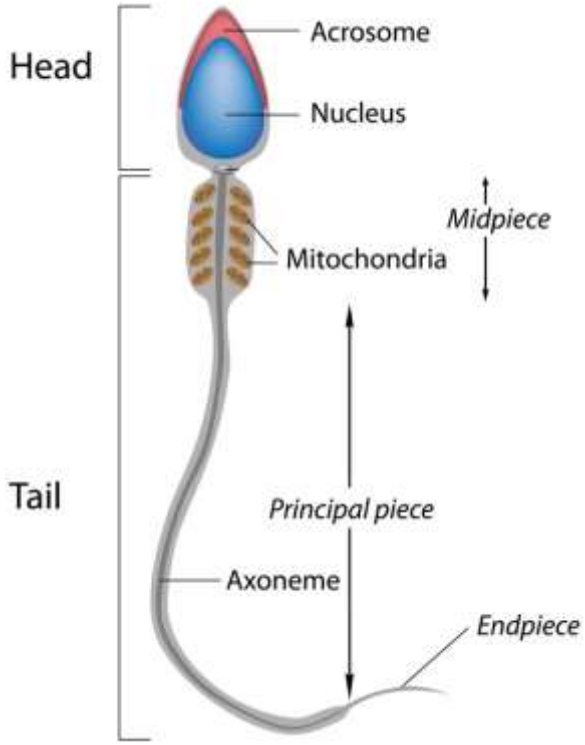

Figure 2: Schematic representation of the human spermatozoon structure. The spermatozoon is constituted by the head where the nucleus and the acrosome vesicle are located and the tail that can be further subdivided in midpiece, principal piece and endpiece. (Adapted from Schuff, 2018)

1.2.1. Head

During the development of the human spermatozoon, the shape of the head suffers several modifications, which includes the formation of the acrosome and chromatin remodelling in parallel with nuclear condensation, to reach a final oval shape with, approximately, 4.0 – 5.5 μm in length and 2.5 – 3.5 μm in width. The sperm head can be divided into an anterior and a posterior region.

1.2.1.1. Anterior region: the acrosome

The anterior region comprises 40 – 70% of the total area of the head and corresponds to the acrosome (Mortimer, 2018). The acrosome is a secretory vesicle that is delimitated by an inner

acrosomal membrane, which is near the nuclear membrane, and the outer acrosomal membrane that is beneath the plasma membrane and covers the acrosome (Frasier & Springate, 2017) (Figure 4). This vesicle comprises two different regions: the anterior portion that undergoes the acrosome reaction and the posterior equatorial segment that does not participate in that event (Mortimer, 2018). The acrosome is indispensable for *in vivo* fertilization since it contains hydrolytic enzymes that allow the sperm to undergo the acrosome reaction and digest the zona pellucida, penetrating into the oocyte (Mack et al., 1983). In fact, before a spermatozoon can fertilize an oocyte, there are a few consecutive steps that the cell must undergo, namely capacitation and acrosome reaction (AR). Reinforcing this, a spermatozoon that doesn't undergo capacitation or AR has no fertilization potential *in vivo* (Fraser, 1998) (Jaiswal et al., 1998). Indeed, although sperm cells present in the ejaculate are morphologically mature and motile, they are fertilizing incompetent (Moody et al., 2017). It is only inside the female reproductive tract (Okabe, 2013) (De Jonge, 2017) that sperm capacitation occurs (Buffone, 2016). The most relevant transformations are associated with cholesterol efflux from the sperm plasma membrane that increases membrane fluidity, variations in intracellular ion concentrations, mostly calcium (Ca^{2+}) and bicarbonate (HCO_3^-) influx, hyperpolarization of the sperm plasma membrane, enhanced activity of the protein kinase A (PKA) and protein phosphorylation, specifically, in tyrosine residues (Buffone, 2016) (Moody et al., 2017) (Molina et al., 2018). Once capacitated, the spermatozoa exhibit a dynamic and energetic swimming pattern – hyperactivation – that is characterized by flagellar beating patterns with higher amplitudes and asymmetries (Frasier & Springate, 2017). The hyperactivation movements allow the sperm to get rid of their bonds with the oviductal epithelium and swim to reach the oocyte (Frasier & Springate, 2017) (Okabe, 2013). In an Andrology lab, capacitation is often achieved *in vitro* by incubating the sperm cells in a capacitating medium which is supplemented with fundamental ions, such as Ca^{2+} and HCO_3^- , energy substrates and albumin (Jaiswal et al., 1998). These capacitated cells are then used in Assisted Reproductive Techniques.

1.2.1.1.1. Acrosome Reaction

Sperm cells move towards the oocyte guided by chemo-attractants, such as progesterone that is secreted by cumulus cells, and oviductal contractions (Frasier & Springate, 2017) (Coy et al., 2014). Progesterone initiates a cascade of reactions in the spermatozoa that start with an increase in the intracellular concentration of Ca^{2+} that, eventually, reach the threshold levels and triggers the AR (De Jonge, 2017). Then, multiple fusion points between the outer acrosomal membrane and the sperm plasma membrane are established resulting in the release of the acrosome content, mainly hyaluronidase and acrosin, and zona pellucida binding proteins, to the environment (Okabe, 2013) (Buffone, 2016). This process turns the spermatozoa able to penetrate the zona pellucida and once it reaches the oocyte plasma membrane, the equatorial segment of the acrosome should remain intact since it is the contact point with the oocyte that will allow the fusion of both sperm and oocyte membranes (Figure 3) (Mortimer, 2018) (Anifandis et al., 2014). Spermatozoa that prematurely undergo AR or that do not possess an acrosome, due to malformation during spermiogenesis, have no fertilizing potential *in vivo* (Fraser, 1998) (Jaiswal et al., 1998).

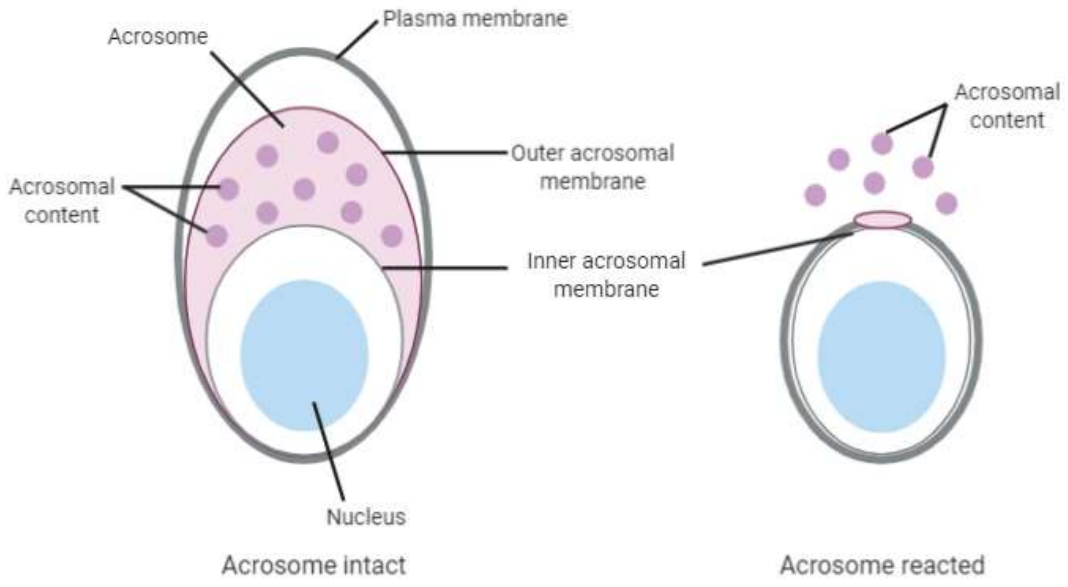

Figure 3: Schematic representation of the acrosome state before (acrosome intact) and after (acrosome reacted) AR. Upon the AR, the outer acrosomal membrane fuses with the plasma membrane and the acrosomal content is released to the exterior via exocytosis (Adapted from Anifandis et al., 2014).

1.2.1.2. Posterior region: the nucleus

The posterior region of the head of the spermatozoon is the core of the cell, where the nucleus is located and the paternal genetic material (23 chromosomes) lies. The nucleus of the spermatozoon is around 10x more compact when compared with the nucleus of somatic cells (Miller et al., 2010). This high level of compaction is achieved by the nucleohistone-nucleoprotamine transition that consists in the replacement of the histones within the nucleosomes by transition proteins and protamines (Oliva, 2006). Protamines are the most common type of proteins in the sperm and are synthesized in the last steps of spermatogenesis (Jodar & Oliva, 2014). They play important roles for the spermatozoon such as the creation of a condensed paternal genome and, consequently, a hydrodynamic nucleus and the protection of the paternal DNA against external assault (Jodar & Oliva, 2014). The process of packaging starts in spermiogenesis where histones are replaced by transition proteins and further by protamines, continuing in the epididymis where the establishment of disulphide bonds between cysteine residues, which is another amino acid commonly found in protamines, takes place (Steger & Balhorn, 2018). The disulphide bonds aid locking the chromatin in its heavily compacted form, thus, contributing to the success of the chromatin packaging that is directly correlated with the DNA integrity. However, still 15% of the histones remain attached to DNA, forming nucleosomes. This DNA encodes for genes that are important for the early stages of development of the zygote. Nevertheless, these may render paternal DNA more susceptible to damage/fragmentation. Damage in the DNA, meaning impaired DNA integrity, is associated with male infertility and difficulties in achieving pregnancy (Miller et al., 2010) (Mali et al., 2016). Infertile patients have been shown to possess higher levels of DNA damage than fertile ones (Chohan, 2006) (Sousa et al., 2008) (Schulte et al., 2010).

1.2.2. Tail

The tail, also known as flagellum, of a morphologically normal human spermatozoon measures 40 – 50 μm in length and its function is mainly to provide motility to the cell. Sperm motility is a crucial factor for the biological function of spermatozoa (Agnihotri et al., 2016) since it is mandatory that these cells actively and efficiently move throughout the female reproductive tract in order to reach the oocyte and fertilize it. Therefore, an impairment in sperm motility may lead to male infertility (Barrell, 2017).

The tail can be divided into midpiece (anterior portion), principal piece and endpiece (posterior portion). The axoneme is the internal cytoskeletal structure of the sperm tail, displaying a 9+2 arrangement, which is constituted by microtubule doublets (Zhao et al., 2017).

The central axoneme is surrounded by sperm tail accessory structures as the outer dense fibers (ODFs) that maintain flagellar elasticity and protect the sperm tail against shear forces during epididymal transport and ejaculation (Zhao et al., 2017), the fibrous sheath and the mitochondrial sheath. Each subdivision of the sperm tail differs in its structure containing different accessory structures apart from the axoneme, all of which important for proper motility (Figure 4) (Lehti & Sironen, 2017).

1.2.2.1. Midpiece

The midpiece is located immediately after the sperm head and establishes the connection between the head and the anterior part of the sperm tail. It should be slim, however thicker than the rest of the tail (Durairajanayagam et al., 2015). The midpiece is constituted by the mitochondrial sheath, which results from 22 – 75 mitochondria tightly packed, and by 9 ODFS surrounding the axoneme (Ramalho-Santos et al., 2009) (Lehti & Sironen, 2017).

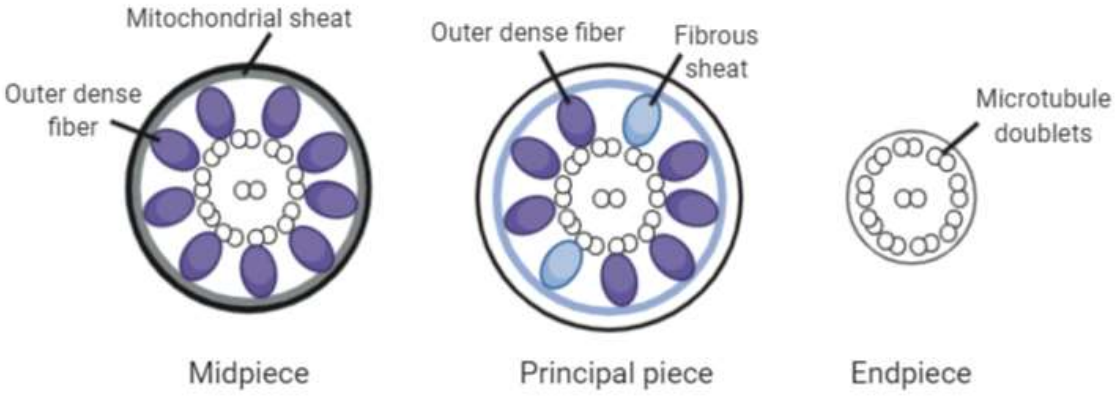

Figure 4: Schematic representation of the sperm tail structure. OFDs are found in the middle and principal piece, mitochondrial sheath is only present in the midpiece and fibrous sheath in principal piece (Adapted from Lehti & Sironen, 2017).

1.2.2.1.1. The role of mitochondria in the spermatozoa

The mitochondria are fundamental organelles required to maintain the homeostasis of every cell, being their most notable function the production of adenosine triphosphate (ATP), besides Ca^{2+} regulation, apoptosis and the production of reactive oxygen species (ROS), which in small physiological levels are beneficial for the spermatozoon, allowing it to undergo capacitation, AR and to interact with the zona pellucida of the oocyte (Cocuzza et al., 2007) (Sanocka & Kurpisz, 2004) (Losano et al., 2018).

The mitochondrion is an oval shaped organelle formed by two membranes, the outer and the inner membrane, and the corresponding intermembrane space (Rajender et al., 2010). The

inner mitochondrial membrane folds several times, creating structures called cristae, that allow to increase its surface area providing large areas for energy production via oxidative phosphorylation (OXPHOS). Therefore, mitochondria are perfectly structured to maximize their productivity (Figure 5) (Rajender et al., 2010) (Van Der Bliek et al., 2017).

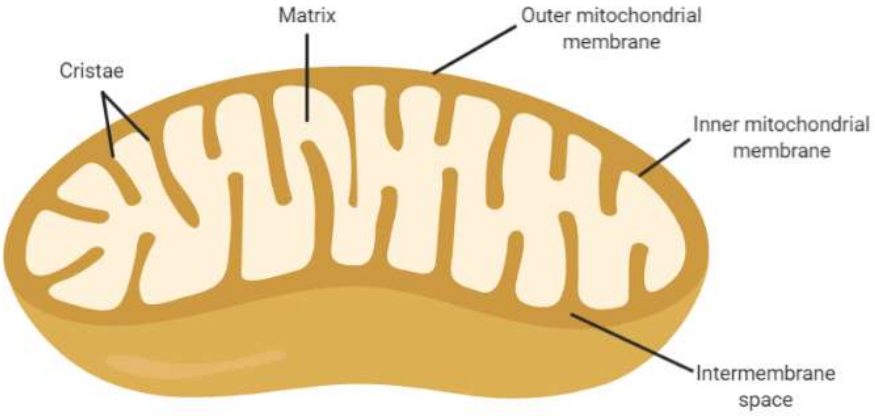

Figure 5: Schematic representation of a mitochondrion. Mitochondrion is constituted by an outer and an inner membrane, being the space between both denominated intermembrane space. The inner membrane folds several times creating structures called cristae. The interior of the organelle, the mitochondrial matrix, is a fluid more viscous than the cytoplasm (Adapted from Rajender et al., 2010).

OXPHOS is a process known for synthetizing ATP by a series of consecutive steps, transferring electrons through the electron transport chain (ETC), which consists in protein complexes and organic molecules localized in the inner mitochondrial membrane (Van Der Bliek et al., 2017) ("Oxidative phosphorylation," 2020). Briefly, the energy generated by the Krebs Cycle is delivered to electrons that are transported by nicotinamide adenine nucleotide in the reduced form (NADH) and flavin adenine dinucleotide ($FADH_2$) to the complex I (nicotinamide dinucleotide-dehydrogenase) and II (succinate dehydrogenase) respectively, initiating the process (Tavares, 2014) ("Oxidative phosphorylation," 2020). Mobile electron carriers such as coenzyme Q and cytochrome c, move electrons between the complex II to complex III (cytochrome c dehydrogenase) and IV (cytochrome c oxidase), respectively (Van Der Bliek et al., 2017). As electrons pass throughout the ETC, the energy released is used to pump protons from the matrix to the intermembrane space creating an electrochemical gradient, the MMP (Yang et al., 2018) ("Oxidative phosphorylation," 2020). This electrochemical gradient drives the synthesis of ATP by complex V (ATP synthase) through the phosphorylation of adenosine diphosphate (ADP) (Tavares, 2014) (Van Der Bliek et al., 2017). Mitochondrial integrity and a high MMP are a prerequisite for the success of fertilization (Espinoza et al., 2009) (Mali et al., 2016). Hence, MMP is considered a good way to assess mitochondrial function and a good

indicator of spermatozoa quality and function since an alteration in MMP is correlated with sperm fertilizing ability (Paoli et al., 2011) (Espinoza et al., 2009).

1.2.2.2. Principal piece

The principal piece is the longest part of the tail and includes most of the propellant machinery. In this segment, 2 of the 9 ODFs are replaced by the columns of the fibrous sheath where several glycolytic enzymes are localized (Nascimento et al., 2008) (Lehti & Sironen, 2017). The fibrous sheath is also responsible for influencing the plane of flagellar beating, the tail flexibility and the shape of the flagellar wave (Mortimer, 2018).

1.2.2.3. Endpiece

The endpiece contains only the axoneme and the plasma membrane (Lehti & Sironen, 2017) and should not have any coil or abnormal bending (over 90º) to allow the proper beating of the sperm flagellum which is the basis of sperm motility. Moreover, sperm motility is indispensable for the passage of sperm through the cervical mucus and to prevent sperm from being phagocytosed inside the female reproductive tract (Durairajanayagam et al., 2015) (Mortimer, 2018).

1.3. Seminal Plasma

Interestingly, only 2-5% of the semen is composed by sperm, being the remaining 95 – 98% the seminal fluid. The secretions from the seminal vesicles account for the majority of the ejaculate (60 – 75%) and contain fructose, citric acid, amino acids, other nutritive substances to the sperm and coagulating proteins. The prostate gland, which is the second major secretory gland in the male reproductive tract, secretes a thin and alkaline fluid (25 – 30%) that not only neutralizes the acidic fluid from the seminal vesicles, increasing the motility of sperm, as also contains acid phosphatase, citric acid, enzymes that liquefy the seminal plasma and zinc (Zn), which aids stabilizing the chromatin and preventing DNA breaks. Finally, a much smaller contributor to seminal plasma volume is the epididymis. Neutral α-glucosidase (NAG) is a biomolecule secreted by epididymal epithelial cells into seminal plasma and is involved in the maturation and motility of spermatozoa, being correlated with semen parameters such as volume, pH and sperm concentration. NAG is considered a marker of epidydimal function. (Qiu et al., 2018) (Li et al., 2019).

