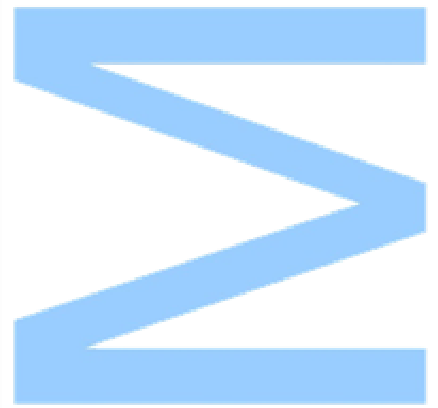

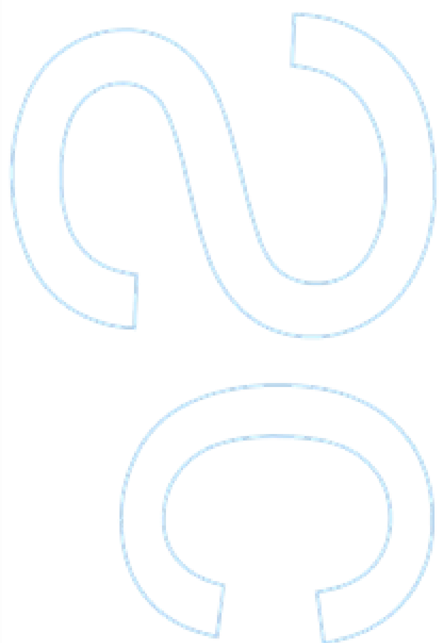

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Impact of hyperoside supplementation on the protection of human sperm against oxidative damage

Mafalda do Vale Ribeiro Moreira

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Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto em
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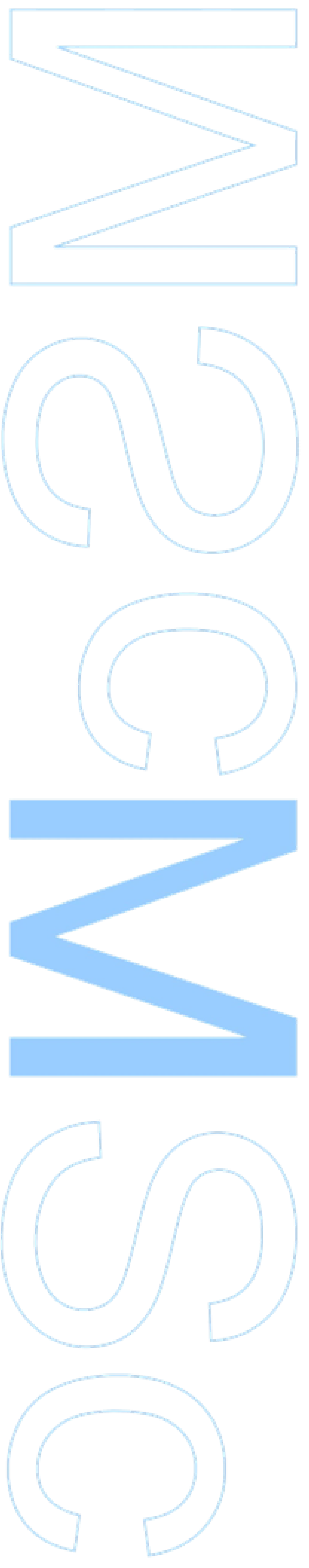

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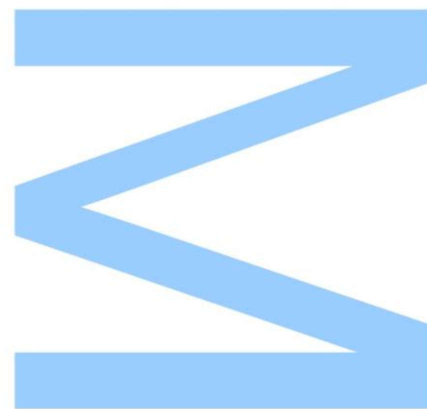
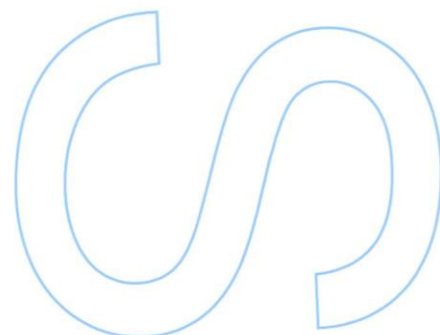
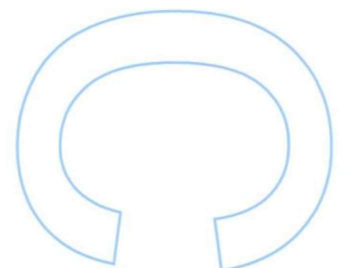

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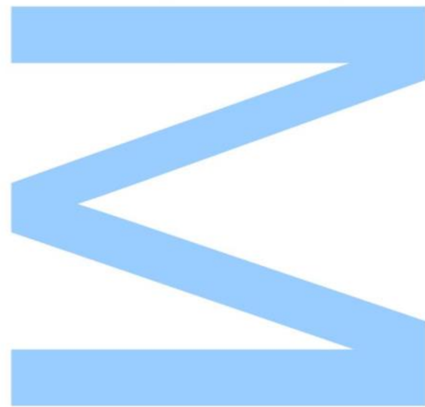

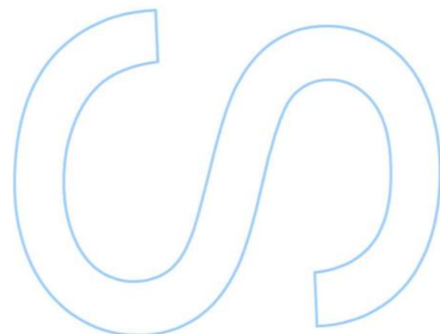

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Resumo

A infertilidade é um problema de saúde global que afeta cerca de 15% dos casais, sendo aproximadamente metade dos casos de infertilidade associados a fatores masculinos. O stress oxidativo (OS) é reportado como uma das principais causas da infertilidade masculina. Os casais inférteis costumam recorrer à tecnologia de reprodução assistida (ART) para conseguir uma gravidez bem-sucedida. No entanto, os protocolos de ART também aumentam a exposição dos gâmetas a condições de OS. Uma estratégia frequentemente utilizada para superar esse problema é a suplementação de meios com antioxidantes. Os flavonóides são um grupo de substâncias naturais que se têm destacado devido às suas propriedades antioxidantes. Assim, este projeto teve como principal objetivo investigar o impacto da suplementação com o flavonóide hiperósido (HYP) na proteção dos espermatozoides humanos contra danos oxidativos. Para isso, foram recolhidas vinte amostras de sémen de pacientes normozoospérmicos que foram submetidas ao tratamento com HYP, por 1 hora, na presença e ausência de peróxido de hidrogénio (H_2O_2). Como controlo positivo, os espermatozoides foram tratados com o antioxidante, vitamina C (VC). Para avaliar os potenciais danos oxidativos dos espermatozoides, foram avaliados os seguintes parâmetros: motilidade total e vitalidade dos espermatozoides, expressão de biomarcadores de OS, capacidade antioxidante total (TAC) do meio, fragmentação de DNA, potencial da membrana mitocondrial (MMP) e identificação e quantificação de metabólitos presentes no meio recorrendo a Ressonância Magnética Nuclear de Protão (^{1}H-NMR). Os nossos resultados demonstraram que a suplementação com HYP não induziu efeitos deletérios na fisiologia e metabolismo dos espermatozoides. Além disso, sob condição de OS-induzido por H_2O_2, o HYP foi capaz de preservar a motilidade dos espermatozoides e diminuir a fragmentação do DNA. Para além disso, o HYP parece também prevenir o aumento da peroxidação lipídica em condições de OS. No entanto, este antioxidante não foi capaz de prevenir a diminuição do MMP e as alterações metabólicas promovidas pelo H_2O_2. Estes resultados foram semelhantes aos obtidos com a suplementação com VC. Esta investigação fornece a primeira evidência de que o HYP protege os espermatozoides humanos contra os danos induzidos por H_2O_2, evitando alguns dos efeitos prejudiciais das ROS. No entanto, mais estudos são necessários para compreender o mecanismo de ação do HYP e o seu potencial para ser usado como suplemento em meios de preparação de espermatozoides em protocolos de ART.