1.4. Endocrine Disruptors

Since the second half of the 20th century that an increasing number in human reproductive diseases and a decline in the human reproductive health worldwide has been observed (Woodruff, 2011) (Virtanen et al., 2017). However, due to the short temporal window, genetic changes cannot explain such observations. Consequently, it was hypothesized that environmental effects could justify those trends since, nowadays, the human being and other animals are continuously exposed to a wide range of chemicals that are released to the environment (Woodruff et al., 2008) (Marques-Pinto & Carvalho, 2013). From this perspective, the concept of EDs appeared and, in 2002, the World Health Organization (WHO) defined it as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" ("WHO | Endocrine Disrupting Chemicals (EDCs)," 2016) (Combarnous, 2017). The endocrine system is composed of a wide range of hormones that together, by acting in loops of stimulation or retraction, coordinate numerous functions of the human body such as development, growth or reproduction (Combarnous, 2017). EDs are a heterogeneous group constituted not only by synthetic chemicals used in (or unintended produced by) industry as lubricants or solvents (polychlorinated biphenyls, polybrominated biphenyls, dioxins), plastics (phthalates, bisphenol A), pesticides (e.g. atrazine, dichlorodiphenyltrichloroethane, vinclozolin), heavy metals (cadmium (Cd), lead (Pb), As, Hg) and pharmaceutical agents, but also by chemicals found in nature such as phytoestrogens and mycotoxins, some of which inclusively ingested by humans (Diamanti-Kandarakis et al., 2009) (Tavares et al., 2016) (Marques-Pinto & Carvalho, 2013). Human beings are exposed to EDs via inhalation of contaminated air, absorption through the skin but, mostly, by the ingestion of contaminated food and water (Balabanič et al., 2011).

Regarding EDs mechanisms of action, they can act in multiple ways, either affecting genomic-based pathways or acting in a non-genomic manner (Tavares et al., 2016) (Iavicoli et al., 2009). EDs can interact directly with hormone receptors acting as an agonist or antagonist of the hormone, can stimulate or inhibit its synthesis, degradation, and transport, and can make the organism more sensitive to the action of the hormone by increasing its temporal window (Combarnous, 2017) (Ramalho-Santos & Tavares, 2019). Nevertheless, nowadays it is also known that they can also exert their effects independently of a hormone receptor.

1.4.1. Heavy metals

Heavy metals are naturally occurring chemical elements characterized by high atomic number, atomic weight or density that can be divided into essential and non-essential metals (Tchounwou et al., 2014). The essential metals, for instance, cobalt (Co), copper (Cu) chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se) and Zn are essential to human health and homeostasis, being part of one or more key enzymes involved in metabolic or biochemical processes (Tchounwou et al., 2014) (Saad & El-Sikaily, 2016). However, despite their need for biological functioning, excess amounts of these metals can cause cellular and tissue damaging (Tchounwou et al., 2014) (Rahman & Singh, 2019). The non-essential metals, such as Hg, Pb, Cd and As, have non- physiological functions in the human body, being considered pollutants that threat human health and the environment. Moreover, they are often detected in cities with a high industrial context in Portugal, and around the world as well (Balabanič et al., 2011) (Saad & El-Sikaily, 2016).

Due to their large applications in industry, agriculture, domestic use, medicine and technology, etc., the human population is continuously exposed to non-essential metals, even though at low concentrations. Nevertheless, the heavy metal toxicity depends on the dose, route of exposure, and chemical species along with the individual intrinsic factors such as age, gender, genetics, etc. (Tchounwou et al., 2014). Yet, some heavy metals, for instance As, Cd, Pb, Hg and Cr are considered non-threshold toxins, causing a negative impact on plants, animals and humans at low doses, and therefore, are ranked as the most problematic heavy metals (Rahman & Singh, 2019). Furthermore, they cannot be degraded, persisting on earth's crust for a long time. Consequently, heavy metals tend to bioaccumulate, i.e. gradually increasing their concentration in an organism. This phenomenon occurs when the organism absorbs the metal in a higher rate of its metabolization/excretion and raises a huge concern for the future (Rahman & Singh, 2019).

Nowadays, the huge development in industry verified in the past century, has led to an increasing demand for heavy metals and, consequently, to higher emission of these pollutants to the environment being thus extremely important to monitor heavily industrialized areas.

1.4.1.1. Arsenic

Arsenic (As) is a naturally occurring metalloid that exists ubiquitously in the environment and causes multiple effects in both animals and human populations (Jana et al., 2006) (Kim & Kim, 2015). It can adopt several chemical states, being the most common forms the trivalent arsenite (As^{3+}) and pentavalent arsenate (As^{5+}) (Wirth & Mijal, 2010) (Kim & Kim, 2015). This

metal is usually used as a component of herbicides, insecticides and fungicides (applied in agriculture), and also in the foundry industry as well as in the combustion of fossil fuels, contributing to the widespread of this environmental contaminant (Chiou et al., 2008) (Iavicoli et al., 2009). Human exposure mostly occurs via oral or inhalation routes (Jana et al., 2006). However, the main source of exposure of the general population is through ingestion of contaminated drinking water where the inorganic forms of As (As^{3+} and As^{5+}) predominate. This raises a huge concern due to the fact that inorganic As is more toxic than the organic counterpart (Jana et al., 2006) (Balabanič et al., 2011). As is a known carcinogenic agent causing tumours in several organs such as lung, skin, liver, prostate and testis (Waalkes et al., 2000) (Waalkes et al., 2003) (Jana et al., 2006).

In 1984, Danielsson et al. assessed the distribution of As in the male reproductive system of rodents using autoradiographic methods and reported a high accumulation of radioactive As in the lumen of the epididymis, especially in corpus epididymis. In testis, the radioactive compound was only slightly uptaken with all the cells displaying a similar level. Besides, after 1-2 weeks of exposure, there was still a high signal in the seminal ducts which indicated that semen may be exposed for a long time to As, which obviously, increased the risk of impaired reproduction (Danielsson et al., 1984). Later on, exposure to sodium arsenite ($NaAsO_2$) via drinking water for 35 days has shown that only the highest concentration (53.39, 133.47, 266.95 and 533.90 μM) significantly decreased sperm count and motility, and induced sperm morphological abnormalities in mice (Pant et al., 2001). Additionally, a significant accumulation of As, except for the lowest concentration (53.39 μM $NaAsO_2$), was observed in testis and accessory sex organs (epididymis, seminal vesicles and ventral prostate). Pant and colleagues reported that since there were no significant effects using 53.39 μM $NaAsO_2$, which is the closest concentration to what humans are exposed to, it would be interestingly to test this dosage for a longer time period (Pant et al., 2001). So, in 2004, the same group released a paper describing the reproductive effects on male mouse that were exposed to 53.39 μM $NaAsO_2$ for 365 days. The chronic exposure to $NaAsO_2$ caused a decrease in the absolute and relative testicular weights as well as on sperm motility and epidydimal count and increased the total percentage of abnormal forms. Furthermore, damage to Leydig, Sertoli and germ cells were detected due to alterations on the activity of 17-beta-hydroxysteroid dehydrogenase, gamma-glutamyl transpeptidase and, sorbitol dehydrogenase and acid phosphatase, respectively, therefore affecting spermatogenesis. Lastly, there was also noticed an accumulation of As in testis, epididymis, seminal vesicles and ventral prostate. Remarkably, the chronic exposure to 53.39 μM $NaAsO_2$ achieved the same results on sperm motility, count, morphology and accumulation

throughout the reproductive tract as the highest concentration of their previous study (533.90 μM $NaAsO_2$) revealing that, the exposure of low levels for longer time periods can be as negative as shorter exposures to higher concentrations. Moreover, the same findings have been found by Jana et al. that employed the same As compound in mature albino rats (5 mg/kg/day) 6 days a week for 4 weeks and Sarkar et al. which investigated the effect of $NaAsO_2$ on Wistar rats spermatogenesis and LH, FSH and testosterone levels by intraperitoneally administering the compound at dosages of 4, 5 or 6 mg/kg/day for 26 days (Sarkar et al., 2003) (Jana et al., 2006). Altered hormonal levels were observed in these studies.

Finally, mice treated with arsenic trioxide (As_2O_3) (0, 0.15, 0.3, 1.5, and 3.0 mg/kg) via subcutaneous injections, for 5 days, with a 2-day interval, for 3 weeks has shown to cause significant histological modifications, reducing the number of mature seminiferous tubules in testis. Furthermore, a decrease in sperm motility and viability was detected as well as altered epididymal sperm number, but the latter only at 3.0 mg/kg. These results are in contradiction to what was reported by others that did not find any toxic effects using the same compound (Omura et al., 1996). Regardless, As_2O_3 reduced the plasma level of testosterone and intratesticular testosterone level as well as the LH levels but only at the higher dosage. Plasma FSH was not significantly affected. Further, since the concentration of testosterone was lower than the controls, the *messenger* ribonucleic acid (mRNA) expression of the enzymes involved in its synthesis were assessed and found out to be decreased too. So, given the fact that the plasma levels of LH and FSH were not severely affected, the treatment was not supressing the hypothalamus-pituitary axis, suggesting that As_2O_3 was acting directly on testis (Chiou et al., 2008).

In humans, though, the data is scarce and somewhat controversial. While As has been associated to a significant impairment in sperm motility but not to sperm concentration or morphology by some (Meeker et al., 2008), others have only found a significant negative correlation between the levels of As and sperm concentration, along with a statistically significant negative correlation between NAG and the levels of As (Li et al., 2012). Furthermore, the levels of As in blood and seminal fluid of low- and normal-quality semen groups, according to the WHO reference values, were found similar within each fluid. However, As was statistically more concentrated in seminal fluid than in blood (Sukhn et al., 2018), suggesting that this metal tends to accumulate in the male reproductive tract.

1.4.1.2. Mercury

Hg is a heavy metal that can be found naturally in the environment either as an elemental Hg form, or as organic and inorganic compounds (Balabanič et al., 2011). The major sources of exposure depend on the form of Hg. Inorganic Hg is more often present in the water, food and air, while elemental Hg is found in dental amalgam in tooth filling. Organic Hg exposure, on the other hand, derives from consumption of fish and other seafood (Atkinson et al., 2001). However, anthropogenic sources such as foundry, mining and manufacturing industries also play a role in releasing this contaminant to the air, water and soil which, ultimately, will cause general toxic effects on animals and humans (Rao & Sharma, 2001) (Bhan & Sarkar, 2005) (Iavicoli et al., 2009).

In humans, one of the first reports describing the deleterious effects of Hg belongs to Popescu that, in 1978, investigated the impact of chronic occupational exposure (between 6 to 8 years) to organic Hg compounds - methylmercury and ethylmercury - on men. Besides several neurological alterations, seminal analysis revealed hypospermia, which is translated to a low volume (< 1.5 mL) of the ejaculate, and an increase in morphologically abnormal forms. Moreover, the authors also showed the results from another group of men chronically exposed (mean duration of occupation 10.5 years) to organic Hg that exhibited higher incidence of hypospermia, asthenozoospermia (reduced sperm motility) and teratozoospermia (predominance of morphologically abnormal sperm forms) and decreased libido (Popescu, 1978). Later on, McGregor and Mason explored the endocrine function, namely the pituitary-testicular axis, on a well characterized male population that was occupationally exposed to Hg vapour and found that among the levels of gonadotrophins (LH and FSH), serum testosterone, free testosterone index and sex-hormone binding globulin analysed, only the latter was different between the Hg workers and an equivalent socio-economic population of non-occupationally exposed men (McGregor & Mason, 1991). Furthermore, in a case report described by Keck and colleagues, a severe oligoasthenoteratozoospermia with only 0.1% and 3.7% of the seminiferous tubules showing intact spermatogenesis on the right and left testis, respectively, elevated serum FSH, lymphocytic infiltration and Hg grains in the lysosomes of Leydig cells and normal levels of the accessory gland markers NAG, fructose and Zn were observed in a patient with a 5-year chronic occupational exposure to Hg vapour. This was the first report proving Hg accumulation in human testis (Keck et al., 1993). In 2016, Martinez et al. also showed accumulation of Hg in the male reproductive system of chronically exposed rats (30 days) to low mercuric chloride ($HgCl_2$) levels (first dose of 4.6 $\mu g\ kg^{-1}$, subsequent doses of 0.07 $\mu g\ kg^{-1}\ day^{-1}$), particularly in the testis, epididymis and prostate, which is in contrast with what was described by others (Khan et

al., 2004). Moreover, the treatment decreased sperm membrane integrity which may be explained by an increase in lipid peroxidation, a phenomenon also reported by others *in vitro* (Arabi & Heydarnejad, 2007), and induced disorganization of the seminal tubules and loss of spermatogenic epithelium in the testis, with only a few tubules exhibiting mature spermatozoa. This revealed the ability of $HgCl_2$ to cross the blood-testis barrier and promote a negative impact on spermatogenesis.

Given this, in an attempt to determine if the effects of $HgCl_2$ on the male reproductive organs in mice are reversible after stopping the treatment, Sharma and colleagues designed two experimental groups in which they orally administered 1.25 mg/kg $HgCl_2$ daily for 30 days, but in the second group the animals were maintained alive for more 45 days, without any $HgCl_2$ treatment, to assess recovery. While no differences between the experimental groups and the control regarding body, testis, *vas deferens* and epididymis weights were observed, a significant decline in the sperm count in testis, *vas deferens* and cauda epididymis after the administration of $HgCl_2$ was detected, along with sperm cells immobilization, a common finding in *in vitro* and *in vivo* studies (Mohamed et al., 1987), and a decline in viability. Cauda epididymal sperm also exhibited kinks and coiled tails and midpiece, decapitation and abnormal heads but these morphological alterations were reverted after the recovery. Serum testosterone levels also remarkably decreased and recovered after the 45 days (Sharma et al., 1996).

Nevertheless, while studies report some effects, others have shown no negative impact of Hg at dietary/environmental levels on human semen quality (Rignell-Hydbom et al., 2007) (Mocevic et al., 2013) (Zeng et al., 2015), particularly on sperm concentration, motility and morphology. Of note, a recent Lebanese study dosed the levels of non-essential metals, including Hg, in blood and seminal fluid and tried to establish associations between these levels and semen quality parameters in a very specific population. The participants were divided in low- and normal-quality semen groups according to the WHO reference values and no significant differences in terms of Hg concentration in blood or in seminal fluid were observed. Controversially, it has been reported that infertile couples present higher blood Hg concentrations than fertile ones, highlighting that Hg may be associated with both male and female infertility (Choy et al., 2002).

Although there is a significant number of papers describing the effects of Hg in the male reproductive system, some of them are controversial and the exact mechanism of action is not fully understood.

1.5. Estarreja

Estarreja is a Portuguese city located in the district of Aveiro that since 1950 has been exposed to high levels of contamination due to the presence of the second largest chemical complex in Portugal. This complex is composed by different industrial units that are known to emit heavy metals into environment (Inácio & Pereira, 2010) (Inácio et al., 2011) (Sociedade de Ciências Agrárias de Portugal et al., 2013). The industrial activity has originated many toxic residues which were deposited in several lands, some of them used for agriculture, and also in liquid effluents, with potentially toxic elements for ecosystems and human health, that circulated without any treatment by drainage ditches that cross agricultural lands (Sociedade de Ciências Agrárias de Portugal et al., 2013) (OHMI, 2019). Fortunately, during the 90s, with the advances in technology and the increasing concern regarding environmental issues, there was a significant technological transformation to reduce the pollutant emissions by the chemical complex (OHMI, 2019). Nevertheless, the industries with greater negative environmental impact remain active since 1950 (Sociedade de Ciências Agrárias de Portugal et al., 2013) and, worryingly enough, no studies evaluating reproductive health were performed in this area despite the numerous studies analysing the quality of the soil, air, and water, and advertising that the chemical complex may potentially jeopardize residents' lives. In 2010, Inácio et al. reported that more than 85% of the soils from an area of 60 km^2 around the chemical complex were contaminated. Forage plants were found to exhibit levels of heavy metals considered excessive or toxic and the concentrations of Cu, Hg, Pb and Zn in the groundwater were above the stipulated limits in the Portuguese legislation, threatening public health (Inácio & Pereira, 2010). Moreover, some cabbage leaves (*Brassica oleracea L*.) and tomato fruits (*Lycopersicon esculentum Mill*) derived from particular horticultural crops presented higher levels of As (up to 3.5 mg/kg) and Hg (up to 0.008 mg/kg) (Inácio et al., 2014), the most prevalent metals in Estarreja (Costa & Jesus-Rydin, 2001) (Inácio et al., 2007) (Inácio et al., 2014), which may lead to an eventual danger situation for daily consumers (Inácio et al., 2014) (Sociedade de Ciências Agrárias de Portugal et al., 2013).

1.6. Objectives

Although there is already some literature describing the impact of the chemical complex of Estarreja, it is crucial to verify if the reproductive health of Estarreja's male inhabitants is being affected, because if so and if control measures are not implemented, the risk of reproductive dysfunction and childless families will increase, causing several social, economic and psychological problems. With this in mind, the aim of this project was dual: 1) to evaluate if the

exposure in Estarreja affects the overall reproductive health status of the adult men population living in the area and compare the results with a control population from the Center region of Portugal, and 2) address the effects of As and Hg, the most prevalent heavy metals at Estarreja, in a complementary *in vitro* approach, aiming to acknowledge possible mechanisms of action in spermatozoa.

CHAPTER II – MATERIALS AND METHODS

2.1. Materials

All reagents were provided by Sigma-Aldrich (St. Louis, United States of America (USA)) unless stated otherwise. For the *in vitro* study, $HgCl_2$ was dissolved in bi-distilled water to yield the primary stock concentration of 10 mM. For As_2O_3, the primary stock concentration (10mg/ml) was prepared by dissolving the metal on a solution of sodium hydroxide (NaOH) 1 M while the secondary stocks were dissolved in capacitating medium (see section 2.3.5).

2.2. Human Biological Samples

Human samples were collected at the Reproductive Medicine Unit from *Centro Hospitalar e Universitário de Coimbra* from men negative for human immunodeficiency virus (HIV)-1, HIV-2, hepatitis B and hepatitis C who were undergoing routine analysis. Each subject signed an informed consent allowing their semen to be used for research purposes. Semen samples were obtained my masturbation after 3-5 days of sexual abstinence. All ethical guidelines were followed.

2.3. *In vivo* study

A total of 172 samples were obtained for the *in vivo* study. Each participant filled in a questionnaire in which overall medical and reproductive history, lifestyle habits, occupational and non-occupational exposure to endocrine disruptors, age, body mass index (BMI), among others, were recorded.

From these, 72 were initially excluded due to male tract problems, such as sexually transmitted diseases or urogenital anomalies (e.g. varicocele, cryptorchidism), hormonal treatments, chemotherapy/radiotherapy, drug abuse and obvious exposure to endocrine disruptors. The remaining 100 samples were then attributed to the control group if had no obvious exposure to endocrine disruptors (n=88) or to the exposed group if belonging to the area of Estarreja (n=12). The spermiogram (section 2.3.1.), as well as sperm viability (section 2.3.2.), DNA/chromatin integrity (section 2.3.3.), mitochondrial function by measurement of MMP (section 2.3.4.), capacitation (section 2.3.5.), acrosome status (section 2.3.6.), and measurement of fructose (section 2.3.7.1.1.), NAG (section 2.3.7.1.2.) and Zn (section 2.3.7.1.3.) levels in seminal plasma were determined, as depicted in Figure 6.

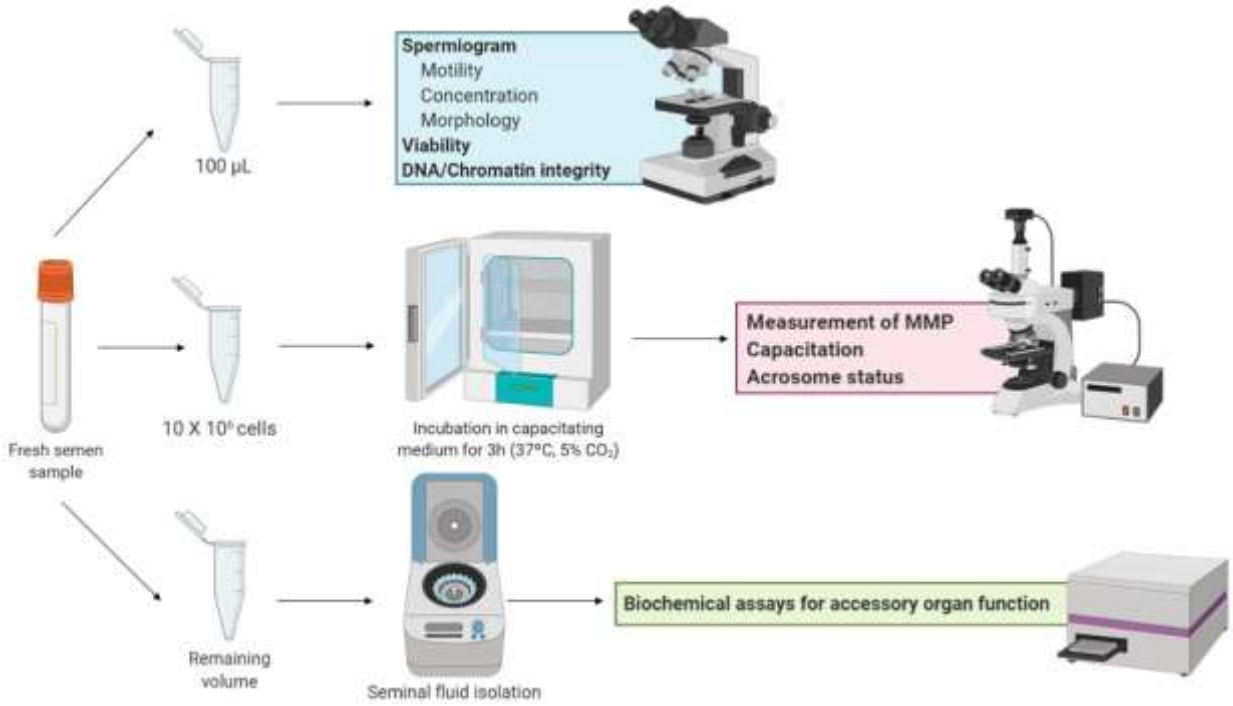

Figure 6: Schematic representation of the procedure for the *in vivo* study. From each fresh sample 100 µl were used to perform the spermiogram, the viability and the DNA/chromatin integrity assays. Given the sample concentration, the volume necessary to contain $10x10^6$ spermatozoa was incubated in capacitating medium for 3h to further assess the MMP, capacitation and acrosome status. The remaining volume of the fresh sample was then used to isolate the seminal fluid required for the biochemical assays.

2.3.1. Spermiogram

2.3.1.1. Macroscopic analysis

Initially, parameters such as volume and pH were analysed for each sample. According to WHO, the volume should be at least 1.5 mL and the pH should be ≥ 7.2 in order for the sample to be considered "normal" (WHO, 2010). Sample volume was evaluated by pipetting while pH was assessed using pH strips.

2.3.1.2. Microscopic analysis

2.3.1.2.1. Motility

Motility was determined under a phase-contrast microscope (200x or 400x magnification) by examining a wet preparation of 10 µl of the fresh sample on a slide. At least 200 spermatozoa were counted in 5 different fields and were divided into three categories of sperm movement: progressive motility (PR) which comprises spermatozoa that are actively moving, either linearly or in large circles, despite of speed, non-progressive motility (NP) that includes spermatozoa that exhibit some type of movement but without progression, such as flagellar beating or

swimming in small circles, and immotility (IM) which is translated in the absence of movement (WHO, 2010). The results are expressed as the percentage of motile spermatozoa (PR+NP).

2.3.1.2.2. Concentration

In order to assess the sperm concentration, it is always first required to determine the dilution factor by examining a wet preparation of the fresh sample. Taking that into account, the sample is diluted in water that helps stopping the sperm movement making easier to count. After that, sperm number is assessed under a phase contrast microscope (400x magnification) using a Neubauer chamber. Each field of the chamber is loaded with 10 μl of the diluted sample and the spermatozoa present in 1 row of the central grid of each field are counted. Sperm concentration (10^6 of cells per mL) is calculated using the formula below (WHO, 2010).

$$C = \frac{N}{n} \times \frac{1}{20} \times dilution\ factor$$

(C = concentration, N = total sperm number, n = total number of rows examined for the replicates)

2.3.1.2.3. Morphology

Sperm morphology was assessed using the Diff-Quik staining (Sousa et al., 2009). A smear of 10 μl of the fresh sample was made and, after air dried, it was sequentially exposed for 15s to three solutions. Briefly, the first solution consists in a fixative reagent, methanol, that preserves the cells in a "live-like state" and prevents its degeneration (Rolls, 2017), whereas the second is eosin and stains basic/positively charged proteins red. Lastly, the third solution, thiazin, stains DNA blue. Afterwards, the slide was rapidly dipped in water to remove excess of dye, allowed to air-dry and then mounted (Sousa et al., 2009). Sperm morphology was assessed under a phase-contrast microscope (x1000 magnification with oil immersion) and at least 200 spermatozoa were counted in 5 different fields. Spermatozoa were categorized into abnormal or normal forms (Figure 7) according to the WHO guidelines (WHO, 2010) and the percentage of each was calculated. The results are expressed as the percentage of normal spermatozoa.

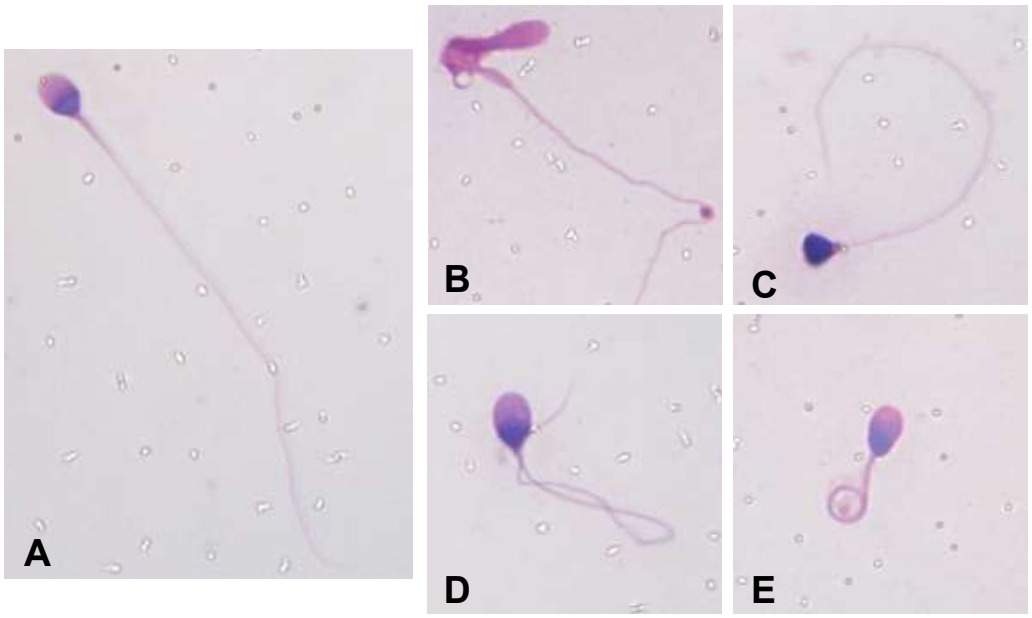

Figure 7: Morphology assessed by the Diff-Quik staining. Normal spermatozoon (A) and defective spermatozoa (B, C, D and E). The spermatozoon represented in **B** contains an amorphous head (head defect), excessive residual cytoplasm (midpiece defect) and a sharply angulated bend (tail defect). The spermatozoon in **C** presents an amorphous head (head defect) and a smooth hairpin bend (tail defect). In **D**, the spermatozoon exhibits a coiled tail (tail defect). In **E,** the sperm head is not oval (head defect), there is an asymmetrical insertion of the midpiece into the head (midpiece defect) and the tail is coiled (tail defect). Images captured by phase-contrast microscopy at 630x magnification.

2.3.2. Viability

Viability was assessed using the eosin exclusion test (WHO, 2010). This method lays in the premise that eosin, which is a membrane-impermeant stain, can only penetrate the cell membrane if the membrane is already compromised/damaged. Hence, dead spermatozoa exhibit a red or dark pink head whereas live spermatozoa will remain unstained with white heads (Figure 8). Briefly, 5 μl of the fresh sample was combined with 5 μl of eosin solution (0.9% (w/v) sodium chloride (NaCl) and 0.5% (w/v) Eosin Y) in a slide. Viability was assessed under a phase-contrast microscope (200x or 400x magnification) and at least 200 spermatozoa were counted in 5 different fields. The results were expressed as the percentage of viable/live spermatozoa.

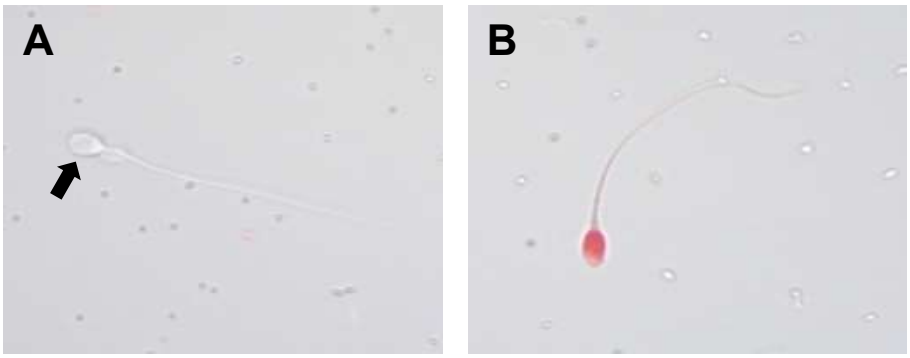

Figure 8: Viability assessment by the eosin exclusion test. The viable spermatozoon (A) remains unstained while the non-viable one (B) allows the entrance of the stain through their plasma membrane, therefore, exhibiting a red head. Images captured by phase-contrast microscopy at 630x magnification. The black arrow indicates the spermatozoon head.

2.3.3. DNA/Chromatin integrity

The assessment of DNA/chromatin integrity was performed using the same method described for sperm morphology evaluation (see section 2.3.1.2.3., the Diff-Quik staining). This assay correlates the intensity of the nuclear stain with the level of DNA fragmentation/chromatin decondensation. Normal sperm heads/nuclei exhibit a light staining, whereas heads/nuclei with fragmented DNA or decondensed chromatin present a darker stain (Figure 9) (Ramalho-Santos et al., 2007) (Sousa et al., 2009). The principle behind this reasoning is that fragmented DNA or decondensed chromatin can bind to the dye more effectively demonstrating a darker staining. For this purpose, at least 200 spermatozoa were counted in 5 different fields and the percentage of spermatozoa displaying a light or a dark sperm head/nucleus was calculated. The results are expressed as the percentage of spermatozoa with abnormal staining.

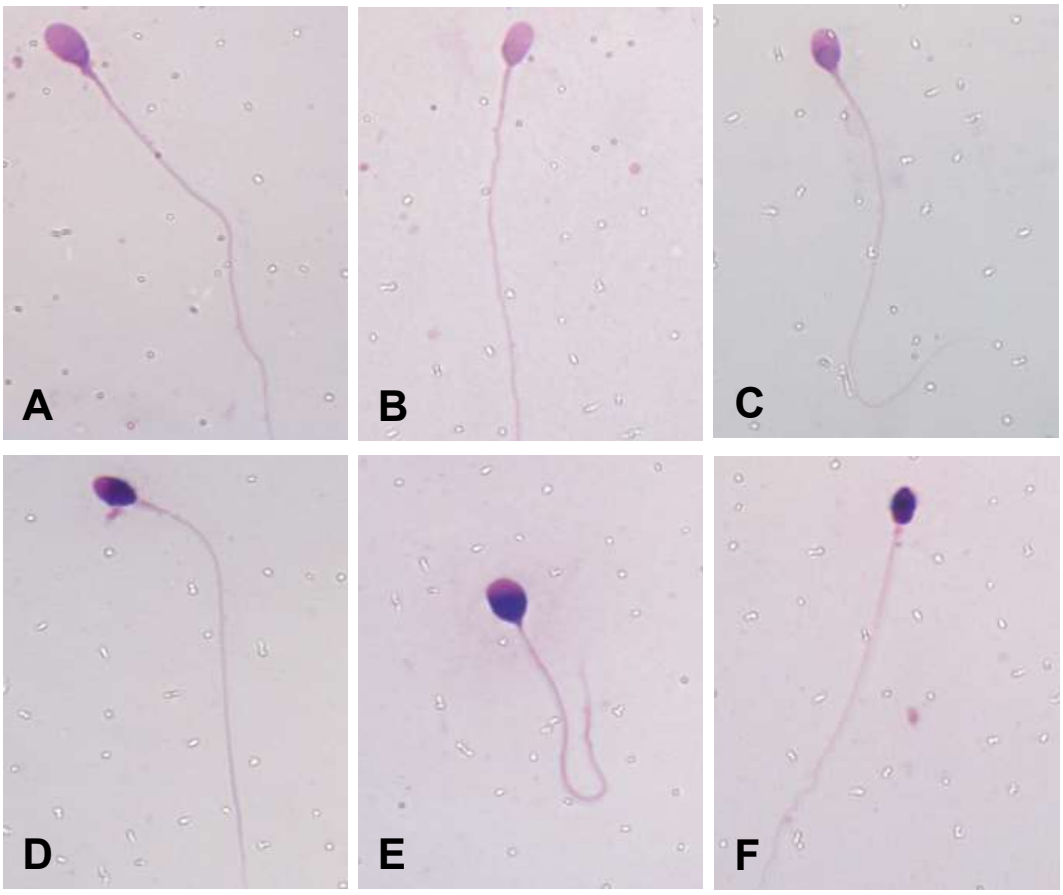

Figure 9: Chromatin/DNA integrity determined by the Diff-Quik staining. Spermatozoa with normal sperm heads (A, B, and C) exhibit a light stain, while spermatozoa with DNA fragmentation or chromatin decondensation (D, E and F) present a darker stain. Images captured by phase-contrast microscopy at 1000x magnification.

2.3.4. Mitochondrial function: measurement of MMP

The MMP, indicative of mitochondrial function, was measured using a lipophilic cationic dye, the JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Ramalho-Santos et al., 2007). This probe exhibits potential-dependent accumulation in mitochondria, changing its fluorescent emission according to the MMP (Ramalho-Santos et al., 2007) (Portela et al., 2015) (Yang et al., 2018). When the MMP is low, JC-1 is present in the form of monomer and exhibits green fluorescence. An increase in the MMP leads to the aggregation of JC-1 that forms polymers and shifts its fluorescence to orange-red. Therefore, this assay allows to distinguish between less active mitochondria and highly active mitochondria, respectively (Figure 10) (Portela et al., 2015) (Yang et al., 2018). Sperm suspensions were incubated with 1.5 μM of JC-1 for 20 minutes at 37ºC in the dark (Amaral & Ramalho-Santos, 2010). After that, a slide with 10 μl of the sperm suspension was mounted and the MMP was assessed by fluorescence microscopy (x630 or x1000 magnification with oil immersion). Sperm cells were

classified according to the fluorescence displayed by their midpiece: green, orange-red, or no fluorescence at all. At least 200 spermatozoa were scored in 5 different fields and the results were expressed as the percentage of spermatozoa with high MMP.

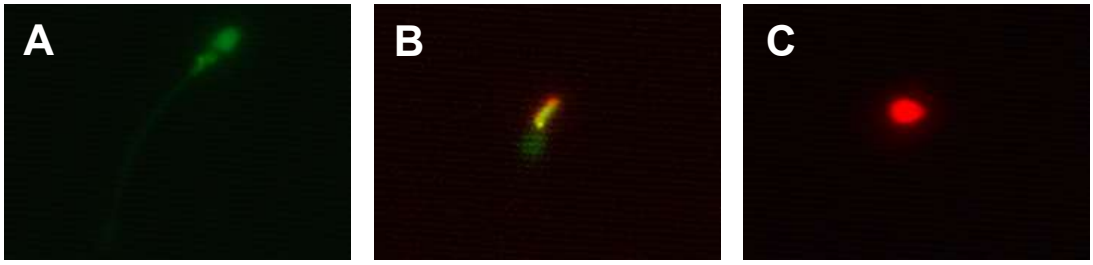

Figure 10: Measurement of MMP using the JC-1 probe. Spermatozoon with less active mitochondria display green fluorescence in the midpiece (A), spermatozoon with highly active mitochondria show orange-red fluoresce in the midpiece (B) and spermatozoon without staining in the midpiece (C). Images captured by fluorescence microscopy at 1000x magnification.

2.3.5. Capacitation

Capacitation status was determined through the detection of phosphorylated tyrosines by immunocytochemistry given the fact that protein phosphorylation, in specific in tyrosine residues, can be considered a marker of late capacitation-like events (Ramalho-Santos, et al. 2007) (Sepúlveda et al., 2016). In sum, spermatozoa were incubated for 3 hours in a capacitating medium that consisted in PBS supplemented with 0.9 mM calcium chloride , 0.5 mM magnesium chloride, 5 mM D-glucose, 0.3% (w/v) bovine serum albumin (BSA), 1mM sodium pyruvate, 10 mM sodium lactate and 1% (v/v) penicillin/streptomycin, pH = 7.2 – 7.4 (Portela et al., 2015). After that sperm cells were fixed with 2% (v/v) formaldehyde in phosphate buffered saline (PBS) for 40 minutes, cell membranes were permeabilized (1% (v/v) Triton X-100 in PBS) for 20 minutes and then, sperm cells were blocked (0.1% (wt/v) BSA and 100 mM glycine in PBS) for 30 minutes. Between each step, spermatozoa were centrifuged at 300g for 5 minutes. Next, cells were incubated overnight, at 37ºC, with a rabbit anti-human phosphotyrosine polyclonal antibody (Zymed, California (CA), USA) diluted 1:10 in blocking solution (Ramalho-Santos, et al. 2007) (Portela et al., 2015). Sperm cells were then washed with 0.1 % Triton X-100 in PBS (washing solution) for 30 minutes at room temperature and incubated with the secondary antibody (Texas Red®-X Goat Anti-Rabbit IgG; Molecular Probes) diluted 1:200 in blocking solution for 1 hour at 37ºC (Tavares, 2014). Once again, samples were washed with the washing solution for 15 min at room temperature. Lastly, slides were mounted with Vectashield antifade mounting medium with 4’,6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA) and examined by fluorescence microscopy (x630 or x1000 magnification with oil immersion)

(Ramalho-Santos, et al. 2007). The marker DAPI was used to counterstain the sperm cell nucleus. Spermatozoa displaying total or partial tail labelling, or exhibiting heterogeneous spots of fluorescence in the tail were considered capacitated whereas, spermatozoa that only displayed fluorescence at the equatorial band or the nuclear DAPI counterstain were classified as non-capacitated (Figure 11) (Portela et al., 2015). At least 200 spermatozoa were scored in 5 different fields and results were expressed as the percentage of capacitated spermatozoa.