Palavras-chave: Infertilidade masculina, stress oxidativo, ROS, ART, hiperósido

Abstract

Infertility is a global health problem that affects about 15% of couples and approximately half of infertility cases are associated with male factors. Oxidative Stress (OS) is reported as one of the major causes of male infertility. Infertile couples often recur to assisted reproductive technology (ART) to achieve a successful pregnancy. However, ART protocols also increase the exposure of gametes to OS conditions. A strategy often used to overcome this problem is the supplementation of media with antioxidants. Flavonoids are a group of natural substances that have been gaining popularity due to their antioxidant properties. Thus, this project aimed to investigate the impact of flavonoid hyperoside (HYP) supplementation on the protection of human sperm against oxidative damages.

For this purpose, sperm samples of twenty normozoospermic patients were supplemented with HYP, for 1 hour, in the presence and absence of hydrogen peroxide (H_2O_2). As a positive control, spermatozoa were supplemented with the well-known antioxidant, vitamin C (VC). To evaluate the potential sperm oxidative damage the following parameters were assessed: total sperm motility and vitality, OS biomarkers expression, total antioxidant capacity (TAC) of the media, DNA fragmentation, mitochondrial membrane potential (MMP), and metabolite identification and quantification of the media by using Proton Nuclear Magnetic Resonance (^{1}H-NMR). Our results demonstrated that supplementation with HYP did not induce any deleterious effects to the physiology and metabolism of the spermatozoa, after 1-hour of treatment. Further, under an H_2O_2-induced OS condition, HYP was able to preserve sperm motility and decrease DNA fragmentation. Furthermore, HYP also appears to prevent the increase in lipid peroxidation under OS conditions. However, this antioxidant could not prevent the decrease of MMP, and the metabolic alterations promoted by H_2O_2. These results were similar to those obtained with VC supplementation.

This investigation provides the first evidence that HYP protects human spermatozoa against H_2O_2 damage, by preventing some of the detrimental effects of ROS. Nevertheless, further studies are required to better understand the mechanism of action of HYP and its potential to be used as a supplement in human sperm preparation media in ART protocols.

Keywords: Male infertility, oxidative stress, ROS, ART, hyperoside

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List of abbreviations

^{1}H-NMR	proton nuclear magnetic resonance
4-HNE	4-hydroxynonenal
8-OHdG	8-hydroxy-2′-deoxyguanosine
AC	adenylate cyclase
ALT	alanine aminotransferase
ART	assisted reproductive technology
BCA	bicinchoninic acid
BER	base excision repair
BSA	bovine serum albumin
BTB	blood-testis barrier
BWW	biggers-whitten-whittingham
cAMP	cyclic adenosine monophosphate
CAT	catalase
CoQ10	coenzyme Q10
dH_2O	distilled water
DHA	dehydroascorbic acid
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
DNPH	2,4-dinitrophenylhydrazine
dUTPs	deoxyuridine triphosphate nucleotides
ESHRE	european society of human reproduction and embryology
ETC	electron transport chain
FDR	false discovery rate
FRAP	ferric reducing antioxidant power

FSH	follicular stimulating hormone
G-6-PD	glucose-6-phosphate dehydrogenase
GnRH	gonadotropin-releasing hormone
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized form of glutathione
HYP	hyperoside
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
JC-1	5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazoylcarbocyanine iodide
LAC	L-acetyl carnitine
LC	L-carnitine
LDH	lactate dehydrogenase
LH	luteinizing hormone
LPO	lipid peroxidation
MAS	malate-aspartate shuttle
MDA	malondialdehyde
MDH	malate dehydrogenase
MMP	mitochondrial membrane potential
NADPH	nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
NOX5	NADPH-oxidase isoform 5
NT	3-nitrotyrosine
OAA	oxaloacetate
OGG1	8-oxoguanine glycosylase

OS	oxidative stress
OXPHOS	oxidative phosphorylation
PBS	phosphate-buffered saline
PHGPx	phospholipid hydroperoxide glutathione peroxidase
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2
PRDXs	peroxiredoxins
PTK	protein tyrosine kinase
PTPase	phosphotyrosine phosphatase
PUFAs	polyunsaturated fatty acids
PVDF	polyvinylidene difluoride
ROS	reactive oxygen species
SOD	superoxide dismutase
ST	seminiferous tubules
TAC	total antioxidant capacity
TCA	tricarboxylic acid
TdT	terminal deoxynucleotidyl transferase
TFA	trifluoroacetic acid
TPTZ	2,4,6-tripyridyl-S-triazine
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UV	ultraviolet
VC	vitamin C
WHO	world health organization
WR	working reagent
ZP	zona pellucida

Chapter I:
Introduction

1. Introduction

1.1. Spermatogenesis

Male fertility is extremely dependent on the success of spermatogenesis, the process by which undifferentiated germ cells (spermatogonia) proliferate by mitotic division, undergo meiotic divisions, and differentiate into specialized haploid cells, the spermatozoa. Spermatogenesis occurs in the seminiferous tubules (ST) of the testis and involves a network of cellular events that are under the control of hormonal regulation and distinct signalling pathways. In humans, it takes approximately 74 days to complete the entire process [1].

Spermatogenesis starts at puberty through hormonal stimulation. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which in turn acts on the anterior pituitary, prompting the release of a follicular stimulating hormone (FSH) and luteinizing hormone (LH) [2]. LH exert its effect by binding to Leydig cell receptors (located in the interstitial spaces of seminiferous tubules), promoting the production and secretion of androgens, mainly testosterone. Testosterone is a powerful endocrine and paracrine stimulator of spermatogenesis [3]. This hormone acts on peritubular cells and Sertoli cells within the testis and it is fundamental for sperm production and the development of the organs of the male reproductive system. The regulation of spermatogenesis also depends on the action of FSH, which acts directly on Sertoli cells (located in ST) [4]. In fact, Sertoli cells (often referred to as “nurse cells”) play a vital role in spermatogenesis as they provide structural, nutritional, survival and immunological support to the developing germ cells. Further, these cells constitute the blood-testicular barrier (BTB), which is formed through basal junctions between adjacent Sertoli cells, dividing the ST into basal and adluminal compartments [5].

During embryonic development, primordial germ cells (PGCs) migrate to the testis, originating the spermatogonia, which immediately start to divide by mitosis. The spermatogonial stem cells proliferation phase occurs in the basal compartment of the seminiferous epithelium. Throughout this phase are produced subpopulations of spermatogonia with different purposes, some will be responsible for the germ cell line maintenance, and others will enter in the spermatogenesis process to become spermatozoa [3]. It is possible to distinguish three types of spermatogonia in the basement membrane of the ST: dark type A (Ad); pale type A (Ap) and type B [6]. Currently, it is accepted that Ad is a reserve of stem cells and Ap are predominant and

have self-renewal properties; in turn, the type B spermatogonia are the one that begins the reproductive cell growth [1, 7]. At puberty, the type B spermatogonia, located in the basal compartment, differentiate into preleptone spermatocytes that later turn into primary spermatocytes. Those primary spermatocytes migrate to the adluminal compartment to initiate meiosis. After crossing the BTB, the primary spermatocyte continues the maturation process by going through leptotene, zygotene, pachytene, and diplotene stages [8]. After completing the first meiotic division the secondary spermatocytes are generated. Then, those undergo the second meiotic division to form the haploid cells, called spermatids (Figure 1).

The round spermatids must face a complex cytodifferentiation to form functional spermatozoa, a process called spermiogenesis. This process begins with nuclear condensation, where the nucleus elongates and condenses its chromatin [9]. Further, occurs the formation of a large granule, resulting from the assembly of small granules in the area of the Golgi apparatus, and once associated with the nuclear membrane the large granule becomes an acrosome [10]. As spermiogenesis continues, the centrioles migrate to a post-nuclear region and one of the centrioles triggers the formation of the flagellum. As the flagellum expands, the mitochondria move to the middle piece to produce the energy necessary for sperm motility [11]. Following these events, the remaining cytoplasm is phagocytized by the surrounding Sertoli cells. The final stage, called spermiation, is characterized by the delivery (*via* Sertoli cells) of spermatozoa into the lumen of ST for their later maturation in the epididymis [1].