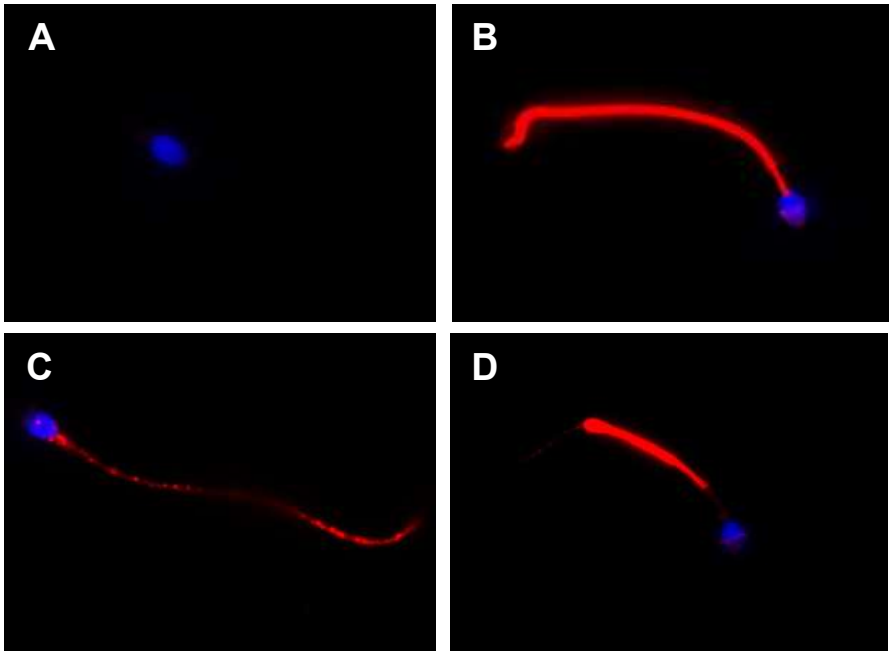

Figure 11: Capacitation categories. In the capacitation assay, spermatozoa can be classified as: non-capacitated (A) or capacitated (B, C and D). This last category includes totally capacitated (B), dotted (C) and partially capacitated (D) spermatozoa (Images captured by fluorescence microscopy at 1000X magnification).

2.3.6. Acrosome status

Acrosomal integrity was determined using the acrosome content marker, PSA-FITC (Pisum sativum agglutinin linked to fluorescein isothiocyanate), that by binding to the acrosomal content, in particular to glycoproteins, allows to establish the presence or the absence of the acrosomal matrix (Liu & Baker, 1988) (Ramalho-Santos et al., 2007). In this experiment, spermatozoa were incubated for 3 hours in capacitating medium (see section 2.3.5.) and, then, fixed, permeabilized and blocked as performed for the capacitation evaluation. Next, spermatozoa were centrifuged (300g for 5 minutes) and incubated with PSA-FITC (diluted 1:200 in blocking solution) for 1 hour at 37ºC followed by another centrifugation (300g for 5 minutes) and a washing step where cells were washed for 15 minutes in a solution containing 0.1% (v/v) Triton X-100 in PBS. Finally, slides were mounted with Vectashield antifade mounting medium with DAPI (Vector Labs, Burlingame, CA) and examined by fluorescence microscopy (x630 or

x1000 magnification with oil immersion). At least 200 spermatozoa were scored in 5 different fields, and only spermatozoa showing a bright green homogenous fluorescence in the acrosome region were considered intact (Figure 12 A). All the other patterns of fluorescence such as heterogeneous spots, fluorescence only in the equatorial segment or no fluorescence at all were considered acrosome-reacted spermatozoa (Figure 12 B) (Portela et al., 2015). Results were expressed as the percentage of cells with intact acrosomes.

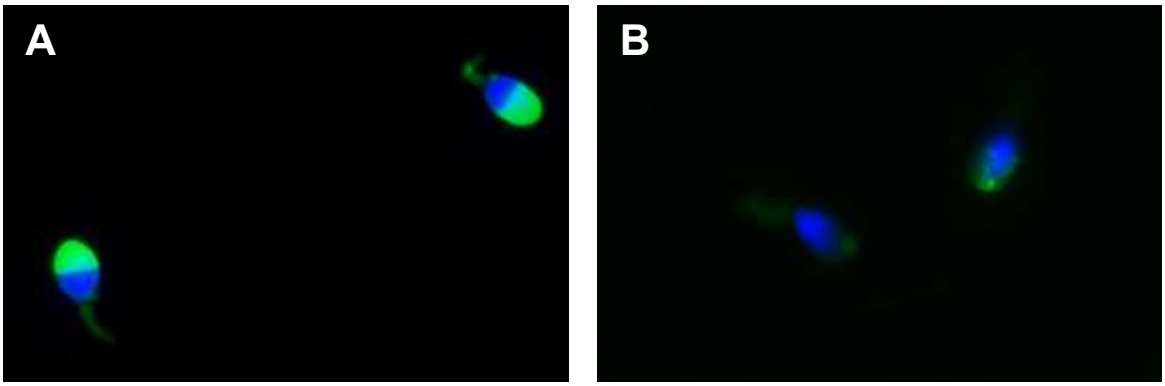

Figure 12: Acrosomal status. Intact (A) and reacted (B). spermatozoa. Images captured by fluorescence microscopy at 1000x magnification.

2.3.7. Biochemical assays for accessory sex organ function

2.3.7.1. Seminal fluid isolation

In order to obtain sperm-free seminal plasma, samples were centrifuged twice for 10 minutes at 1000g and, between each centrifugation, the supernatant (seminal plasma) was transferred to another test tube. To confirm that the segregation between seminal plasma-spermatozoa went effectively, a slide with 10 μl of the seminal plasma was examined under a phase-contrast microscope (200x or 400x magnification) and, in case of the presence of any spermatozoon, the procedure was repeated until a sperm-free seminal plasma was obtained. Then, seminal plasma was stored at -20ºC until a significant number of samples were collected to run the assays.

2.3.7.1.1. Measurement of fructose levels in seminal plasma (seminal vesicles function)

Fructose levels were measured according to the WHO guidelines (WHO, 2010). Initially, sperm-free seminal plasma samples were thawed and mixed on a vortex and dilutions (1:10) of each sample were prepared as well as a pool of seminal plasma for internal quality control. Afterwards, samples and the pool were deproteinized in a sequential step with 63 μmol/L zinc sulfate ($ZnSO_4$) followed by 1 mol/L NaOH. After 15 minutes, all the tubes were centrifuged at

8000g for 5 minutes and 50 μl of the supernatant of each were transferred to a new test tube. At that point, the 50 μl fructose standards as well the blanks were made to build the standard curve for the assay. The blank consists in purified water and the fructose standards consist in successive dilutions of 2.24 mmol/L D-glucose that yield the concentrations of 1.12, 0.56, 0.28 and 0.14 mmol/L. Next, 50 μl of colour reagent (indole 2 μmol/L in benzoate preservative 16 μmol/L) and 500 μl of hydrochloric acid (HCl) 32% v/v, were sequentially added to each tube and mixed well. Then, tubes were heated at 50ºC for 20 minutes in a water bath and cooled on ice for 15 minutes. The premise is that fructose, under the influence of heat and low pH, forms a complex with indole that, by having colorimetric properties, can be measured using a spectrophotometric method. Finally, a 96-multiwell plate was loaded with 250 μl of each tube (samples and standards) and read at 490 nm wavelength. The concentration of fructose in the ejaculate (μmol/l) was obtained by extrapolating the value given by the standard curve, multiplying the results of the sample by the dilution factor of 16 and again by the whole volume of the sample (ejaculate) (WHO, 2010).

2.3.7.1.2. **Measurement of neutral α-glucosidase activity in seminal plasma (epididymal function**)

The activity of the enzyme NAG was measured according to the WHO guidelines with only a few alterations in the protocol (WHO, 2010). Although seminal plasma contains either NAG and its acidic isoenzyme that are secreted by the epididymis and the prostate, respectively, the acidic isoform can be selectively inhibited by sodium dodecyl sulfate (SDS) allowing to detect only the activity of NAG and consequently, the epididymal function.

Sperm-free seminal plasma samples were thawed and mixed on a vortex, and while removing 15 μl of each sample to new test tubes, blanks [15 μl of colour reagent 2 (0.1g of SDS dissolved in 100 mL of 0.1mol/L sodium carbonate (Na_2CO_3) solution)] and semen pools for internal quality control purposes were made. In the semen pools, 8 μl of castanospermine (1 mmol/L), which is a glucosidase inhibitor, were added to provide the seminal plasma blank value. Afterwards, 100 μl of synthetic glucopyranoside (PNPG) substrate solution (5 mg/mL) at 37ºC were transferred to each tube, followed by an incubation of 2 hours at 37ºC. During this time, the standards of p-nitrophenol (PNP) for the standard curve were prepared. Successive dilutions of 200 μmol/L PNP (obtained by adding 80 μl of 5 mmol/L stock PNP in 2 mL with colour reagent 2) yielded the concentrations of 160, 120, 80 and 40μmol/L. After incubation, 1 mL of colour reagent 1 (0.1mol/L Na_2CO_3 solution) was added to each tube to stop the reaction and the samples were centrifuged for 10 min at 1000g. The reasoning behind this procedure is that

the enzyme glucosidase in the presence of its substrate – PNPG – converts this compound in PNP that turns yellow by adding Na_2CO_3, allowing its detection by spectrophotometry. Afterwards, 96-multiwell plates were loaded with 250 μl of either the standards and the samples and read at 450 nm wavelength, using the blank to set the zero. The activity of NAG in undiluted seminal plasma was obtained by multiplying the values obtained from each sample by the correction factor (0.6194) and subtracting the activity of the castanospermine seminal plasma blank to those values (IU/l). Then, to achieve the correct glucosidase activity in the ejaculate (mU), the value obtained for the glucosidase activity in undiluted seminal plasma was multiplied by the whole volume of semen (mL) (WHO, 2010).

2.3.7.1.3. Measurement of zinc levels in seminal plasma (prostate function)

Zinc (Zn) levels were determined in seminal plasma using the Wako Zinc test kit (FUJIFILM Wako Chemicals Europe GmbH, Germany) and following the procedure described in the WHO laboratory manual (*WHO*, 2010). The principle of this assay is that the compound 2-(5-bromo-2-pyridylazo)-5-[N-propyl-N-sulfopropyl)amino]-phenol (5-Br-PAPS), which is present in one of the reagents of the kit (Reagent A), binds with Zn and forms a complex that absorbs light at 560 nm and is directly proportional to the amount of Zn.

The first step of the procedure was to prepare the standards for the standard curve of the assay. They consist in successive dilutions of 100 μmol/L $ZnSO_4 \cdot 7H_2O$ that yielded the concentrations of 80, 60, 40, 20 and 10 μmol/L. Additionally, a blank with only purified water was also prepared. Then, after being thawed and mixed well, sperm-free seminal plasma samples were diluted by adding 5 μl of the sample in 300 μl of purified water on a test tube. After that, samples were mixed on a vortex by 5 s and the 96-multiwell plate was loaded with 40 μl of the blank, the standards and the samples. Next, 200 μl of colour reagent [20 μl Reagent A + 5 μl Reagent B (Salicylaldoxime 29 mmol/L)] was added to each well and the plate was mixed for 5 min on a plate shaker. After that time, the plate was read at 570 nm wavelength and the blank was used to set the zero of the standard curve. Zn concentration in the ejaculate (μmol/l) was obtained by extrapolating the value given by the standard curve, followed by multiplying the results of the sample by the dilution factor of 61 and again by the whole volume of the sample ejaculate (*WHO*, 2010).

2.4. *In vitro* study

2.4.1. Rationale for the selection of $HgCl_2$ and As_2O_3 concentrations and times of exposure

The concentrations and times of exposure used in this study were selected given what was previously described in the literature for both metals.

$HgCl_2$ (0.5 μM, 1 μM , 50 μM, 100 μM) and As_2O_3 (0.5 μM, 0.8 μM, 1.3 μM, 50μM) doses were selected with the intention of having environmental concentrations similar to a non-obvious exposed scenario (Li et al., 2012) (Sukhn et al., 2018), intermediary concentrations (higher exposure scenarios in which the city of Estarreja is potentially included) and higher ones that would exert a deleterious effect acting like positive controls for the experiments (Ernst & Lauritsen, 1991) (Eggert-Kruse et al., 1992) (Arabi & Heydarnejad, 2007) (Castellini et al., 2009) (Slivkova et al., 2010) (Martinez et al., 2016). Lastly, as both Hg and As co-exist in Estarreja, mixtures of $HgCl_2$ and As_2O_3 concentrations (0.5 μM $HgCl_2$ + 0.5 μM As_2O_3, 1 μM $HgCl_2$ + 0.8 μM As_2O_3) were also considered in the present study, although not for all parameters evaluated.

Moreover, concerning the times of exposure (t=0h, t=3h and t=24h), they were chosen not only to verify the immediate effect of $HgCl_2$, As_2O_3, or both on spermatozoa (t=0h) but also their prolonged effect (t=24h) since some literature reported that, in rodents, both metals tend to accumulate in testis, epididymis where sperm is stored and other accessory sex organs (Martinez et al., 2016) (Danielsson et al., 1984), (Pant et al., 2001) (Pant et al., 2004) (Chiou et al., 2008). Additionally, a paper performed with human samples also described a significant accumulation of As on human seminal fluid (Sukhn et al., 2018).

2.4.2. Exposure to heavy metals ($HgCl_2$ and As_2O_3)

To avoid masking the results, only normozoospermic samples i.e. exhibiting PR ≥ 32% or PR+NP ≥ 40%, sperm concentration ≥ $15X10^6$ sperm/mL and morphologically normal forms ≥ 4% were considered for this study. Samples were washed 3X with PBS, discarding the supernatant-containing seminal plasma, other cells and debris. $10x10^6$ Sperm cells were then exposed to either an untreated control, $HgCl_2$, As_2O_3 or $HgCl_2$ + As_2O_3 mixtures at specific time points (t=0h, t=3h, t=24h) and numerous functional sperm parameters were evaluated as follows:

2.4.2.1. Motility

Motility was determined under a phase contrast microscope (200x or 400x magnification) similarly to what was described in section 2.3.1.2.1.. At least 200 spermatozoa were counted

after exposure to each metal (n=8) or mixture (n=4) and at each timepoint. The results are expressed as the percentage of motile (PR + NP) spermatozoa.

2.4.2.2. Viability

After exposure, sperm were incubated with 100 nM SYBR14 and 240 nM propidium iodide (PI; LIVE/DEAD Sperm Vitality kit, Molecular Probes, Eugene, USA) for 20 minutes at 37ºC, in the dark (Amaral & Ramalho-Santos, 2010) (Tavares, 2014). While SYBR14 is a membrane-permeant fluorescent dye that stains all sperm nucleus green, PI is a membrane-impermeant fluorescent stain (red fluorescence), that can only penetrate compromised/damaged sperm cell membrane. In essence, the red fluorescence overlaps the green fluorescence allowing to differentiate the dead from the live spermatozoa, respectively (Amaral & Ramalho-Santos, 2010). Spermatozoa were then classified by fluorescence microscopy (x630 or x1000 magnification with oil immersion) according to their nuclear stain: alive if displaying green fluorescence, dead if presenting red fluorescence (Figure 13). At least 200 spermatozoa were scored in 5 different fields after exposure to each metal (n=8) or mixture (n=4) and at each timepoint. The results were expressed as the percentage of live spermatozoa.

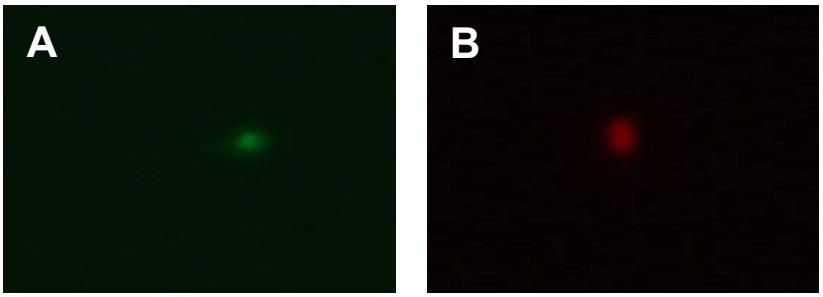

Figure 13: Viability assay using the Live/Dead kit. A live spermatozoon presents green fluorescence on the head (A), while the dead one exhibits red fluorescence (B). Images captured by fluorescence microscopy at 1000x magnification.

2.4.2.3. Mitochondrial function: measurement of MMP

MMP was assessed by fluorescence microscopy (x630 or x1000 magnification with oil immersion) as described in section 2.3.4. . At least 200 spermatozoa were scored after exposure to each metal (n=8) or mixture (n=4) and at each timepoint. The results were expressed as the percentage of spermatozoa with high MMP.

2.4.2.4. Capacitation

Capacitation status was assessed by fluorescence microscopy (x630 or x1000 magnification with oil immersion) as mentioned in section 2.3.5. . At least 200 spermatozoa were scored after exposure to each metal and at each timepoint (n=8). The results were expressed as the percentage of capacitated spermatozoa.

2.4.2.5. Acrosome status

Acrosome status was determined by fluorescence microscopy (x630 or x1000 magnification with oil immersion) as referred in section 2.3.6. . At least 200 spermatozoa were scored after exposure to each metal and at each timepoint (n=8). The results were expressed in the percentage of cells with intact acrosomes.

2.5. Statistical Analysis

Statistical analysis was performed using the SPSS software (Statistical Package for the Social Sciences Program) version 20.0 for windows (SPSS inc, Chicago, USA). All variables were evaluated for normal distribution through Shapiro-Wilk or Kolmorogov-Smirnov test, according with the number of experiments. Linear and logistic regression analyses were performed to predict if variables could affect the outcomes obtained in different populations. Comparisons between groups were carried out either by one-way ANOVA and related post hoc tests or the the Kruskall-Wallis and the Mann-Whitney tests if variables did not follow normal distribution. $p \leq 0.05$ were considered statistically significant and results were expressed as mean $\pm$ standard error of the mean (SEM).