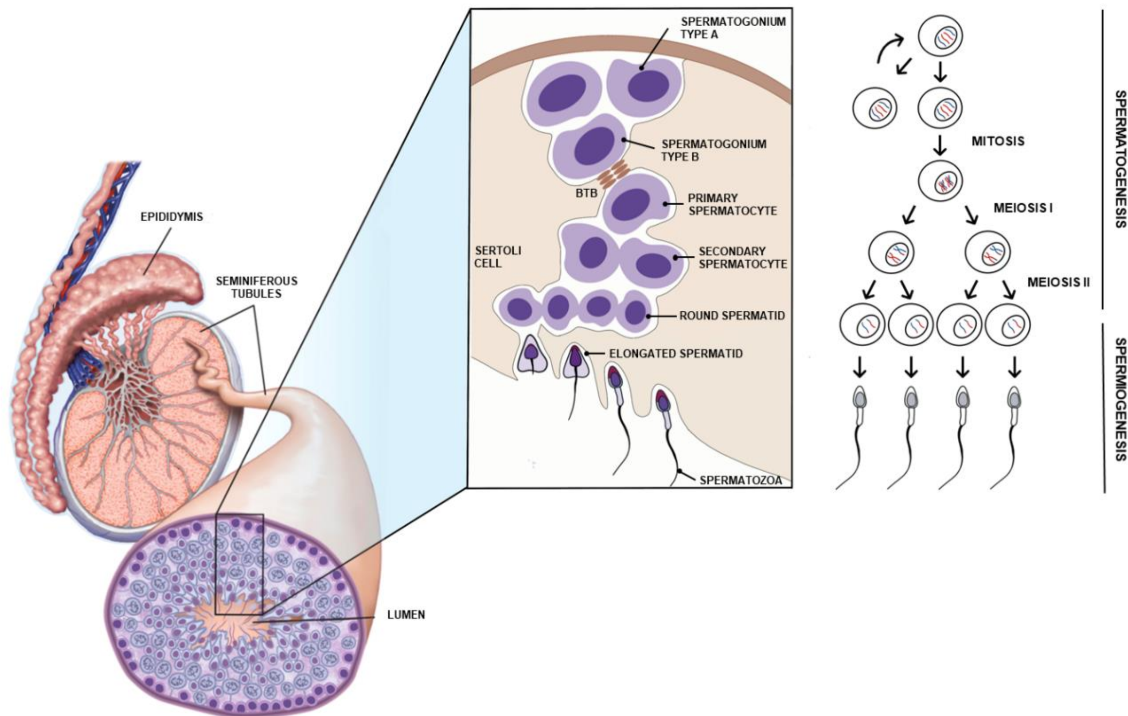

Figure 1: Schematic illustration of spermatogenesis. Sperm cells are produced within the seminiferous tubules in the testis. The spermatogonial cells, located at the basement membrane, proliferate by mitotic divisions giving rise to a new population of stem cells (spermatogonia type A), which are responsible to guarantee the germ cell line. In turn, spermatogonia type A can also divide and develop into spermatogonia type B, which will later differentiate into primary spermatocytes. Then, those spermatocytes undergo meiosis I (separation of the homologous pairs of chromosomes and crossing-over events) and form the haploid secondary spermatocytes. Following, secondary spermatocytes undergo meiosis II (separation of sister chromatids) and form the round spermatids. At last, spermatids must face a complex cytodifferentiation to form the functional spermatozoa (spermiogenesis). (Adapted from [12]) .

1.2. Male infertility: a growing concern

The decline in male fertility is an emerging problem in modern societies. It is recognized that in our society there is an increasing trend for couples to become parents at a later age. Additionally, unhealthy lifestyles and environmental factors are significant contributors to the decrease in fertility rates. The combination of these factors not only affect the fertility rates but also contribute to a higher incidence of congenital birth defects [13] and fetal birth [14].

Infertility can be defined as the inability to conceive after at least 12 months of regular and unprotected sexual activity [15]. This health issue affects approximately 15% of couples worldwide and is thought that the male factor is associated with around half of these cases [16]. A study conducted between 1990 and 2017 reported an increase in the global disease burden of infertility during this period and demonstrated that, on a

global scale, there is an increase in the age-standardized infertility prevalence rate of 0.291% in men, *per* year [17].

Multiple causes affect male reproductive potential, however, about 30-50% of cases of male infertility have unexplained causes and are classified as idiopathic [18]. Several sperm alterations present in idiopathic infertility have commonly been associated with the presence of high levels of reactive oxygen species (ROS) [19]. MacLeod [20], was the first to report a relation between high levels of ROS and male infertility, and since then there has been a growing concern regarding the role of oxidative stress (OS) in the etiology of male infertility. OS is a condition characterized by the imbalance between antioxidant defences and ROS production and this state is extremely prejudicial to cellular systems, affecting essential metabolic processes [21]. There is evidence that ROS-mediated damage contributes to about 30-80% of male infertility cases [22]. In fact, human spermatozoa is a cell especially vulnerable to ROS attack, mainly due to the abundance of polyunsaturated fatty acids (PUFAs) in their plasma membrane [23]. In addition, sperm cells are ineffective in repairing ROS-induced damage because they lack the necessary cytoplasmic-enzymes repair systems [24].

1.3. Free radicals and reactive oxygen species

Free radicals are a group of extremely reactive molecules with unpaired electrons that can modify biomolecules through oxidation, in an attempt to become in a stable state [25]. Their structure makes them react almost instantly with substances nearby. The terms free radical and ROS are often used interchangeably, however not all ROS are free radicals. ROS is a collective term that includes oxygen radicals, such as hydroxyl radical (•OH), superoxide ion ($O_2^{\bullet-}$), hydroperoxyl radical ($HO_2^{\bullet}$), lipid peroxyl (LOO•), and nonradical oxygen derivatives such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3) [25-27]. Radicals are less stable and more reactive than non-radical derivatives. Notwithstanding, the last ones can be converted into free radicals by diverse reactions in living organisms.

In cellular systems, when the level of ROS exceeds the antioxidant capacity, the cell turns into a state of OS. The overproduction of ROS can cause cellular damage throughout the oxidation of amino acids in proteins, lipids in membranes, and carbohydrates in nucleic acids [28]. However, ROS are known for playing a dual role in biological systems, having detrimental and beneficial effects [29]. Some of the mentioned ROS play an important role in spermatozoa functions, including •OH, $O_2^{\bullet-}$ and H_2O_2 [30]. In particular, H_2O_2 is considered a major ROS due to its important role

as a cellular signalling intermediate [31]. However, high concentrations of H_2O_2 have proven to be prejudicial to spermatozoa functions [32]. Despite the lower reactivity of this molecule, H_2O_2 can easily penetrate spermatozoa membranes because it is electronically not charged. Moreover, H_2O_2 and $O_2{\bullet}^-$ can undergo a series of chemical reactions, known as "Fenton/Haber-Weiss chemistry", to generate the •OH. In biological systems, ferric ion (Fe^{3+}) in the presence of $O_2{\bullet}^-$ can be reduced to ferrous ion (Fe^{2+}) (Haber-Weiss reaction). In turn, Fe^{2+} can react with H_2O_2 and form a highly reactive •OH (Fenton reaction) [33] (Figure 2).

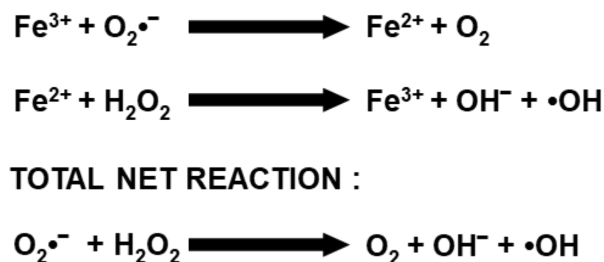

Figure 2: Fenton and Haber-Weiss reactions. Free iron (Fe^{2+}) can react with hydrogen peroxide (H_2O_2), leading to the generation of the highly reactive hydroxyl radical (•OH), through the Fenton reaction. Superoxide ($O_2{\bullet}^-$) can also react with ferric ion (Fe^{3+}) in the Haber-Weiss reaction leading to the production of Fe^{2+}.