CHAPTER III – RESULTS

3.1. *In vivo* study

3.1.1. Characterization of the study groups

To ensure that the results obtained for this *in vivo* study were derived from the exposure to heavy metals and not from eventual confounders, each participant had to fill in a questionnaire as indicated in the materials section. After initially excluding several samples, a careful analysis was performed with factors such as age, BMI and lifestyle habits such as tobacco and alcohol consumption, coffee intake and eating habits besides supplementation intake that could be confounder variables in this study. The data retrieved indicate that the groups were not statistically different ($p > 0.05$, Table 1), thus any observed effect in male reproductive health could be related to the exposure to heavy metals.

Table 3: Background data of the study groups. Results represent mean concentration ± SEM.

	Control Group	Exposed Group	Significance
Age (range)	35.27 ± 0.58 (25-54) n=88	34.08 ± 1.31 (26-42) n=12	> 0.05
BMI (kg/m^2) (range)	26.11 ± 0.42 (19.38-35.64) n=88	27.27 ± 1.50 (21.20-35.08) n=12	> 0.05
Cigarettes/day (range)	4.67 ± 1.00 (0-60) n=88	5.83 ± 2.81 (0-25) n=12	> 0.05
Alcoholic beverages/day (range)	0.47 ± 0.07 (0-3) n=79	0.43 ± 0.27 (0-3) n=11	> 0.05
Coffee/day (range)	2.97 ± 0.27 (0-20) n=86	2.74 ± 0.75 (0-9) n=12	> 0.05
Supplement intake with metals in last 3 mo (nº of subjects/total)	12/82	1/12	> 0.05
Daily intake of: (nº of subjects/total)			
Dairy products	47/86	9/12	> 0.05
Red meat	40/86	9/12	> 0.05
Fat fish	14/86	5/12	> 0.05
Soy and other related products	3/86	0/12	> 0.05

3.1.2. Spermiogram

The spermiogram is the classical procedure used in any Andrology clinic to evaluate sperm quality worldwide using fast, simple and low-cost procedures established by the WHO (WHO; 2010).

In this study, no statistically significant differences were observed between the control and the exposed groups in what concerns semen volume and pH, nor among sperm concentration, motility and morphology ($p > 0.05$, Table 2). Of note, according to the WHO criteria, the mean values of all parameters in both groups were considered normal, presenting ≥ 1.5 mL, pH ≥ 7.2, ≥ $15X10^6$ sperm/mL, PR+NP ≥ 40% and ≥ 4% morphologically normal forms (WHO, 2010) (Table 2). Nevertheless, there were samples in both groups presenting values for concentration, motility and normal morphology below the WHO criteria as easily observed by the range values given in brackets (Table 2).

Table 4: Spermiogram data from the control and exposed groups. Results represent mean concentration ± SEM.

	Control Group	Exposed Group	Significance
Spermiogram			
Semen volume (mL) (range)	3.58 ± 0.12 (1.5-7.0) n=88	3.30 ± 0.31 (1.8-5.0) n=10	> 0.05
Semen pH (range)	8.21 ± 0.03 (7.5-9.0) n=88	8.12 ± 0.07 (7.7-8.5) n=12	> 0.05
Concentration ($x10^6$/mL) (range)	54.10 ± 6.02 (0-227.5) n=88	35.34 ± 8.88 (0-90) n=12	> 0.05
Total motility (%) (range)	42.2 ± 2.09 (0-77) n=88	51.50 ± 6.07 (29-78) n=10	> 0.05
Normal forms (%) (range)	5.12 ± 0.47 (0-27) n=81	8.31 ± 1.92 (1-18) n=9	> 0.05

3.1.3. Viability and MMP

Despite not being a parameter often evaluated in this type of studies, sperm viability was found unaffected in the exposed group, being its mean percentage similar to the percentage of viable cells found in the control group ($p > 0.05$, n=81 and n=8 for the control and exposed groups, respectively, Figure 14 A). Furthermore, the same outcome was observed for MMP, a good indicator of mitochondrial function. Indeed, no differences were observed in the percentage of cells with high MMP among the control and exposed groups ($p > 0.05$, n=50 and n=7 for the control and exposed groups, respectively, Figure 14 B).

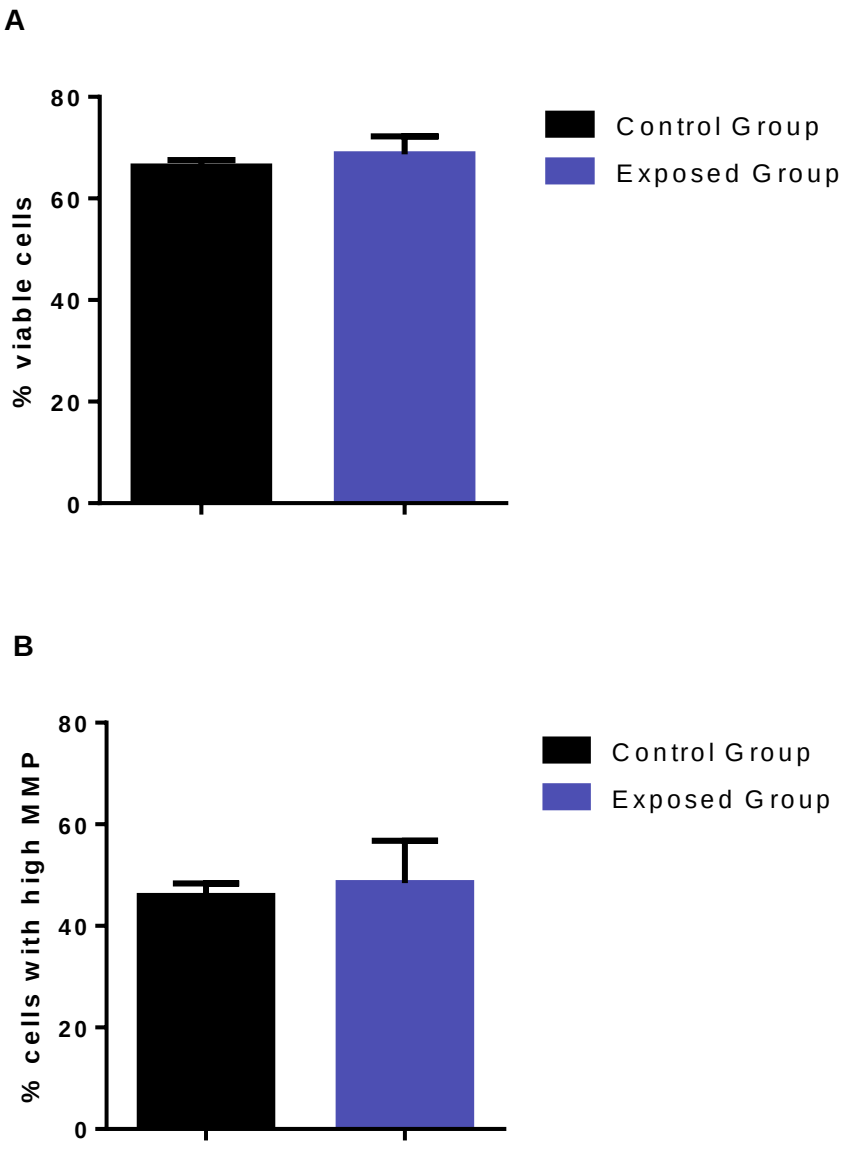

Figure 14: Percentage of viable cells (A) and cells with high MMP (B) in the control and exposed groups. Results represent mean percentage ± SEM.

3.1.4. DNA/Chromatin integrity

In the present study, no differences in the percentage of sperm cells with abnormal DNA/chromatin integrity were detected among the control and exposed groups ($p > 0.05$, n=81 and n=9 for the control and exposed groups, respectively, Figure 15).

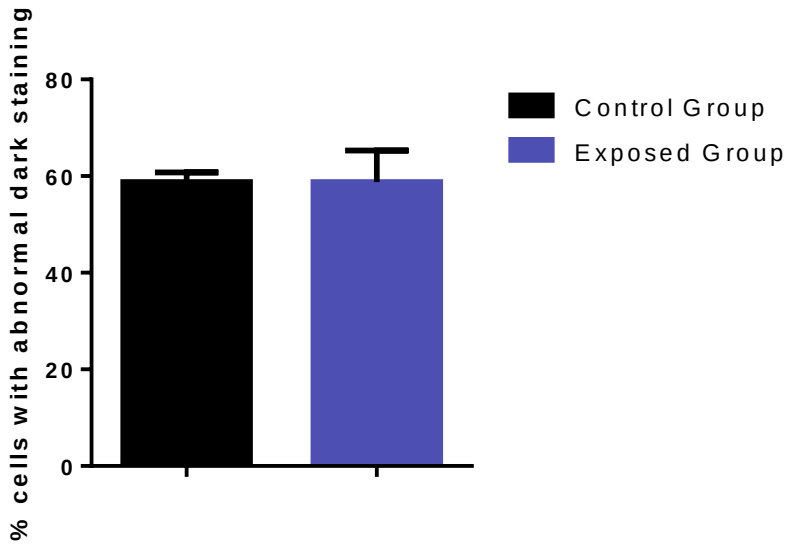

Figure 15: Percentage of cells with abnormal dark staining in the control and exposed groups. Results represent mean percentage ± SEM.

3.1.5. Capacitation and acrosome status

In the present study, significant differences were observed for capacitation and acrosome integrity. In fact, the percentage of capacitated cells were lower in the exposed group ($p < 0.05$, n=43 and n=6 for the control and exposed groups, respectively, Figure 16 A), which also presented a lower percentage of acrosome-intact spermatozoa (p=0.01, n=45 and n=6 for the control and exposed groups, respectively Figure 16 B).

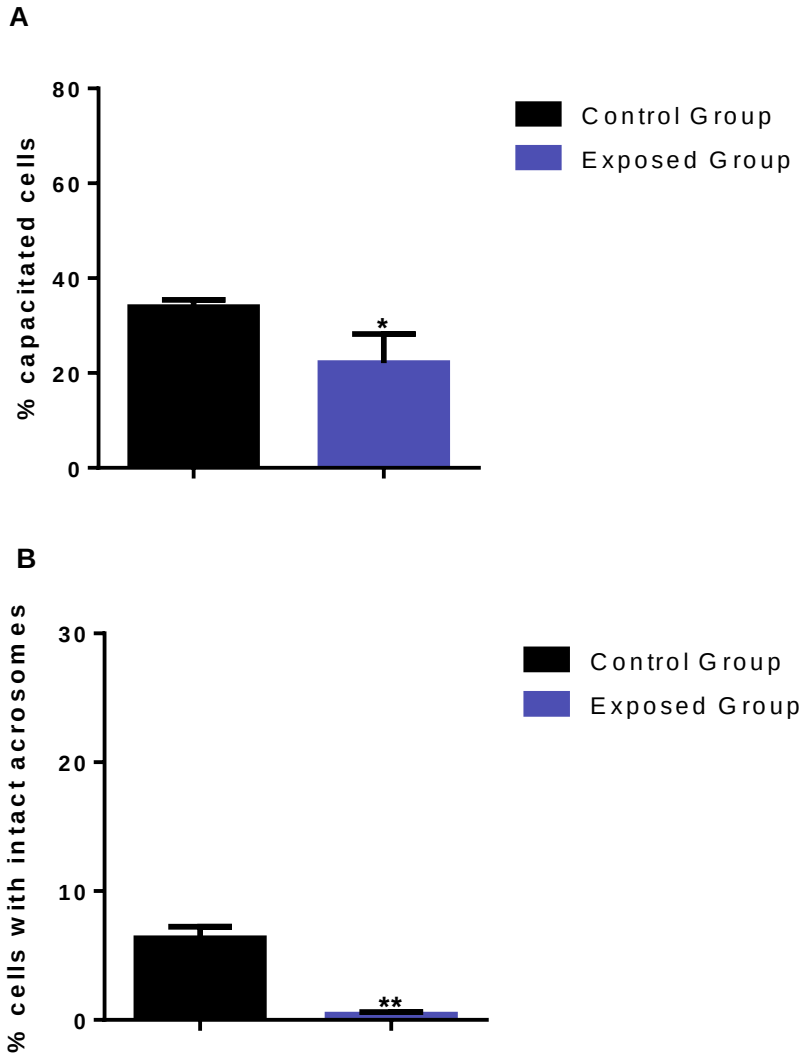

Figure 16: Percentage of capacitated cells (A) and intact acrosomes (B) in the control and exposed groups. Results represent mean percentage ± SEM. * $p < 0.05$ and ** $p = 0.01$ indicate significant differences when compared to the control group (black).

3.1.6. Biochemical assays

Finally, when looking for the accessory sex glands function, there were no significant differences regarding fructose, NAG and Zn levels in seminal plasma between the control and the exposed groups ($p > 0.05$, Table 3). Of note, according to the WHO criteria, the mean values of these indicators in both groups were considered normal, presenting ≥ 13 µM fructose/ejaculate, ≥ 20 mU NAG/ejaculate and ≥ 2.4 µM Zn/ejaculate (WHO, 2010) (Table 3). Yet, there were samples in both groups presenting values for NAG and Zn (and therefore, for

epididymis and prostate function) below the WHO criteria as easily observed by the range values given in brackets (Table 3).

Table 3: Biochemical marker concentrations in seminal fluid from each group. Results represent mean concentration ± SEM.

	Control Group	Exposed Group	Significance
Biochemical markers			
Fructose (μM/ejaculate) (range)	63.57 ± 5.16 (13.0-205.4) n=67	97.7 ± 31.12 (13.47-163.41) n=5	> 0.05
NAG (mU/ejaculate) (range)	90.36 ± 8.23 (2.5-364.6) n=65	54.78 ± 10.38 (16.78-76.93) n=5	> 0.05
Zn (μM/ejaculate) (range)	6.12 ± 0.51 (0.20-16.80) n=68	4.73 ± 0.78 (2.32-6.91) n=5	> 0.05

3.2. *In vitro* study

3.2.1. Viability

For this *in vitro* study, it was important to address if $HgCl_2$, As_2O_3 and their mixtures could affect sperm viability.

The two lower concentrations of $HgCl_2$ (0.5 μM and 1 μM) did not significantly affect the percentage of viable cells when compared to the control ($p > 0.05$, Figure 17 A). Nevertheless, at 50 μM a statistically relevant decline in sperm viability already at 0h was observed in relation to the control ($p < 0.01$), with a more obvious effect detected after 3h ($p < 0.01$) and 24h of exposure ($p < 0.001$). At 100 μM $HgCl_2$, the percentage of viable cells was drastically decreased at all times, reaching values of or about 0% ($p < 0.001$, Figure 17 A). Additionally, the percentage of viable cells after exposure to the lower concentrations (0.5 μM and 1 μM $HgCl_2$) statistically differed from the percentage of viable cells treated with the highest concentrations (50 μM and 100 μM $HgCl_2$), an observation that was constant throughout time (Figure 17 A). Differences were also observed between 50 and 100 μM $HgCl_2$ at t=0h and t=3h, but not after 24h where both promoted complete viability loss (Figure 17 A).

Exposure to As_2O_3 revealed that this metal does not significantly affect sperm viability at the concentrations and timepoints used in this study ($p > 0.05$, Figure 17 B) with the exception of 50

μM As_2O_3 that significantly decreased the percentage of viable cells after 24h of incubation ($p < 0.05$, Figure 17 B) . Moreover, statistical differences were detected in the percentage of viable cells between 0.5 μM As_2O_3 ($p < 0.001$), 0.8 μM As_2O_3 ($p < 0.01$) and 1.3 μM As_2O_3 ($p < 0.01$) with 50 μM As_2O_3 at t=24h (Figure 17 B).

Regarding the combination of $HgCl_2$ and As_2O_3, both mixtures (0.5 μM $HgCl_2$ with 0.5 μM As_2O_3 and 1 μM $HgCl_2$ with 0.8 μM As_2O_3) reduced sperm viability after 24h of incubation ($p < 0.05$ per each mixture, Figure 17 C). No differences were noticed between mixtures ($p > 0.05$, Figure 17 C).

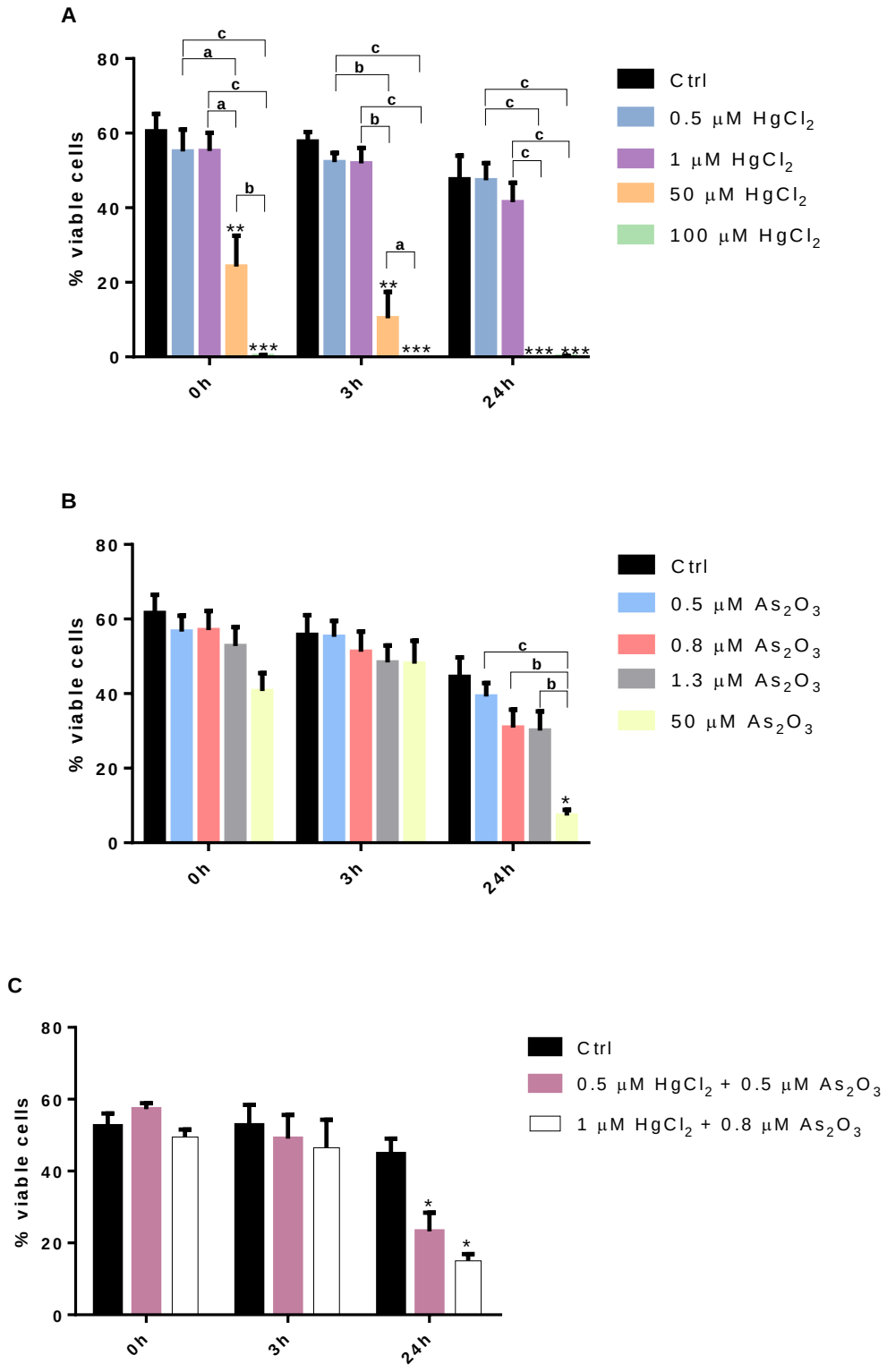

Figure 17: Percentage of viable cells after 0h, 3h and 24h of incubation with (A) $HgCl_2$ (n=8), (B) As_2O_3 (n=8) and (C) $HgCl_2$ + As_2O_3 mixtures (n=4). Results represent mean percentage ± SEM. ** $p < 0.01$ and *** $p < 0.001$ represent significant differences when compared to the control (black) within the same time of incubation. **a** $p < 0.05$, **b** $p < 0.01$ and **c** $p < 0.001$ represent significant differences among concentrations within the same time of incubation.