1.4. Sources of ROS in human semen

Multiple sources contribute to the production of ROS in the human semen, including intra- and extracellular sources within the semen, and external sources (such as lifestyle habits). The spermatozoa itself is considered a source of ROS, mainly due to their metabolic activity. They can generate ROS through two pathways: β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) system, at the plasma membrane and/or NADH-dependent oxidoreductase (diaphorase), at mitochondria [34, 35]. The latter mechanism is often pointed as a major source of ROS. Mitochondria generate ATP through the oxidative phosphorylation (OXPHOS) process, and NADH diaphorase is a key participant in this process. During mitochondrial respiration occurs the leakage of electrons, which further react with oxygen molecules, forming $O_2{\bullet}^-$ [36]. Later, this radical dismutate, spontaneously or by the enzymatic activity of superoxide dismutase (SOD), to produce H_2O_2. In fact, $O_2{\bullet}^-$ is the predominant ROS produced by human spermatozoa. In the course of spermatogenesis, there are spermatozoa unable to complete maturation, resulting in immature sperm cells. The incomplete maturation results in a retention of excess cytoplasmic residues in the mid-piece. Cytoplasmic

residues contain high levels of glucose-6-phosphate dehydrogenase (G-6-PD), an enzyme that controls the production of NADPH, through the hexose monophosphate shunt. It means that higher levels of G6PD yield greater NADPH production. In turn, intracellular NADPH is processed *via* NOX (enzyme located in the plasma membrane) and acts as a substrate for ROS production [37]. In addition, it was recently described that human spermatozoa present a calcium-dependent NADPH-oxidase isoform 5 (NOX5) [38]. It was found that NOX5 protein expression was higher in asthenozoospermic men compared to normozoospermic men. Along with this finding, it was reported a negative correlation between NOX5 protein expression and sperm motility, and a positive correlation with intracellular production of $O_2^{\bullet-}$ and H_2O_2, in human spermatozoa [39].

Regarding extracellular sources within semen, leukocytes are the major source of ROS, producing about 1000 times more ROS than spermatozoa [40]. Leukocytes are naturally found in a normal ejaculation, however, when they are present in concentrations above $1x10^6$ leukocytes/mL, it is defined as leukocytospermia [15]. The high number of leukocytes in the seminal fluid is often associated with the presence of inflammation or infection in the reproductive tract [41]. However, it can also have other causes, such as varicocele and smoking. Similar to spermatozoa, leukocytes produce $O_2^{\bullet-}$ through NADPH oxidation (*via* NOX) [42]. However, leukocytes have the particularity to release large amounts of $O_2^{\bullet-}$ as a mechanism to kill pathogens, making these cells the prevailing producer of seminal ROS [30]. Several studies have reported the negative effects of leukocytospermia on sperm function [43, 44]. Besides, the presence of a higher number of leukocytes in the seminal fluid is also related to *in vitro* fertilization failures [45].

Another contributor to ROS production is the pathological condition of varicocele. Currently, varicocele is one of the major causes of male infertility with an incidence of 15% in the male population and 40% among infertile men [46]. Varicocele is defined as an abnormal dilatation of the pampiniform plexus veins of the spermatic cord [47]. The pathophysiology of this condition is strictly related to OS, mainly due to the increase in testicular temperature and intratesticular pressure combined with testicular hypoxia [48]. According to previous studies, patients with varicocele show high levels of ROS, increased DNA fragmentation, decreased seminal antioxidant defence and reduced sperm quality [49, 50].

Lifestyle habits, as well as the surrounding environment, are external key factors toward human semen quality. Diet and nutrition have been frequently associated with

the maintenance of reproductive functions and semen quality [51, 52]. However, the previous data point that a hypercaloric diet, excessive intake of saturated fats and trans-fatty acids and a high glycaemic index are factors closely associated with increased OS, which is one of the underlying causes of several disorders, such as obesity and type 2 diabetes [53, 54]. Both increased OS and metabolic disorders affect sperm functions and increase the risk of male infertility. Smoking is also a habit that is known to increase OS, not only by inducing excessive production of ROS but also by impairing the antioxidant defences. Cigarettes contain several toxic substances that contribute to free radical generation, such as nicotine and other alkaloids, and nitrosamines [55]. A study conducted on infertile patients revealed that smoking patients had higher levels of seminal OS than non-smokers [56]. Alcoholism is another element linked with ROS production and reduced antioxidant capacity. Ethanol metabolism generates acetaldehyde as a by-product, which produces ROS by interacting with proteins and lipids [57]. A clinical study from Muthusami *et al.* on 66 alcoholic men, evidence that chronic alcohol consumption has a negative effect on male reproductive hormones and semen quality [58]. Exposure to pollution, radiation and toxins have been also related to OS exacerbating conditions [37]. Pant *et al.* found a negative correlation between phthalate levels (a chemical often used in plastics) and sperm quality, and conversely a positive association with high levels of ROS [59]. Radiation emitted by mobile phones has been proved to be associated with increased OS in human semen, affecting sperm motility and vitality [60].

1.5. Physiological role of ROS in semen

At physiological levels, ROS plays a vital role in intracellular signalling cascades, mediating important sperm functions. Once in the female reproductive tract, spermatozoa have to undergo several biochemical and morphological changes to acquire the ability to bind the zona pellucida (ZP) and fertilize the oocyte, which is called sperm capacitation [61]. The capacitation comprises changes in the plasma membrane, such as the removal of cholesterol and modifications of glycoproteins present on the surface of the membrane [62]. These events are followed by hyperactivation, which is characterized by changes in the motility and amplitude of the flagellar movement [63]. After hyperactivation, the sperm penetrates the cumulus oophorus and achieves the ZP. This connection results in an acrosome reaction that consists on the release of proteolytic enzymes that will cause a pore in the extracellular matrix of the ZP [26]. The initial molecular mechanisms behind capacitation and hyperactivation involve the influx of Ca^{2+} and HCO^{3-}, and cytosol alkalization. The

influx of Ca^{2+} along with ROS (particularly $O_2^{\bullet -}$), stimulates the activation of adenylate cyclase (AC), which increases the production of cyclic adenosine monophosphate (cAMP) and activates protein kinase A (PKA). In turn, PKA triggers NADPH oxidase inducing ROS generation. Further, PKA also participates in the activation of protein tyrosine kinase (PTK), which is responsible for the phosphorylation of tyrosine [64] (Figure 3). In addition, H_2O_2 also plays an important role in PTK activity, inhibiting phosphotyrosine phosphatase (PTPase) activity, which dephosphorylates tyrosine residues [65]. Thus, ROS are involved in the increased tyrosine phosphorylation in the fibrous sheath around the flagellum axoneme, an essential event in sperm capacitation to acquire the potential to undergo acrosome reaction [66]. A study conducted by Aitken *et al.* demonstrated that *in vitro* inhibition of ROS resulted in a decrease in phosphorylation of tyrosine residues, supporting the importance of ROS in the aforementioned mechanisms [67]. The molecular events of the acrosome reaction present similarities to those observed on capacitation, (such as phosphorylation of tyrosine proteins, an influx of Ca^{2+}, and cAMP and PKA activity) hence ROS are also implicated in this process [68]. During the acrosome reaction, the Ca^{2+} generated on capacitation promotes the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP2). The by-products of this cleavage are involved in the fusion of the acrosomal and plasma membranes and in the activation of protein kinase C (PKC) [64]. These last events prompt the influx of Ca^{2+} and the activation of phospholipase A2 (PLA2), an enzyme that catalyses the release of fatty acids. It was shown that H_2O_2 also contributes to PLA2 activation [69] (Figure 3). Griveau *et al.* demonstrated that ROS appears to play an important role in acrosome reaction through the possible de-esterification of the membrane phospholipids, thus increasing membrane fluidity [70]. Further, *in vivo* acrosome reaction is initiated with sperm-ZP binding, and ROS have been shown to increase spermatozoa affinity to ZP, probably by the phosphorylation of plasma membrane proteins [68]. After crossing ZP, spermatozoa must fuse with the oocyte and this event is highly dependent on the membrane fluidity [71]. The mechanism whereby ROS enhances membrane fluidity occurs during the biochemical cascade of capacitation and acrosome reaction. As mentioned above, PLA2 cleaves the secondary fatty acid in the triglycerol structure of the membrane phospholipid, thus increasing plasma membrane fluidity and enhancing sperm-oocyte fusion [68].