3.2.2. Motility

Exposure to low levels of $HgCl_2$, namely 0.5 μM and 1 μM, did not significantly affect the percentage of motile cells throughout time in relation to control ($p > 0.05$, Figure 18 A). However, at higher dosages, $HgCl_2$ significantly reduced sperm motility (Figure 18 A). 50 μM, despite not having an immediate effect ($p > 0.05$ at t=0h), significantly reduced total sperm motility at 3h ($p < 0.01$) and 24h ($p < 0.001$). In contrast, the highest concentration (100 μM $HgCl_2$) instantly caused a decrease in the percentage of motile cells ($p < 0.05$ at t=0h) with a more pronounced effect at later time points (t=3h and t=24h, $p < 0.001$, Figure 18 A). Furthermore, when comparing concentrations, differences were only detected after 3h and 24h of incubation and mainly between the lowest (0.5 μM and 1 μM $HgCl_2$) and the highest ones (50 μM and 100 μM $HgCl_2$) (Figure 18 A).

Crossing these results with the previous ones regarding sperm viability after incubation with $HgCl_2$, it is quite intriguing and contradictory to verify that at t=0h, apparently, there are no viable cells at 100 μM but there are 15% of motile spermatozoa. In fact, since the motility is determined immediately after the exposure to the metal (t=0h) while viability is only determined 20 min later due to technical specificities, this could explain the strong decrease in viability at 100μM $HgCl_2$. To address this, 3 normozoospermic samples were used to monitor sperm motility every 5 minutes in order to establish if at t=20min there were no motile cells and if this effect was still maintained after 25 minutes (Figure 19). As one may observe, no motility was observed following 20 min of exposure (Figure 19), further validating our hypothesis.

In what concerns As_2O_3 exposure, significant alterations were detected but only after 24h of exposure. At this time point, 0.8 μM As_2O_3 ($p < 0.05$, Figure 18 B), 1.3 μM As_2O_3 ($p < 0.001$, Figure 19 B) and 50 μM As_2O_3 ($p < 0.01$, Figure 18 B) decreased the percentage of motile cells when compared to the control. Furthermore, no differences were observed among concentrations within the selected time points ($p > 0.05$, Figure 18 B). However, a tendency was observed between 0.5 μM As_2O_3 and 1.3 μM As_2O_3 ($p = 0.069$, Figure 18 B) as well as 0.5 μM As_2O_3 and 50 μM As_2O_3 ($p = 0.052$, Figure 18 B).

In relation to the incubation with $HgCl_2$ and As_2O_3 mixtures, both doses (0.5 μM $HgCl_2$ +0.5 μM As_2O_3; 1 μM $HgCl_2$ + 0.8 μM As_2O_3) significantly decreased the percentage of motile cells after 24h of incubation ($p > 0.05$, Figure 18 C). No differences between mixtures were noticed ($p > 0.05$, Figure 18 C).

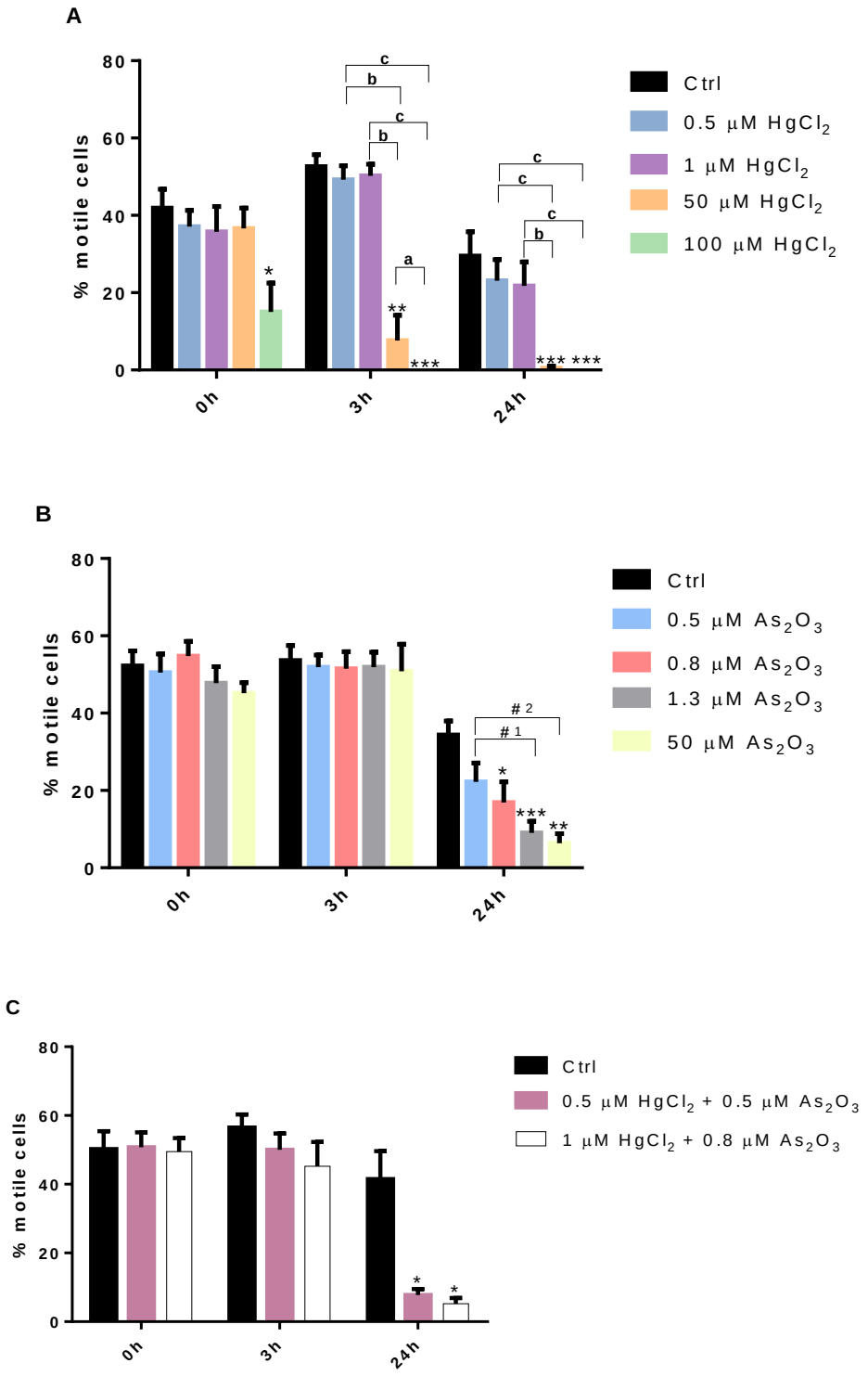

Figure 18: Total sperm motility after 0h, 3h and 24h of incubation with (A) $HgCl_2$ (n=8), (B) As_2O_3 (n=8) and (C) $HgCl_2$ + As_2O_3 mixtures (n=4). Results represent mean percentage ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ represent significant differences when compared to the control (black) within the same time of incubation. **a** $p < 0.05$, **b** $p < 0.01$ and **c** $p < 0.001$ represent significant differences among concentrations within the same time of incubation. **#** represent a tendency (**#1** $p = 0.069$ and **#2** $p = 0.052$).

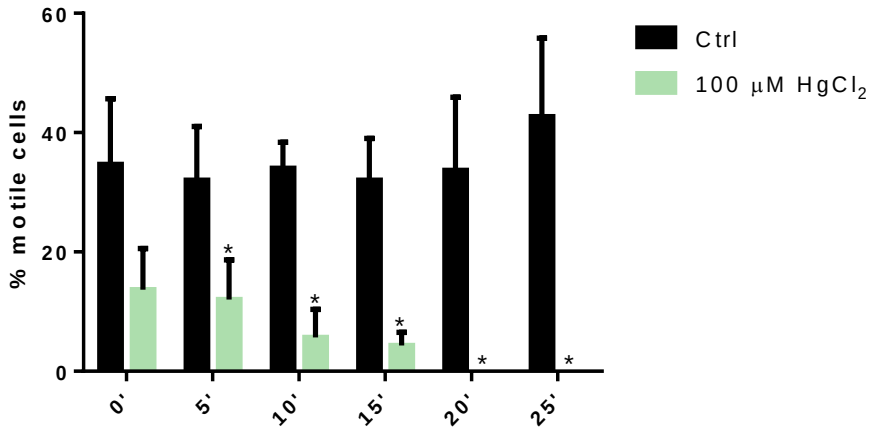

Figure 19: Percentage of total motile cells after incubation with the highest $HgCl_2$ concentration (n=3) for up to 25 minutes. Results represent mean percentage ± SEM. * $p < 0.05$ represent significant differences when compared to the control (black) within the same time of incubation.

3.2.3. MMP

MMP is strongly correlated with sperm quality and function (Espinoza et al., 2009).

Exposure to 0.5 μM and 1 μM $HgCl_2$ did not significantly modify the percentage of cells with high MMP, i.e. with highly active mitochondria, when compared to the control ($p > 0.05$, Figure 20 A). However, at 50 μM $HgCl_2$, a clear decline in the percentage of cells with high MMP at 0h was observed ($p < 0.05$), as well as following 3h ($p < 0.05$) and 24h of exposure ($p < 0.001$, Figure 20 A). The highest $HgCl_2$ dose (100 μM) remarkably decreased the percentage of cells with high MMP at all times when compared to control ($p < 0.001$, Figure 20 A). Comparisons among concentrations showed differences mainly between the lowest (0.5 and 1 μM) and highest ones used in the present study (50 and 100 μM) (Figure 20 A). These differences were more pronounced at t=24h ($p < 0.001$, Figure 20 A). Furthermore, and as observed for viability, differences between 50 and 100 μM $HgCl_2$ at t=0h and t=3h were detected, but not after 24h where both induced total loss of high MMP (Figure 20 A).

On the other hand, only 50 μM As_2O_3 significantly impaired high MMP after 24h when compared to control ($p < 0.001$, Figure 20 B). Additionally, the percentage of cells with high MMP significantly differed when comparing the highest concentrations with all the others after 24h of exposure ($p < 0.001$, $p < 0.01$, $p < 0.01$ respectively, Figure 20 B).

Furthermore, when the spermatozoa where exposed to $HgCl_2$ and As_2O_3 mixtures, a decline in the percentage of cells with high MMP was observed in comparison with the control (t=24h), being the difference exerted by the mixture with the two intermediary concentrations (1 μM $HgCl_2$ and 0.8 μM As_2O_3) more pronounced ($p < 0.01$, Figure 20 C) than the effect caused by the

mixture with the lowest concentrations (0.5 μM $HgCl_2$ and 0.5 μM As_2O_3) ($p < 0.05$, Figure 20 C). No differences between mixtures were noticed ($p > 0.05$, Figure 20 C).

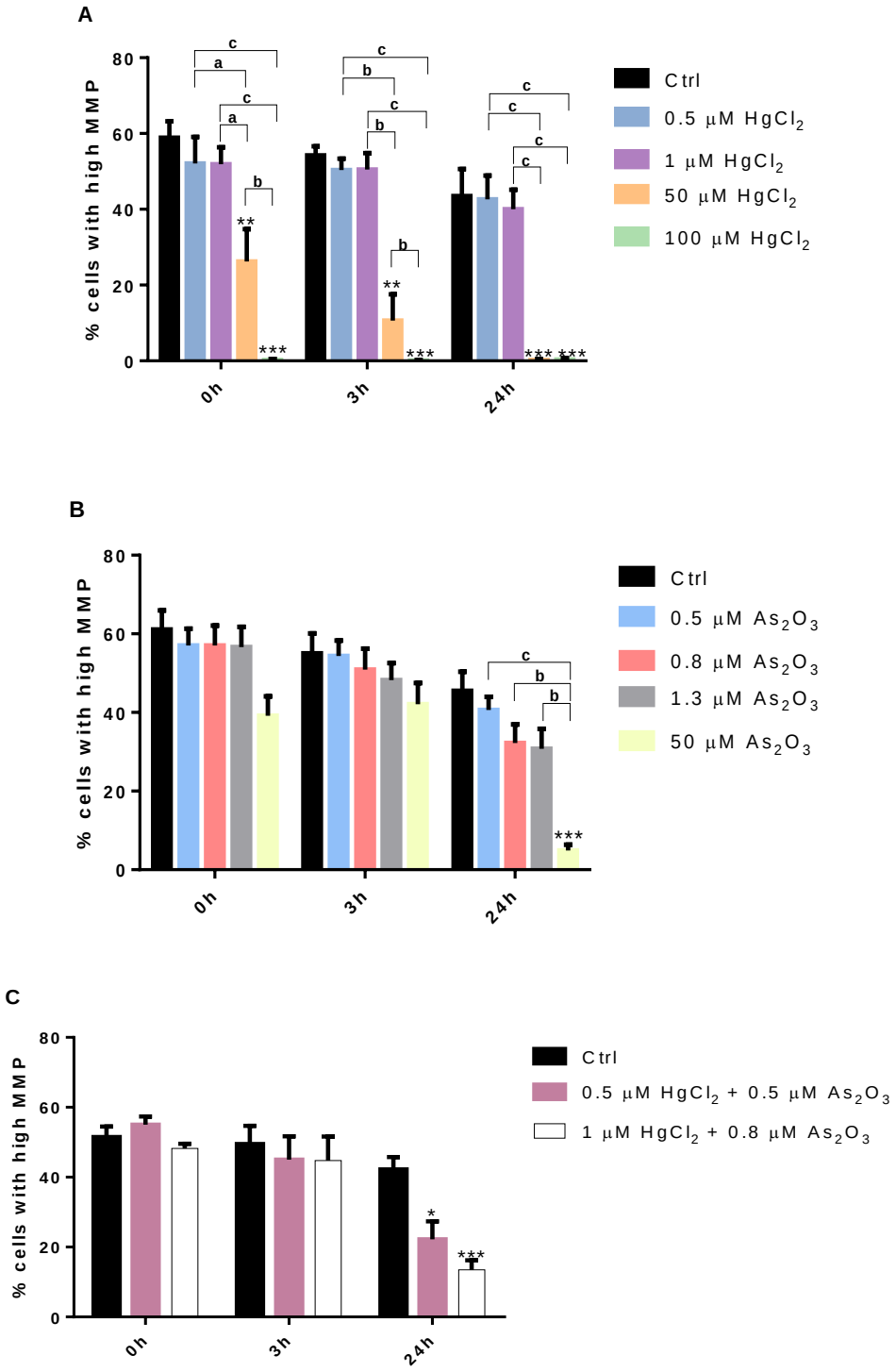

Figure 20: Percentage of cells with high MMP after 0h, 3h and 24h of incubation with (A) $HgCl_2$ (n=8), (B) As_2O_3 (n=8) and (C) $HgCl_2$ + As_2O_3 mixtures (n=4). Results represent mean percentage ± SEM. ** $p < 0.01$ and *** $p < 0.001$ represent significant differences when compared to the control (black) within the same time of incubation. **a** $p < 0.05$, **b** $p < 0.01$ and **c** $p < 0.001$ represent significant differences among concentrations within the same time of incubation.

3.2.4. Capacitation

100 μM $HgCl_2$ jeopardized the process of sperm capacitation, reaching a statistically significant difference at t=3h when compared to the control ($p < 0.05$) and further kept after 24h ($p < 0.01$, Figure 21 A). At t=24h, 50 μM $HgCl_2$ also started to exert a deleterious effect, decreasing the percentage of capacitated cells ($p < 0.05$, Figure 21 A). Furthermore, after 3h of exposure 0.5, 1 and 50 μM $HgCl_2$ presented significantly higher percentages of capacitated cells when compared to 100 μM $HgCl_2$. Following 24h, besides the usual statistical differences between the lowest (0.5 and 1 μM $HgCl_2$) and the highest (50 and 100 μM $HgCl_2$) doses studied (Figure 21 A), more capacitated cells were observed after exposure to 50 μM when compared to 100 μM $HgCl_2$ ($p < 0.05$, Figure 21 A).

Incubation with As_2O_3 did not affect the percentage of capacitated cells when comparing the control with the different As_2O_3 concentrations ($p > 0.05$, Figure 21 B). Similarly, no differences were observed when concentrations were compared within the same time of incubation ($p > 0.05$, Figure 21 B).

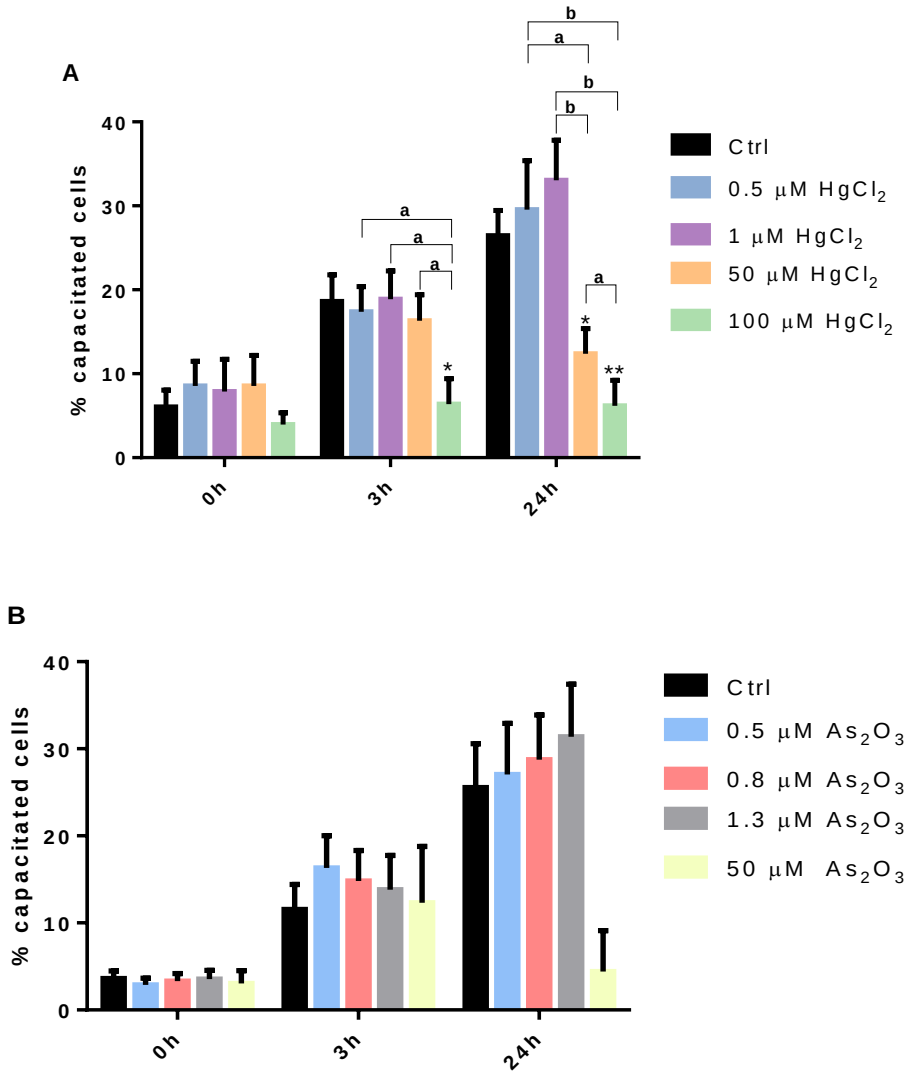

Figure 21: Percentage of capacitated cells after 0h, 3h and 24h of incubation with (A) $HgCl_2$ (n=8) and (B) As_2O_3 (n=8). Results represent mean percentage ± SEM. * $p < 0.05$ and ** $p < 0.01$ represent significant differences when compared to the control (black) within the same time of incubation. **a** $p < 0.05$ and **b** $p < 0.01$ represent significant differences among concentrations within the same time of incubation.

3.2.5. Acrosome status

Regarding the acrosome status, $HgCl_2$ only significantly altered the percentage of cells with intact acrosomes after 3h and 24h of exposure when compared to the control (Figure 22 A). For the intermediary timepoint, only 100µM $HgCl_2$ significantly affected the acrosome integrity ($p < 0.001$, Figure 22 A). However, after 24h, both 50 µM and 100 µM $HgCl_2$ significantly reduced the percentage of cells with intact acrosomes ($p < 0.05$, Figure 22 A). Moreover, the images obtained by fluorescence microscopy revealed the presence of small granules that were visible at 50 µM after 24h of exposure and 100 µM $HgCl_2$ after 3 and 24h of incubation, suggesting premature

acrosome reaction. Differences among concentrations were also found following 3h and 24h of exposure. Percentages of cells with intact acrosomes were statistically different when comparisons were performed between the lower (0.5 and 1 μM $HgCl_2$) and the higher concentrations (50 and 100 μM $HgCl_2$) (Figure 22 A). A statistical tendency was also observed between 50 and 100 μM $HgCl_2$ following 3h of incubation ($p = 0.064$, Figure 22 A).

In what concerns As_2O_3 treatment, no significant differences in the percentage of cells with intact acrosomes were detected between the control and the chosen concentrations at any time point ($p > 0.05$, Figure 22 B). Additionally, acrosome integrity did not differ among concentrations within the same time of incubation ($p > 0.05$, Figure 22 B).

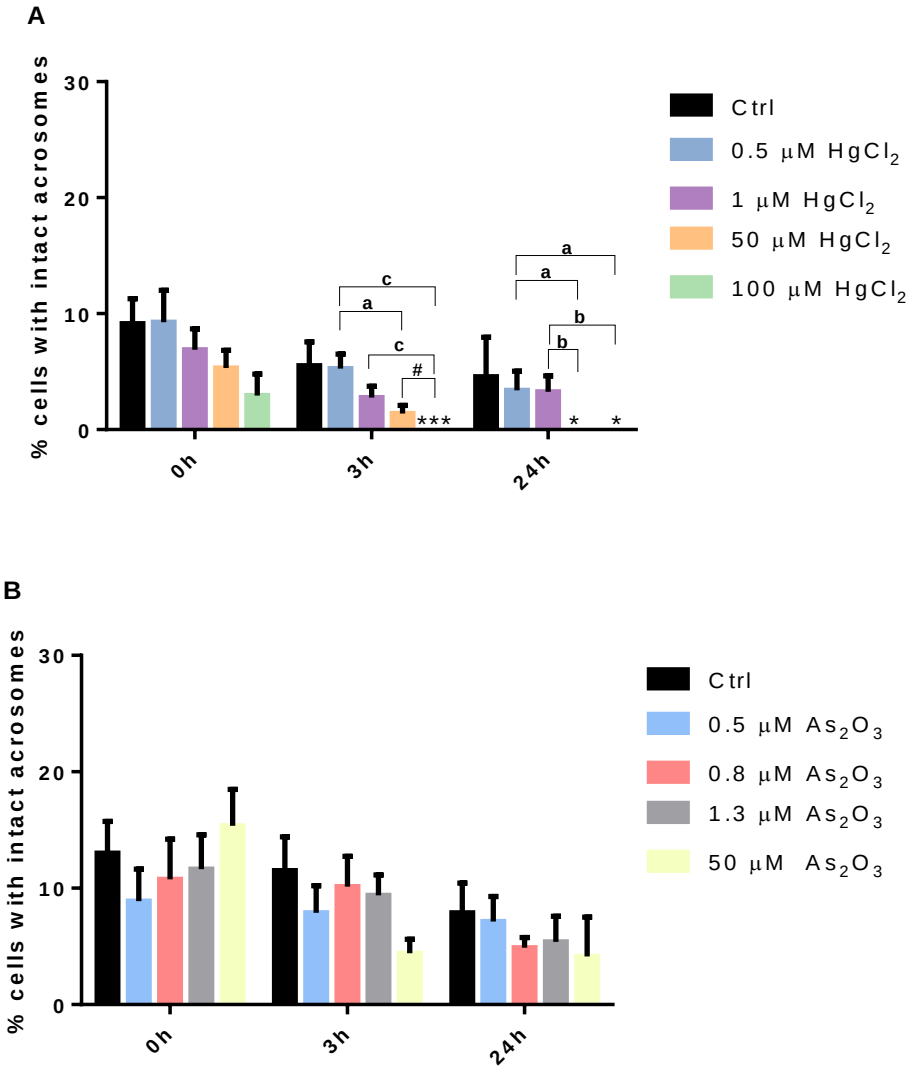

Figure 22: Percentage of cells with intact acrosomes after 0h, 3h and 24h of incubation with (A) $HgCl_2$ (n=8) and (B) As_2O_3 (n=8). Results represent mean percentage ± SEM. * $p < 0.05$ and *** $p < 0.001$ represent significant differences when compared to the control (black) within the same time of incubation. **a** $p < 0.05$, **b** $p < 0.01$ and **c** $p < 0.001$ represent significant differences among concentrations within the same time of incubation. **#** represent a tendency ($p = 0.064$).

3.2.6. Summary of the *in vitro* results

For a better comprehension of the *in vitro* data the following table summarizes the results obtained (Table 4).

Table 4: Overview of the *in vitro* results. The symbols used refer to the results obtained between the control and heavy metal exposure. The absence of significant results is represented by X, while the presence of statistically significant differences is represented by ✓.

$HgCl_2$												
	t=0h				t=3h				t=24h			
	0.5 µM	1 µM	50 µM	100 µM	0.5 µM	1 µM	50 µM	100 µM	0.5 µM	1 µM	50 µM	100 µM
Motility	X	X	X	✓	X	X	✓	✓	X	X	✓	✓
Viability	X	X	✓	✓	X	X	✓	✓	X	X	✓	✓
MMP	X	X	✓	✓	X	X	✓	✓	X	X	✓	✓
Capacitation	X	X	X	X	X	X	X	✓	X	X	✓	✓
Acrosome status	X	X	X	X	X	X	X	✓	X	X	✓	✓

As_2O_3												
	t=0h				t=3h				t=24h			
	0.5 µM	0.8 µM	1.3 µM	50 µM	0.5 µM	0.8 µM	1.3 µM	50 µM	0.5 µM	0.8 µM	1.3 µM	50 µM
Motility	X	X	X	X	X	X	X	X	X	✓	✓	✓
Viability	X	X	X	X	X	X	X	X	X	X	X	✓
MMP	X	X	X	X	X	X	X	X	X	X	X	✓
Capacitation	X	X	X	X	X	X	X	X	X	X	X	X
Acrosome status	X	X	X	X	X	X	X	X	X	X	X	X

Mixtures						
	t=0h		t=3h		t=24h	
	0.5 µM $HgCl_2$ + 0.5 µM As_2O_3	1 µM $HgCl_2$ + 0.8 µM As_2O_3	0.5 µM $HgCl_2$ + 0.5 µM As_2O_3	1 µM $HgCl_2$ + 0.8 µM As_2O_3	0.5 µM $HgCl_2$ + 0.5 µM As_2O_3	1 µM $HgCl_2$ + 0.8 µM As_2O_3
Motility	X	X	X	X	✓	✓
Viability	X	X	X	X	✓	✓
MMP	X	X	X	X	✓	✓

CHAPTER IV – DISCUSSION

4.1. *In vivo* study

Over the past decades, a decline in the male reproductive health, i.e. a decrease in the seminal quality and an increase in the incidence of testicular cancer, cryptorchidism and hypospadias, has been reported worldwide (Carlsen et al., 1992) (Auger et al., 1995) (Swan et al., 2000) (Agarwal & Allamaneni, 2005) (Iwamoto et al., 2006) (Iwamoto et al., 2007) (Geoffroy-Siraudin et al., 2012) (Sengupta et al., 2017) (Virtanen et al., 2017) (Mishra et al., 2018) (Rehman et al., 2018). EDs levels have been rising as well as the human exposure to such contaminants, which supports the idea that EDs may be involved in deterioration of the male reproductive health (Bliatka et al., 2017) (Virtanen et al., 2017) (Rehman et al., 2018). However, despite the unquestionable importance of investigating such association, there is still lack of research in this field due to conflicting interests from chemical and pharmacological industries as well as agriculture, governmental entities, non-governmental organizations and consumer organizations (Rajpert-De Meyts & Carrell, 2016). Nevertheless, some literature has been published addressing the impact of EDs exposure on male reproductive health; however, the vast majority use animals as models in a well-controlled environment (Sarkar et al., 2003) (Chiou et al., 2008) (Martinez et al., 2016) (Mohammadi et al., 2018) that do not mimic the conditions that human beings are exposed on a daily basis, being, therefore, essential to perform human studies to draw more solid conclusions and to raise awareness of the risks of EDs (Bliatka et al., 2017) (Viguié & Zalko, 2020).Taking this into account, this study aimed to describe the sperm quality and function and overall reproductive health status from men living in a heavily industrialized area (Estarreja) and compare them with an adult male population living in a non-industrialized context.

In order to draw valid conclusions, it was necessary to ensure that the outcomes that we have obtained in the *in vivo* study were derived from the exposure to heavy metals and not due to other factors that could be biasing the results. So, each participant of the study had to fill in a questionnaire and after proper statistical analysis, the results demonstrated that the exposed and the control groups were not statistically different in terms of age, BMI, smoking habits and alcohol consumption (number of cigarettes and ingestion of alcoholic drinks per day, respectively), number of coffees per day and food habits (ingestion of supplements and consumption of dairy products, red meat, fat fish, soy and other related products). This validation was fundamental for the quality of the results taken here, since it is already described in the literature that, for example, alcohol consumption and smoking habits can impair sperm quality, and, not so unanimously, caffeine intake as well (Meri et al., 2013) (Jensen et al., 2014) (Ricci et al., 2017). Furthermore, the ingestion of supplements could potentially constitute a

secondary source of metals, such as Se and Mg, and might have some benefits, modulating sperm quality parameters as described by Salas-Huetos and colleagues on their review (Salas-Huetos et al., 2018), therefore, being a relevant parameter to be addressed. Similarly, the consumption of, for instance, red meat and fat fish could potentially contain the presence of other EDs (Rignell-Hydbom et al., 2007) (Law et al., 2012), that could bias the effects derived from the heavy metal exposure associated with the proximity to the chemical complex in Estarreja. Furthermore, ageing and obesity are other factors associated with decreased male fertility, although some controversy may exist (Almeida et al., 2017) (Liu & Ding, 2017). Fortunately, the lack of statistically significant differences among groups allowed us to guarantee that any posterior result related to sperm quality and accessory organ function was likely derived from heavy metals exposure.

Starting with the spermiogram, this is usually the first evaluation being performed in the male when there is the suspicion of infertility in a couple, mainly because it is a fast, simple and low-cost evaluation widely employed to determine the seminal quality of an individual. As a result, in many papers it ends up being the only one executed (Auger et al., 1995) (Iwamoto et al., 2006) (Meeker et al., 2008) (Geoffroy-Siraudin et al., 2012) (Zeng et al., 2015). However, there is growing evidence that this is a bit outdated and cannot accurately predict sperm fertilizing ability, giving just a coarse overview of the seminal quality on the moment and letting out more subtle alterations that may be related to infertility as well (Agarwal & Allamaneni, 2005) (Sousa et al., 2008) (Moody et al., 2017). In the present study, none of the parameters addressed (semen volume, semen pH, sperm concentration, % total motility and % normal forms) were different among groups. Moreover, it is important to highlight that the mean values for each parameter assessed were considered normal according the reference values of the WHO guidelines (*WHO*, 2010). Hence, it is possible to conclude that the heavy metal exposure is not affecting the most assessed parameters which, at first sight, could led to the hasty conclusion that the exposure wasn't affecting sperm quality. Yet, we performed a very complete work that comprises more accurate functional indicators of sperm fertilizing ability (viability, MMP, DNA/chromatin integrity, capacitation and acrosome status), often disregarded in this type of studies, that provided deeper insights on this topic.

Regarding sperm viability, it is quite intuitive to think that the less viable sperm cells, less the chances to fertilize the oocyte. Even more, when we are aware that only a small percentage of the initial pool can reach the oocyte (Georgadaki et al., 2016) (Nucleus Medical Media, 2020). Thus, a sperm sample with poor viability can *a priori* have lower chances to achieve fertilization. However, in the present study, the control and the exposed groups did not differ between them, suggesting that the environmental exposure to the hazardous

contaminants in Estarreja are not inducing sperm death. Moreover, considering the reference value of the WHO, which is 58% of viable cells, both groups are considered normal in terms of sperm viability (*WHO*, 2010).

Another parameter very useful to evaluate sperm function is MMP. Several studies report that, in the ejaculate, the subpopulation of spermatozoa with highly active mitochondria (high MMP), simultaneously, tend to have higher percentage of morphologically normal cells, higher motility, more capacitated and with intact acrosome cells and less percentage of chromatin decondensation/DNA damage, turning the MMP a strong predictor of sperm functionality (Gallon et al., 2006) (Espinoza et al., 2009) (Marchetti et al., 2012) (Sousa et al., 2011) (Paoli et al., 2011) (Agnihotri et al., 2016). Nevertheless, this parameter was not affected in this study. Both the control and the exposed groups presented similar percentages of cells with high MMP. Consequently, it is possible to infer that the heavy metal exposure is not affecting mitochondrial functionality, at least in terms of MMP.

Chromatin/DNA integrity is crucial for the correct transmission of the paternal genetic material (Chohan, 2006). In fact, after the fertilization, sperm DNA must be able to decondense in a suitable time point in order to transmit its genetic information without any defects (Agarwal & Allamaneni, 2005). Furthermore, it is described in the literature that sperm DNA integrity is correlated with fertility outcomes, for instance, fertilization, implantation and pregnancy rate in a way that, higher levels of DNA fragmentation are often associated with poor seminal quality and ultimately, infertile men usually have higher levels of DNA damage when compared with fertile individuals (Chohan, 2006) (Sousa et al., 2008) (Schulte et al., 2010). The DNA fragmentation can be derived from defects in the chromatin packaging during spermatogenesis, defective apoptosis or excessive production of ROS in the ejaculate (Agarwal & Allamaneni, 2005) (Chohan, 2006). However, environmental and/or occupational exposure to toxicants, genetic factors, smoking and oxidative stress may also lead to this phenomenon (Chohan, 2006). Thus, it could be expected that the proximity to the 2nd largest chemical complex of Portugal, was interfering with the DNA integrity of the exposed individuals. Yet, the results indicate that it is not the case since there are not statistically differences in the percentage of cells with abnormal DNA between groups. Thus, this finding suggests that at the levels seen in Estarreja, the toxicants are not affecting DNA integrity.

In 2007, Rignell-Hydbom and co-workers have evaluated 195 Swedish fishermen that were exposed to MeHg through diet in order to assess if there was a negative impact on semen quality. Here, we evaluated men that were exposed to heavy metals with the same purpose. Rignell-Hydbom et al. addressed sperm motility, sperm concentration, total sperm count and sperm chromatin integrity and no statistically significant differences were observed, which is

similar to our findings (Rignell-Hydbom et al., 2007). Furthermore, Mocevic et al. evaluated blood concentrations of Hg from 529 subjects living in environmentally exposed countries (Greenland, Poland and Ukraine) to establish if there was a correlation between blood Hg concentrations and semen quality. The findings showed no significant correlations between blood Hg concentrations and semen parameters (semen volume, total sperm count, sperm concentration, count, morphology and motility), concluding that there was no evidence that Hg exposure in Greenland, Poland and Ukraine may lead to adverse effects on male reproductive health (Mocevic et al., 2013). In 2015, Zeng and colleagues, in a paper that was quite comparable to the previous one (Mocevic et al., 2013), attempted to determine if the urinary concentration, instead of the blood Hg concentration, was correlated with semen quality parameters (sperm concentration, motility and morphology). Yet, the results did not show any association between Hg in urine and an impairment in semen quality (Zeng et al., 2015). Indeed, until now, it has been difficult to correlate the heavy metal exposure or blood/urinary concentration from a certain metal with an aggravation of the seminal parameters. However, this project explored other relevant parameters to assess male reproductive health, such as sperm capacitation and acrosome status. Capacitation is the last maturation process that spermatozoa must undergo before fertilization (Kerns et al., 2018). Surprisingly, in this study, the capacitation process was found to be significantly affected, with the exposed group presenting a lower number of capacitated cells. This is actually the first reported evidence that, heavy metal exposure adversely impacts male reproductive health, disrupting a fundamental process for sperm functionality. Taking into account that, in the present study, the capacitation was quantified via the detection of phosphorylated tyrosines, it is plausible to assume that the exposure of heavy metals by the individuals living in Estarreja, is interfering with tyrosine phosphorylation. In the literature, several papers describe the capacitation as a signal transduction pathway involving a redox-regulated, cyclic adenosine monophosphate (cAMP)-mediated, tyrosine phosphorylation cascade (Aitken et al., 1995) (Aitken et al., 2004) (O'Flaherty, 2015). Indeed, that are studies in other different types of cells that report the importance of the cellular redox status in regulating the levels of tyrosine phosphorylation (O'Shea et al., 1992) (Schieven et al., 1993) (Fialkow et al., 1993) (Aitken et al., 1995). Thus, it is believed that low levels of ROS stimulate cAMP production that further increase tyrosine phosphorylation, in a mechanism mediated by PKA. Consequently, if ROS are scavenged, cAMP production is inhibited and capacitation is supressed (Aitken et al., 2004). On the contrary, high levels of ROS can lead to premature capacitation and acrosome reaction (Escada-Rebelo et al., 2020). Thus, it would have been quite convenient to measure the levels of ROS in both the control and the exposed groups, using the most appropriate fluorescent probes by flow-cytometry and/or fluorescence microscopy (Escada-

Rebelo et al., 2020), in order to determine if there were significant differences between groups that could explain the lower number of capacitated cells in the exposed group.