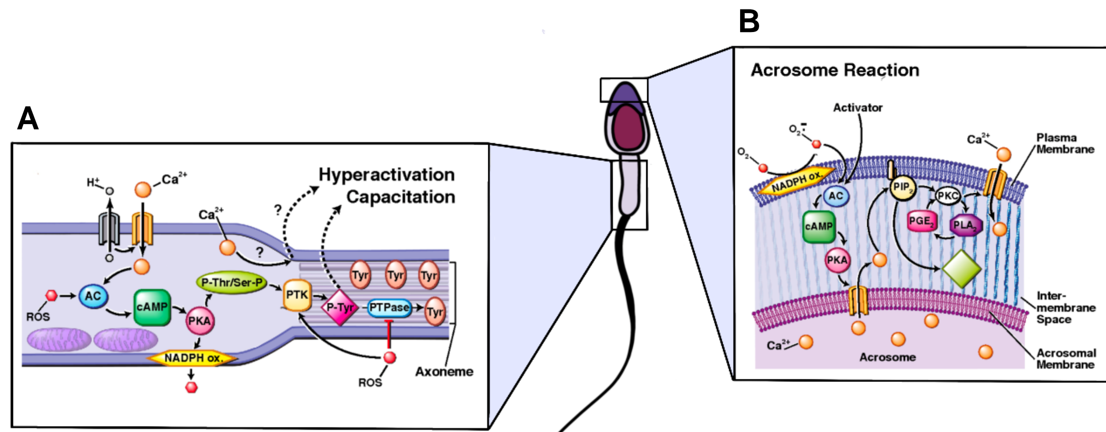

Figure 3: Schematic illustration of the involvement of ROS in the biochemical pathways of sperm capacitation and hyperactivation (A) and acrosome reaction (B). (A) In sperm capacitation and hyperactivation, reactive oxygen species (ROS) (particularly O2•⁻) along with Ca^{2+}, can activate adenylate cyclase (AC) and produce cyclic adenosine monophosphate (cAMP). In turn, cAMP activates protein kinase A (PKA), triggering a higher production of ROS by NADPH oxidase (NOX). PKA also triggers phosphorylation of serine and tyrosine residues that, along with other pathways, lead to the activation of protein tyrosine kinase (PTK). PTK phosphorylates tyrosine residues of the fibrous sheath surrounding the axoneme, which is an important event in sperm capacitation. (B) Acrosome reaction can be induced by ROS. The release of Ca^{2+} from the acrosomal calcium store may induce the cleavage of phosphatidylinosital-4,5-bisphosphate (PIP2), promoting the activation of actin-severing proteins. This phenom leads to the fusion of the acrosomal and plasma membranes. The cleavage of PIP2 also triggers PKC activation, resulting in a second influx of Ca^{2+} and activation of phospholipase A2 (PLA2). PLA2 leads to increased fluidity of the sperm plasma membrane. (Adapted from [64]).

1.6. Pathological role of ROS in semen

As mentioned above, certain levels of ROS are required for male reproductive functions, however, high levels of ROS may lead to cellular OS and trigger pathological conditions. Further, the intricate architecture of spermatozoa including the composition of sperm membranes and the absence of cytoplasm, make spermatozoa particularly vulnerable to OS.

1.6.1. Lipid peroxidation

Lipid peroxidation (LPO) is an oxidative self-propagating chemical reaction, characterized by the attack of ROS to lipids, essentially those that have double bonds, such as polyunsaturated fatty acids (PUFAs). Sperm cells are especially sensitive to damage caused by ROS, essentially due to the presence of high concentrations of PUFAs in their membranes [72]. In these fatty acids, the presence of a double bond next to a methylene group makes the methylene C-H bond weaker, and as a consequence hydrogen is more vulnerable to abstraction [73].

The LPO process can be divided into three phases: initiation, propagation and termination. The presence of the •OH, as well as the HO_2•, enhances the initiation phase [74]. In biological systems, those ROS can be generated through the Fenton/Haber-Weiss pathway [33]. During the initiation phase, both the •OH and HO_2• are capable to remove hydrogen from the membrane creating a carbon-centred lipid radical (L•). The propagation phase is characterized by the reaction of the lipid radical with molecular oxygen to form lipid peroxyl radical (LOO•), which in turn can abstract another hydrogen from the membrane and generate a lipid hydroperoxide (LOOH) and a new L•, that can continue the chain reaction [75, 76]. In the termination phase, two radicals can react with each other, thus eliminating the radical to form a stable product. Alternatively, the presence of antioxidants, such as vitamin E, can donate a hydrogen atom to a LOO• and generate LOOH and the relatively inert vitamin E radical [77] (Figure 4).

As a result of lipid peroxidation, a wide variety of oxidation products are generated. Lipid hydroperoxides are the main primary products of lipid peroxidation. Aldehydes are also generated as secondary products of this process, mainly malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [77]. MDA is a mutagenic by-product and is widely used as a biomarker for lipid peroxidation due to its reaction with thiobarbituric acid (TBA) [78]. In contrast, 4-HNE is a highly toxic by-product and acts as a signalling molecule [79]. Its toxicity can be explained by its high reactivity towards thiols and amino groups [80].

Several pathological effects of LPO are associated with the loss of sperm function. LPO affects membrane fluidity, increases non-specific permeability to ions, and inactivate membrane-bound receptors and enzymes [23]. Further, several studies have shown that LPO negatively impacts sperm concentration, morphology and especially have a detrimental effect on sperm motility [81, 82]. A previous study found higher MDA levels in infertile patients, in comparison with normozoospermic men. Also, the rates of seminal MDA were negatively associated with sperm motility and sperm concentration [83]. On the other hand, it has been shown that 4-HNE can bind to mitochondrial proteins in human spermatozoa and promote electron leakage. The OS resulting from the latter event is associated with loss of mitochondrial membrane potential and oxidative DNA adducts formation [84].

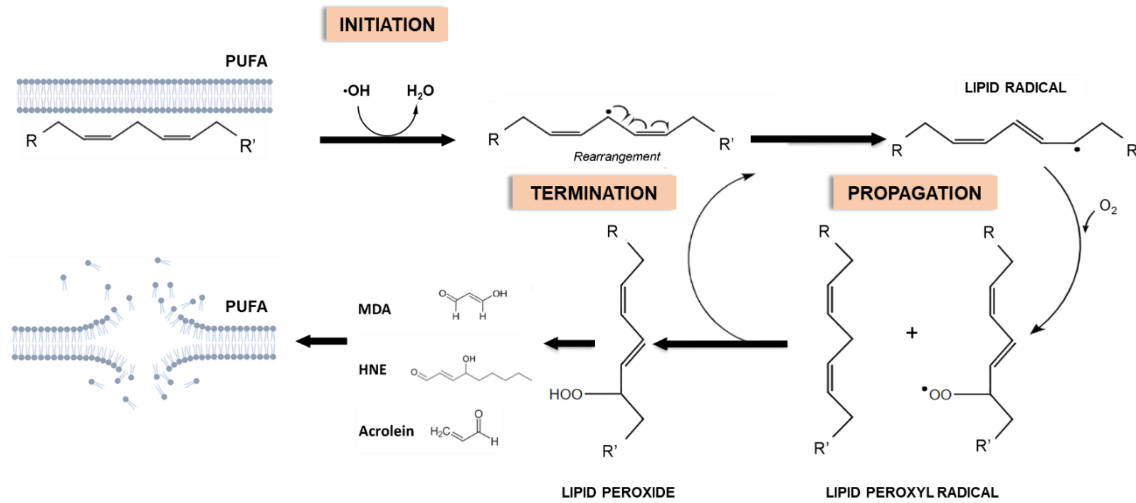

Figure 4: Schematic representation of ROS-mediated lipid peroxidation chain reaction. The lipid peroxidation process involves sequential phases of initiation, propagation, and termination. In the initiation phase, prooxidants abstract a hydrogen from the membrane forming the carbon-centred lipid radical (L•). In the propagation phase, lipid radical can quickly react with oxygen and generate a lipid peroxyl radical (LOO•), which then can abstract a hydrogen from another lipid molecule generating a new lipid radical and a lipid hydroperoxide (LOOH). In the termination phase, antioxidants can donate a hydrogen atom to the lipid peroxyl radical species resulting in the formation of nonradical products. These products may also undergo fragmentation to produce reactive by-products, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), which can generate irreversibly covalent adducts with proteins, DNA, and phospholipids, leading to cell damage.