Finally, the last step that separates the spermatozoon from the oocyte is the AR, an event that needs to occur at the right time, i.e., in the vicinity of the oocyte. Otherwise, the sperm cells can prematurely lose the ability to successfully interact with the oocyte and to fertilize it (Fraser, 1998). As a result, this study evaluated the acrosome status in order to observe if in the presence of any stimuli, the acrosome would remain intact (as desirable) or it would prematurely react. The results showed that the exposed group have a significant lower number of cells with intact acrosomes when compared with the control. This is a remarkable finding that proves that the individuals that live in Estarreja and, consequently, are more exposed to environmental pollutants, such as heavy metals, not only have the capacitation process diminished, but also have the acrosomal integrity compromised.

After the analysis of the parameters related with sperm quality and functionality, since the goal of this study was also to give an overview of the overall reproductive status of the men living in Estarreja, the functionality of the seminal vesicles, epididymis and prostate was also assessed. The seminal fluid provides energy, immune defence and other contributions to sperm cells motility, transport, capacitation and fertilising ability (Said et al., 2009) (Hopkins et al., 2017). Therefore, poor seminal outcomes may be a consequence of abnormal accessory sex organs secretions (*WHO*, 2010). The seminal vesicles secrete, among others, fructose that is required for sperm cells metabolism and motility (Thi Trang et al., 2018). Moreover, low levels of fructose were correlated with men with asthenozoospermia (reduced sperm motility) (Gonzales et al., 1989) (Said et al., 2009) and genital inflammation (Comhaire et al., 1989). The results obtained in this study demonstrated that both groups do not differ between them and present mean values of fructose considered normal according to the WHO guidelines (*WHO*, 2010), suggesting that seminal vesicles function is not compromised. Remarkably, Thi Trang et al. have described that fructose levels were inversely correlated with sperm concentration, viability and motility arguing that since fructose is a primary source of energy, the higher concentration, viability and motility, require more energy, so fructose is lower (Thi Trang et al., 2018). Interestingly, in this study, this was not observed. Further, regarding the epididymis, they play a crucial role in the process of sperm maturation and their acquisition of progressive motility (Mahmoud et al., 1998). Lower levels of NAG activity are often an indicator of epididymitis, genital tract inflammation, epidydimal obstruction and defects in the sperm maturation process (Mahmoud et al., 1998) (Said et al., 2009) (Eertmans et al., 2014). Our results indicate that there was no difference among groups and, besides that, both groups presented mean normal values of NAG activity (*WHO*, 2010), suggesting that exposure is not having a

deleterious effect on the epididymal function as well. Lastly, the prostate gland secretes Zn which is a fundamental factor for the male reproductive system being involved in the normal development of the gonads, the prostate and in sperm motility and viability (Fuse et al., 1999) (Chia et al., 2000) (Thi Trang et al., 2018). Zn has also been reported to play an important role on sperm membrane stabilization, prevention of nuclear chromatin damage and in the antibacterial activity of the seminal plasma (Chia et al., 2000) (Saleh et al., 2008). Furthermore, low concentrations of Zn are suggested to be associated with poorer fertility outcomes, in males, in a way that, after Zn therapy, studies demonstrate an increase in sperm concentration, progressive motility and improved conception and pregnancy outcome (Tikkiwal et al., 1987) (Kynaston et al., 1988) (Omu et al., 1998) (Chia et al., 2000) (Saleh et al., 2008). However, although several papers describe a positive correlation between Zn concentration in seminal plasma and sperm concentration and motility (Fuse et al., 1999) (Chia et al., 2000) (Saleh et al., 2008) (Thi Trang et al., 2018) others did not find any statistically relevant correlation (Mankad et al., 2006) (Eliasson & Lindholmer, 2009). Yet, the clinical relevance of Zn on male fertility is unquestionable. Hence, the concentration of Zn in the seminal plasma of both groups was determined and the results showed no differences between them. Also, both mean values are considered normal according to the WHO guidelines (*WHO*, 2010).

4.2. *In vitro* study

The *in vitro* study was planned to determine the possible mechanisms of action of toxicants in spermatozoa, since it is known that different substances may act differently. Thus, the first aspect to take into consideration was which heavy metals should we include in the study. Since the *in vivo* study was focused on the city of Estarreja, as a model of a heavily industrialized area, the two heavy metals were chosen accordingly to the predominance on that region, therefore the selection of As and Hg (Costa & Jesus-Rydin, 2001) (Inácio et al., 2007) (Inácio et al., 2014). Actually, although Hg has been more widely studied than As, the majority of the papers have described its effect in rodents and there is still limited information regarding its effects on human sperm and related mechanisms of action. Afterwards, the next step was to determine the concentrations for each metal that were more suitable to include in the present project. As explained before, the concentrations were determined considering what was described in the literature, given that we were not able to determine the levels of both metals in samples of men from Estarreja due to these challenging times. However, the plan was having the lowest concentration closer to the physiological levels of both metals in seminal plasma, although that was more difficult than expected since the literature determining the levels of Hg and As is very

scarce. In relation to As, only two papers referred its concentration in seminal plasma, yet both papers focused on a very specific non-obvious exposed population. Li et al. established the levels of As on seminal plasma from 28 Chinese men with normozoospermic samples, according to the WHO guidelines, reaching values from 36.77 to 69.55 ng/mL (Li et al., 2012). Sukhn et al. used the same strategy but on a different population (55 Lebanese men) and reported values ranging from 26.37 to35.93 ng/mL (Sukhn et al., 2018). Therefore, the lowest value found in seminal plasma from normozoospermic men was 26.37 ng/mL and the highest value found was 69.55 ng/mL. Hence, the lowest concentration used in this study for As_2O_3 was 0.5μM which is equivalent to 100 ng/mL. For Hg, only one paper described the physiological levels of this metal on seminal plasma and is the first aforementioned for As. Li et al. measured the levels of Hg in the same population reaching values of 29.27 to 80.51 ng/mL (Li et al., 2012). In the present study, the lowest concentration used for $HgCl_2$ was 0.5 μM which corresponds to 135.75 ng/ml, a value slightly above to what was established in the literature. However, it is expected that the concentrations of both metals in reproductive fluids of men from areas strongly contaminated with these compounds, such as in the city of Estarreja, are higher than in other places with no obvious exposure.

Both $HgCl_2$ and As_2O_3 did not affect sperm viability at the lowest dosages (0.5 μM and 1 μM and 0.5 μM and 0.8 μM, respectively) throughout time. This finding is in agreement with the sperm viability results from the *in vivo* study, since we believe these concentrations are the closest to the ones found in men from Estarreja, although there is no data available indicating the concentrations of Hg and As in this population. Regarding the two highest concentrations of $HgCl_2$ (50 μM and 100 μM) it was quite expected that they would exert a negative effect on sperm viability given the intention for them to act as a positive control for the experiment. Moreover, it was fascinating to observe that for 50 μM $HgCl_2$ the effect was intensified with time, achieving values closer to 100 μM $HgCl_2$ (which is the double) after 24h of incubation. However, in a study from 2007, Arabi and Heydarnejad assessed the immediate effect of $HgCl_2$ on sperm viability using five different concentrations of this compound (50, 100, 200, 400 and 800 μM) and only noticed a statistically significant effect when the highest concentration was applied (Arabi & Heydarnejad, 2007). In our case, at t=0h, 100 μM $HgCl_2$ was sufficient to induce a statistically significant decrease in sperm viability, which is a concentration 8x lower. For the 1.3 and 50 μM As_2O_3, it was quite exciting to see that only 50 μM As_2O_3 could decrease sperm viability but only after 24h, existing significant differences in the % of viable cells between the three other concentrations and 50 μM As_2O_3 at this timepoint. Overall, the *in vitro* results

suggest that Hg is more toxic than As since 50 μM $HgCl_2$ induce cell death immediately after exposure and the same concentration of As_2O_3 only induced the same outcome after 24h.

In what concerns sperm motility, the lowest concentration of Hg and As did not reduce the % of motile cells in the timepoints tested, which matches the *in vivo* study. For $HgCl_2$, despite the 50 μM did not immobilize the spermatozoa immediately, after 3h and 24h of incubation it gradually decreased sperm motility reaching values of approximately 0% of motile cells at t=24h. Nevertheless, using the same compound, Ernst and Lauritsen reported a significant decrease in sperm motility *in vitro* right after 15 min of exposure to 40 and 80 μM $HgCl_2$ and, after 90 min of exposure (the maximum applied in this paper) 20, 40 and 80 μM $HgCl_2$ provoked a total immobilization of the spermatozoa (Ernst & Lauritsen, 1991). Conversely, in the present study, after 3h of incubation there was still 6.51% of motile cells in the 50 μM $HgCl_2$ concentration and the total immobilization was only observed after 24h of exposure to 50 and 100 μM $HgCl_2$. The highest dosage, 100 μM $HgCl_2$, caused an immediate decline in the % of motile cells, causing a complete immobilization subsequently at t=3h and t=24h as mentioned before. Regarding As_2O_3 exposure, similarly to what happened with sperm viability, only significant results were found after 24h of incubation. However, in this case, besides the highest concentration (50μM), all other doses significantly affected sperm motility with the exception of. 0.5 μM As_2O_3. Therefore, it is possible to conclude that As is more toxic for the spermatozoa, causing its immobilization, since 0.8 and 1.3 μM As_2O_3 could reduce the % of motile cells after 24h whereas, after the same exposure time, 1 μM $HgCl_2$ did not significantly affect sperm motility.

MMP was unaffected by 0.5 and 1 μM $HgCl_2$ as well as 0.5 and 0.8 μM As_2O_3 throughout time, being these results convergent with the ones found in the *in vivo* study. Nevertheless, the analysis of the results regarding the highest concentrations of each metal (50 and 100 μM $HgCl_2$ and 1.3 and 50 μM As_2O_3) reveals an exciting information regarding their mechanism of action. While 50 and 100 μM $HgCl_2$ caused an immediate decline in the % cells with high MMP, that was more accentuated over time, 1.3 and 50 μM As_2O_3 did not affect mitochondrial functionality except for 50 μM As_2O_3, that significantly decreased the % cells with high MMP at t=24h. Focusing only on the 50 μM concentration of both $HgCl_2$ and As_2O_3 in order to establish a comparison, it is clear that while 50 μM $HgCl_2$ caused a reduction in the % cells with high MMP at all the timepoints, 50 μM As_2O_3 only showed a significant effect after 24h of incubation. Thus, it is possible to suggest that the Hg affects mitochondria in a larger extent when compared to As.

Considering the capacitation results, it is evident that this process is enhanced over time due to the rise in the bars length visible in both $HgCl_2$ and As_2O_3 graphs. The lowest

concentrations of Hg and As (0.5 μM and 1 μM and 0.5 μM and 0.8 μM, respectively) did not alter the % of capacitated cells in the selected timepoints, but when we analysed the results regarding the highest concentrations, they were quite distinct among metals. On one hand, $HgCl_2$ did not exhibit an effect at t=0h, but at later times, 100 μM and 50 $HgCl_2$ decreased the % of capacitated cells. On the other hand, As_2O_3 did not affect the % of capacitated cells over time, even in the highest concentrations, which reinforces the theory that each metal has different reproductive signatures.

Finally, in relation to the acrosome status, the percentage of cells with intact acrosomes was not altered by the incubation with 0.5 and 1 μM $HgCl_2$ and 0.5 and 0.8 μM As_2O_3 over time. These results, along with the ones of capacitation, are divergent from the results that were obtained in the *in vivo* study. However, it is important to take into consideration that in the *in vivo* study, the individuals are exposed to various toxicants at the same time which may lead to synergistic effects between them. Moreover, regarding the highest concentrations, both 50 and 100 μM $HgCl_2$ decreased the % of cells with intact acrosomes after 24h of incubation but only 100 μM $HgCl_2$ could exert the same effect after only 3h of incubation (although the results demonstrate a tendency for 50 μM $HgCl_2$). Furthermore, under the microscope it was possible to observe some granules, that are indicators of premature acrosome reaction. Curiously, Castellini et al. also described the appearance of microvesicles with different sizes in the acrosome region that lead to acrosome breakage but using rabbit spermatozoa and methylmercury (MeHg) for 4h (Castellini et al., 2009). As_2O_3 failed to induce AR, even at the highest concentrations (1.3 and 50 μM As_2O_3) over time.

Although the toxicity of a single heavy metal has been well-described in the literature, giving clues about its mechanisms of action (Pant et al., 2001) (Slivkova et al., 2010) (Ayoka et al., 2016) (Zhao et al., 2017) (Zhang et al., 2018), only few papers have described the combined toxicity of multi heavy metals (Zhou et al., 2017) (Karri et al., 2018) (Wang et al., 2020). Nevertheless, human beings are constantly exposed to complex mixtures of heavy metals that due to their long lasting properties tend to bioaccumulate, raising a huge concern regarding their harmful effects on human lives (Zhou et al., 2017) (Wang et al., 2020). Therefore, to deepen the knowledge acquired by this project, and in an attempt to reflect and mimic the conditions that the human beings face daily, mixtures of $HgCl_2$ and As_2O_3 were also incorporated in the *in vitro* study. The goal was to verify if, by using the lowest concentrations of each metal (0.5 μM $HgCl_2$ + 0.5 μM As_2O_3 and 1 μM $HgCl_2$ + 0.8 μM As_2O_3, respectively), that were previously tested individually and did not show any effect at any timepoint in the parameters that were evaluated, the results would be different indicating that by adding the two metals the effect of each one

could be potentiated by the presence of the other – synergistic effect (Andersson et al., 2016). Hence, the effect of the mixtures was evaluated on sperm motility, viability and MMP. Although both mixtures did not affect sperm motility, viability and MMP after 3h of exposure, the results concerning the 24h incubation were unequivocal. The results indicated that only after 24h of exposure to either 0.5 μM $HgCl_2$ + 0.5 μM As_2O_3 or1 μM $HgCl_2$ + 0.8 μM As_2O_3 was sufficient to significantly decrease sperm motility, sperm viability and mitochondrial functionality, the latter traduced by a decreased number of cells with high MMP. These findings are in accordance to what was described by Andersson et al. on their paper, where the authors describe that mixtures can have serious effects that must be taken into consideration and that usually evolve a dose-additive mechanism even when different modes of action are involved. Moreover, the authors also refer that a given endocrine disruptor might have different mechanisms of action accordingly to its dosage (Andersson et al., 2016). Overall, the data provided by the mixtures raise a huge concern because it showed that lower dosages can be as dangerous as higher dosages in the presence of other contaminant species, which is precisely what happens in an *in vivo* scenario. Furthermore, if only 24h of exposure to the mixtures was sufficient to induce negative consequences in sperm parameters, it is difficult to predict what can happen to the male reproductive health of men that permanently live in a heavily contaminated industrialized area.

CHAPTER V – CONCLUSIONS AND FUTURE PERSPECTIVES

Industrialization is an indispensable part of our daily lives, however, it is mandatory to know, at least, the price that we may pay for living in such a developed world if no safe and adequate measures are taken. Therefore, using the city of Estarreja as a model of a heavily industrialized area, known to be contaminated with heavy metals, this study evaluated exhaustively the impact of heavy metals exposure on male reproductive health by addressing several parameters that are not often evaluated in this type of studies worldwide. In sum, the results suggested that the daily exposure to heavy metals may affect sperm functionality in the individuals living in the area of Estarreja, potentially jeopardizing their fertility. Importantly, this *in vivo* study also shows that by only analysing conventional sperm parameters such as concentration, motility and morphology (which are a hallmark in these studies), we are missing a lot of information regarding sperm fertilizing potential which consequently, may lead to the conclusion that certain EDS may not be affecting a certain population when, in fact, that may not be the case. However, a larger sample size is required to have more certainty on such association. Besides, although we recognize that the sample size is a limitation of this study, due to the pandemic conjecture that we live nowadays, it was impossible to collect more samples. Additionally, it is also relevant to address in the future if this exposure is affecting the HPG-axis by addressing LH, FSH and testosterone levels in these individuals or if the heavy metals directly target the male reproductive tract and/or sperm.

We are aware that is not possible to associate the results obtained *in vivo* only with the exposure to As and Hg since, although these are the most prevalent, there are other metals and compounds present in the environment in Estarreja. Therefore, to overcome that issue, it was initially planned to conduct the dosing of these two metals in the seminal plasma of the control and exposed participants by inductively coupled plasma mass spectrometry, in a collaboration established with Prof Maria Eduarda Pereira from the Chemistry Department of the University of Aveiro, so that we could associate the *in vivo* results with these two metals. Unfortunately, due to the pandemic we could not have this data on time, but we believe to have it in a near future. Thus, the doses selected to address the direct mechanisms of action of these metals in human spermatozoa were established by previous literature and not by our own data.

Considering the *in vitro* study, it is possible to affirm that As and Hg, alone, cause direct negative effects on sperm functionality, even though at not environmentally-relevant concentrations. Yet, this was the first study describing such a wide range of alterations, derived from $HgCl_2$ and As_2O_3 exposure on human spermatozoa. Moreover, these two compounds when combined, potentiated each other and negatively affected the sperm cells functionality, strikingly, at physiological doses. This is particularly important given that we are daily exposed to a mixture of compounds and these results indicate that male fertility may be compromised.

Nevertheless, more studies are required to realize the extension of the damage, since only sperm motility, viability and mitochondrial functionality were evaluated. Of note, these two compounds have never been tested simultaneously, and this data is therefore totally novel.

Overall, it is important to reinforce that this was the first study performed in Portugal, in a heavily industrialized scenario, that aimed to evaluate the impact on the male reproductive health and one of the most complete studies performed so far on this thematic. Unquestionably, a strong point of this project was the variety of parameters evaluated, incorporating the analysis of more accurate functional indicators of sperm fertilizing ability such as mitochondrial function, capacitation and acrosome status, often disregarded in this type of studies. Despite the limitations referred above, this study will create awareness and serve as a starting point for further larger studies.

CHAPTER VI – REFERENCES

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