1.6.2. DNA damage

OS is considered one of the major causes of DNA damage in human sperm. Normally, sperm DNA is tightly packaged by protamines (protecting the DNA from free radicals assault), although infertile patients sometimes present deficient protamination, which increases spermatozoa exposure to DNA damage [85]. Several studies reported that free radicals attack the integrity of DNA resulting in a wide variety of anomalies, including single-stranded and double-stranded DNA breaks, base modifications, DNA cross-links among others [86, 87].

DNA repair in spermatozoa is inefficient and only occurs during specific stages of spermiogenesis [88]. A mature sperm cell holds the first enzyme in the base excision repair (BER) pathway, the DNA 8-oxoguanine glycosylase (OGG1), but are devoid of other enzymes downstream of the BER pathway (such as apurinic/apyrimidinic endonuclease 1). Due to this incomplete DNA repair mechanism, the excess of ROS may result in the creation of vulnerable abasic sites, leading to DNA strand fragments [89]. A common by-product of DNA oxidation is 8-hydroxy-2-deoxyguanosine (8-

OHdG), which results from the damage generated by •OH attack. Evidence suggests that the last event not only destabilize the DNA structure but may also eventually result in the formation of DNA strand breaks [90]. De Iuliis and colleagues found a powerful correlation between increased 8-OHdG concentration and DNA fragmentation in human spermatozoa, highlighting the relevance of OS in the induction of sperm DNA damage [91]. Furthermore, DNA damage may also occur as a consequence of lipid peroxidation, since the by-products of that event are highly mutagenic and genotoxic to DNA. Actually, in a previous study, DNA fragmentation was positively correlated with MDA levels and positively associated with abnormal sperm morphology [92]. Nevertheless, at fecundation, there is the possibility of repairing sperm DNA damage through repair mechanisms of the oocyte or early embryo. Particularly, the abasic sites remaining from the incomplete repair of single-strand breaks can easily be repaired by the oocyte through the BER pathway [93].

Increased DNA damage is also an emerging problem during assisted reproductive technology (ART) procedures. In the ART context, spermatozoa experience *in vitro* handling, being more exposed to OS conditions and consequently carrying a greater risk of DNA damage. A meta-analysis demonstrated that high levels of sperm DNA fragmentation have a prejudicial impact on the outcome of *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI), resulting in decreased pregnancy rates and increased miscarriage rates [94].

1.6.3. Apoptosis

Apoptosis, also known as programmed cell death, is a controlled mechanism that allows cells to self-destruct. This process seems to be regulated by extrinsic and intrinsic factors and can be triggered by a broad range of stimuli. Apoptosis of germ cells is crucial to retain the optimal germ cell to Sertoli cell ratio and to remove abnormal germ cells. However, sperm apoptosis remains a controversial and poorly understood subject [95]. It is not clear if ejaculated spermatozoa retain the ability to activate the apoptotic signalling cascade or if the apoptotic markers detected in ejaculated spermatozoa are a result of an abortive apoptotic process started before ejaculation [96, 97]. An *in vitro* study conducted by Lachaud and colleagues showed that ejaculated spermatozoa lack the capacity of initiating the apoptotic pathway of cell death, and their death occurs mostly by necrosis [96, 98]. Notwithstanding also has been reported that mature sperm cells express different markers of apoptosis, such as

caspase activation, externalization of phosphatidylserine, alteration in mitochondrial membrane potential (MMP) and DNA fragmentation [99-101].

A few studies have investigated the involvement of ROS in the apoptosis pathways of spermatozoa. High levels of ROS can disrupt mitochondrial membranes, thus resulting in the release of cytochrome C, which activates caspases 3 and 9, thus leading to apoptosis [95, 102]. Caspases are a family of cysteine proteases that play a key role in regulating apoptosis. Agarwal *et al.* found a positive correlation between levels of seminal ROS and the levels of cytochrome C ($r=.43$; $P<.03$), caspase 9 ($r=.56$; $P<.001$), and caspase 3 ($r=.65$; $P<.01$), suggesting that ROS-mediated damages may lead to sperm apoptosis in patients with male factor infertility [103]. Interestingly, other authors demonstrated that H_2O_2 stimulate annexin V-binding to phosphatidylserine on the outer leaflet of the membrane, and DNA fragmentation, in human semen of infertile patients. Additionally, H_2O_2 shown to increase caspase-3 enzyme activity in those cells [104].

Apoptosis in sperm may also be initiated by ROS-independent pathways involving the cell surface protein Fas. This protein is known to mediate apoptosis through the binding to Fas-ligand or anti-Fas antibody [95]. Although, there is evidence that some of the Fas-labelled cells escape apoptosis through abortive apoptosis. Patients with abnormal semen parameters, exhibit a large percentage (about 50%) of Fas-positive spermatozoa, suggesting a failure in the process of apoptosis. It has been hypothesized that the presence of ejaculated spermatozoa showing apoptotic markers indicates that, in some subfertile men, an “abortive apoptosis” may occur [105].

1.6.4. Protein oxidation

Sperm proteins are targets of redox reactions that often result in the activation or inactivation of certain signalling pathways. Depending on the type and level of ROS produced, different protein modifications can be important for physiological processes (“redox signalling”) or might be prejudicial contributing to the inactivation of protein functions [106]. Some of the major oxidative modifications include thiol oxidation, tyrosine nitration, and the formation of carbonyl groups [107]. Oxidative assaults on proteins usually result in the oxidation of amino acid side chains, oxidation of the protein backbone, fragmentation of the peptide chain, altered electric charge and increased susceptibility or extreme tolerance to proteolysis [108].

Several amino acids can be directly modified *via* side-chain reactions with ROS. The most vulnerable to ROS attack are those with aromatic side chain groups and those containing sulfhydryl groups (i.e., cysteine and methionine) [27]. Particularly, cysteine has a thiol (-SH) group, which is highly reactive and easily oxidized. An *in vitro* study demonstrated that the presence of peroxynitrite (highly reactive nitrogen species) increases thiol oxidation in human spermatozoa, resulting in a reduction of sperm motility [109]. In accordance with these findings, other investigations have found an association between the oxidation of thiol groups and impairment of sperm function [110, 111]. Piomboni *et al.* detected higher levels of oxidized thiol groups in azoospermic and leukocytospermic samples when compared with normozoospermic samples [110]. Also, a previous study found high levels of thiol oxidation in peroxiredoxins (PRDXs) (antioxidant enzymes), in sperm samples from infertile patients. Additionally, the thiol oxidation of PRDXs was positively correlated with sperm DNA damage and lipid peroxidation, and negatively correlated with sperm motility [111]. It is important to note that PRDXs are antioxidant enzymes that contain cysteine at their active site, which make them potential targets for ROS.

Tyrosine nitration is also a protein modification involving reactive nitrogen species and is characterized by the addition of a NO_2 group to the *ortho* position of the aromatic ring of tyrosine [112]. The increase of OS can promote the reaction between $O_2{\bullet}^-$ and nitric oxide (NO), forming peroxynitrite ($ONOO^-$). Under physiological conditions, the $ONOO^-$ can react with carbon dioxide (CO_2) and be later decomposed into $CO_3{\bullet}^-$ and NO_2. The nitration of tyrosine, *via* attack of NO_2, forms the irreversible modification 3-nitrotyrosine (NT) [113]. A study conducted by Salvolini and co-workers found higher content of nitrotyrosine in asthenozoospermic samples, raising the hypothesis that an excess of tyrosine nitration may be involved in the pathogenesis of idiopathic asthenozoospermia that causes male infertility [114].

Protein carbonylation is another common and irreversible chemical modification prompted by ROS. Carbonyl groups can be generated by the attack of ROS in amino acid side chains of proline, arginine, lysine, and threonine, promoting the generation of reactive ketones or aldehydes. The last event is known as primary protein carbonylation [115]. The secondary protein carbonylation event includes the reaction of nucleophilic side chains of lysine, histidine, and cysteine residues with aldehydes produced during lipid peroxidation (such as 4-HNE and MDA) or with reactive carbonyl derivatives generated as a result of the reaction of reducing sugars (ketoamines, ketoaldehydes) [116]. The determination of protein carbonyl groups can be performed using the chemical probe 2,4-dinitrophenylhydrazine (DNPH). The reaction of this

probe with a protein carbonyl leads to the formation of a stable 2,4-dinitrophenol (DNP) product, which can later be detected and quantified [115]. A previous study investigated the effect of protein carbonyl concentration on sperm motility and in the results of ICSI in the laboratory. The results demonstrated that the high content of protein carbonyls in semen negatively affects sperm motility and ICSI results [117].

1.6.5. Impaired sperm motility

Spermatozoa motility is an important predictor of sperm quality and fertilization success. High levels of ROS have been frequently correlated with decreased sperm motility [118, 119]. However, the mechanism by which ROS affect sperm motility is not fully understood. Griveau *et al.* hypothesized that H_2O_2 can cross the membranes and inhibit enzymes such as G6PD, leading to reduced availability of NADPH and a concomitant accumulation of antioxidant enzymes. The latter events may affect the levels of antioxidant defences and lead to the peroxidation of membrane phospholipids [120]. Another hypothesis is that H_2O_2 can trigger a cascade of chemical reactions that result in a decrease in phosphorylation of axonemal proteins and reduced sperm motility. These events are associated with reduced membrane fluidity and sperm-oocyte fusion [120, 121]. As mentioned above, motility loss often occurs as a consequence of lipid peroxidation. An *in vitro* study demonstrated that the addition of lipid aldehydes, such as 4-HNE and acrolein, resulted in a significant motility loss, in human spermatozoa [84].

1.7. Antioxidants in semen

Antioxidants are compounds with the ability to combat OS, especially through their scavenging properties. These types of compounds are able to donate an electron to neutralize free radicals, thereby reducing its potential to cause cell damage [122]. Moreover, antioxidants can also act as metal chelators to prevent the formation of ROS. In order to counterbalance the possibility of cellular ROS-mediated damage, humans have developed an organized and complex antioxidant defence system. This system involves endogenous and exogenous components that act synergistically to counteract the effect of free radicals [123]. In human semen, the protection against oxidative damage is afforded by enzymatic and nonenzymatic antioxidants. These antioxidants can have a physiological source or can be obtained through the diet [124]. However, spermatozoa have a minimal amount of antioxidants due to the lack of

cytoplasm, which is why most of the antioxidant capacity is contained in seminal plasma [125].

1.7.1. Enzymatic factors

The antioxidant enzymatic system in human semen is constituted by an enzyme triad, which includes SOD, catalase (CAT), and glutathione peroxidase (GPx). SODs are metal-containing enzymes that catalyse the dismutation of $O_2•^-$ into molecular oxygen and H_2O_2 [126] (Figure 5). Three SOD isoforms can be distinguished in aerobic cells, and they exist in both extra- and intracellular forms. The intracellular isoforms of SOD differ in their composition, active centres, and location in the cell. In cytoplasm is found an isoform that contains copper and zinc in the active centre, known as Cu-ZnSOD or SOD-1, while in mitochondria is found a manganese-containing enzyme, called SOD-2 [127, 128]. The SOD-3 (also known as EC-SOD) is the extracellular form of SOD and is associated with surface polysaccharides. The seminal plasma contemplates a high activity of the SOD isoforms, with 75% of the activity related to SOD-1 and with 25% related to SOD-3 [129]. The SOD activity in seminal plasma was positively correlated with sperm concentration and total motility [130].

CAT, an enzyme found in peroxisomes, converts H_2O_2 into water and molecular oxygen [131] (Figure 5). The structure of this enzyme is composed of a heme group with a centrally located iron atom [132]. An earlier investigation reported a significant decrease in catalase activity in asthenozoospermic patients compared to normozoospermic men. In addition, the levels of catalase were positively correlated with sperm motility and morphology [133].

GPx is an enzyme that also acts on the degradation of H_2O_2 using reduced glutathione (GSH). The by-products of this reaction are water and the oxidized form of GSH (GSSG) [127] (Figure 5). Furthermore, GPxs can metabolize organic peroxidized molecules [134]. The GPx family includes multiple isoenzymes that are distributed in different tissues and diverge between species [135]. Most forms of GPx are selenium-dependent, and the selenoprotein phospholipid hydroperoxide GPx (PHGPx), also known as GPx4, is one of the most important forms of GPx in spermatozoa [136]. GPx4 is predominant in testis and constitutes about 50% of the midpiece protein content, localized in the mitochondrial helix [137]. A previous study demonstrated a strong positive correlation between PHGPx deficiency and male infertility, suggesting that insufficient expression of mitochondrial PHGPx can be a possible cause of

oligoasthenozoospermia in infertile men [138]. Also, it was shown that GPx plays an essential role in the integrity and function of spermatozoa membranes and reduces LPO [139].

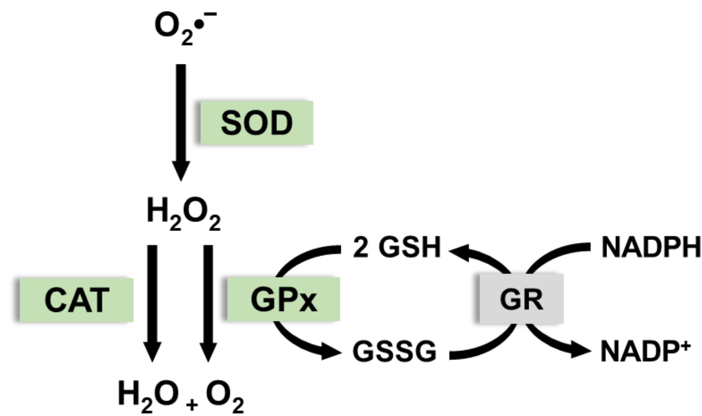

Figure 5: The synchronized function of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzyme activities. SOD catalyses the dismutation of superoxide radical ($O2•^{-}$) to hydrogen peroxide (H_2O_2). Catalase converts the H_2O_2 into water and molecular oxygen. GPx also neutralizes H_2O_2 by taking hydrogens from two glutathione (GSH) molecules resulting in H_2O and an oxidized form of glutathione (GSSG). Glutathione reductase (GR) then regenerates GSH from GSSG through NADPH oxidation.

1.7.2. Non-enzymatic factors

Non-enzymatic antioxidants can be produced endogenously or obtained through the consumption of foods or dietary supplements. The non-enzymatic antioxidants usually found in human semen include vitamins (A, C and E), carnitines, coenzymes Q10, among others.

Vitamin C (VC), also known as L-ascorbic acid, is a water-soluble substance with low-molecular-weight. This micronutrient is a reducing agent and acts as a scavenger of ROS, being an electron donor that reacts with substrates like H_2O_2, generating ascorbyl radicals and further dehydroascorbic acid (DHA). DHA in turn can be converted into the reduced ascorbic acid form by a chemical reaction involving GSH [140]. L-ascorbic acid is also efficient in recycling the oxidized form of vitamin E by reducing tocopheroxyl radicals, contributing to the maintenance of the α-tocopherol redox state within biological membranes [141]. The human body is not able to synthesize L-ascorbic acid, being dependent on the intake of foods with VC to obtain this micronutrient. The recommended daily intake is 65 to 90 mg/day [142]. The VC circulating levels found in the human serum are relatively low, while in the seminal fluid the levels are 10 times higher, with values ranging from 200-700 μM [143-147]. VC is commonly prescribed in oral antioxidant therapies for infertile men, usually in

combination with other compounds, such as vitamin E, coenzyme Q10 and L-carnitine [148, 149]. Several clinical trials reported that oral supplementation of vitamin C decrease sperm DNA fragmentation and improve sperm-quality parameters [150-153]. Additionally, *in vitro* studies also report that the supplementation of sperm medium with VC was able to decrease the negative effects of vitrification on sperm parameters and chromatin quality [154], and improve DNA integrity [155, 156]. This essential micronutrient plays an important role in physiological processes such as in the metabolic processes of tyrosine, tryptophan and folic acid and also in preventing lipid peroxidation [157, 158]. Vitamin E (α-tocopherol) is another vitamin found in human semen and is one of the major lipophilic chain-breaking antioxidants [159]. Vitamin E is located primarily within the phospholipid bilayer of biological membranes, due to the presence of a hydrophobic side chain acting as a protector against lipid peroxidation of PUFAs [160]. An *in vivo* study demonstrated that vitamin E and selenium oral supplementation produced a significant decrease in MDA concentrations and result in an improvement of sperm motility [161].

Carnitines (L-carnitine (LC) and L-acetyl carnitine (LAC)) are synthesized by the organism and are involved in the mitochondrial β-oxidation process, providing energy to fuel spermatozoa. They are essential to sperm metabolism and are highly concentrated in the epididymis [162]. Carnitines are also known as water-soluble antioxidants as they have a protective role against ROS [162]. A trial study demonstrated that administration of both LC and LAC improves sperm motility and total oxyradical scavenging capacity of the seminal fluid, in patients affected by idiopathic asthenozoospermia [163]. Furthermore, a meta-analysis regarding the use of carnitines as oral antioxidant therapy concluded that carnitines may be efficient in improving pregnancy rates and sperm functions in male patients affected by infertility problems [164].

Coenzyme Q10 (CoQ10) is a naturally occurring nutrient in the body and is a crucial component of the mitochondrial respiratory chain [165]. In sperm cells, the majority of CoQ10 is concentrated in the midpiece [166]. The reduced form (ubiquinol) represents approximately 90% of the total CoQ10 in the body and acts as a powerful antioxidant preventing lipid peroxidation [167, 168]. It has been demonstrated that supplementation with CoQ10 in infertile men with idiopathic oligoasthenoteratozoospermia can attenuate OS in seminal plasma and improve semen parameters [169].

Zinc and selenium are trace elements that act as cofactors for antioxidant enzymes, exerting antioxidant properties in seminal fluid. Zinc is a key element in DNA transcription and protein synthesis, and due to the importance of these factors in the

development of germ cells, this mineral plays a vital role in the maintenance of spermatogenesis and testicular development [170]. A clinical study showed that oral zinc supplementation improved sperm parameters in men with asthenozoospermia [171]. Selenium is also an essential micronutrient for testicular development and spermatogenesis. It has been shown that selenium deficiency is associated with epithelium atrophy of ST and morphological abnormalities of spermatozoa head and midpiece [172].

1.8. Flavonoids

Currently, there is a high demand for natural products, mainly by the pharmaceutical industries in order to produce new drugs. Their chemical diversity and their multiple bioactivities make them potent biomedical agents. Worldwide, about 50% of pharmaceutical products contain natural products and about 25% of prescription drugs are derived from natural bioactive compounds [173]. Plant-derived compounds are commonly referred to as phytochemicals and recently they have gained popularity due to their antioxidant properties. Among phytochemicals, flavonoids are one of the most important classes. Flavonoids belong to a large group of polyphenolic compounds; more specifically they belong to a class of plant secondary metabolites having a polyphenolic structure [174]. They are usually found in leaves, flowers, roots and fruits of plants. The basic structure of a flavonoid has three rings (C6–C3–C6), consisting of two benzene rings, (A and B), and a heterocyclic pyrene ring (C) [175]. Variations on the pattern of substitution of the C ring and the degree of oxidation define the different subclasses of flavonoids known as flavones, flavonols, flavanones, isoflavones, flavanols or catechins, and anthocyanins [176] (Figure 6). In plants, flavonoids play different roles such as pigmentation and aroma of flowers, protection against UV radiation and are often synthesized in response to microbial infections [177, 178]. Nonetheless, the properties of flavonoids differ according to their chemical structure. As phytochemicals, flavonoids are produced in plants by the phenylpropanoid pathway, therefore, their synthesis cannot occur in humans and animals [179]. Nevertheless, flavonoids are part of the human diet and present pharmacological properties that allow them to act on biological systems. Flavonoids hold a broad spectrum of therapeutic properties, such as antioxidant, cardioprotective, anti-diabetic, anti-ageing, anti-inflammatory, and anti-cancer properties [179, 180].

Flavonoid compounds have proven to be powerful antioxidants *in vitro*. However, i*n vivo*, their effects are minimized due to their low water-solubility, bioavailability, and

weak absorption [181]. Flavonoids can occur as aglycones, glycosides, and methylated derivatives, but predominantly they appear as glycosides. Several sugar moieties can bind to the different hydroxyl groups of the flavonoid aglycone, thus resulting in a wide variety of flavonoids (the flavonoid aglycones and their different glycosides). The flavonoid glycosides are mainly found as O-glycosides with the glycosidic linkage at the C-3 or C-7 position, although less often C-glycosides can also be found [179]. The nature of glycosylation is known to influence the absorption, metabolism, and bioavailability of flavonoids [182].

Among the sundry biological properties of all the flavonoids groups, the foremost reported property is their antioxidant capacity. The antioxidant activity of flavonoids is highly dependent on the arrangement of functional groups in the nuclear structure, being mostly influenced by the configuration, substitution and the number of hydroxyl groups [183]. In general, the antioxidant mechanisms of action of flavonoids include the inhibition of ROS formation; the direct scavenging of ROS and the activation of antioxidant defences [184]. Flavonoids can prevent ROS generation through the interaction and inhibition of enzyme functions, such as NOX and/or by chelating metal ions involved in free radical formation [179, 185]. On the other hand, flavonoids are also efficient in scavenging ROS due to the presence of functional hydroxyl groups which enable hydrogen atom transfer and electron transfer to neutralize powerful radicals [186]. In addition, flavonoids can also counteract ROS through the upregulation of antioxidant enzymes with radical scavenging capacity [187].

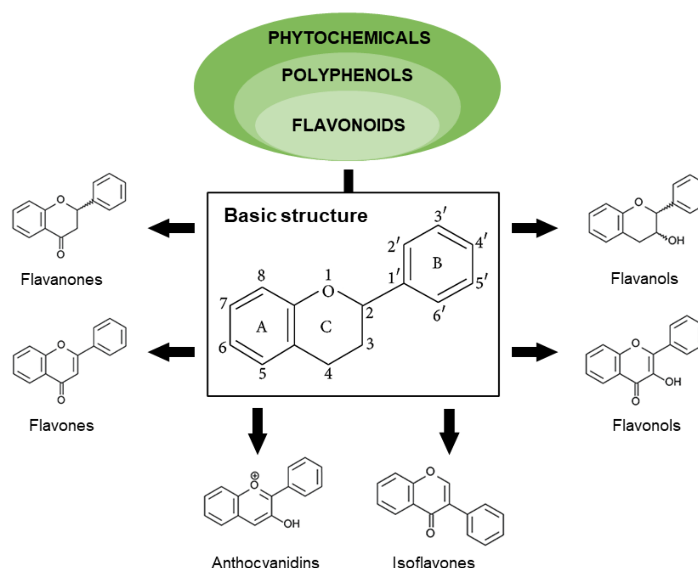

Figure 6: Basic skeleton structure of flavonoids and their subclasses.

1.8.1. Hyperoside

One of the most widespread flavonoids in nature is quercetin. Particularly, quercetin belongs to the flavonols subclass and is broadly distributed in plants, fruits, and vegetables. As mentioned above, flavonoids often exist in the form of conjugated sugar derivatives. More than 100 glycosides of quercetin are known, among which we will highlight the hyperoside (quercetin 3-*O*-β-D-galactoside) [188]. The synthesis of hyperoside occurs through the glycosylation of quercetin at the hydroxyl group in C3, with a galactoside group [189] (Figure 7). In plants, this process is catalysed by glycosyltransferases. Hyperoside is mainly found in plants of the genera *Hypericum* and *Crataegus*, including a diversity of vegetables and fruits [190, 191]. This phytochemical is commonly present at low concentrations and, therefore, its extraction and isolation have proven difficult [192]. Hyperoside can also be synthesized from the quercetin derivative-rutin, but the process is more costly. Whether natural or synthetic, the chemical structure of hyperoside has multiple isomers, hence the complexity in the isolation and extraction of this compound. The biochemical properties and the toxicity of these isomers can be very different, so it is important to find methods that allow hyperoside extraction with a higher degree of purity and efficiency [192].

Several pharmacological activities have been associated with hyperoside including anti-cancer, anti-inflammatory, anti-thrombotic and anti-depressant properties [193-196]. Although, in the past years, the focus of hyperoside research has been on its antioxidative effects. A previous study has demonstrated that hyperoside possesses cytoprotective properties against OS in lung fibroblast cells [197]. Also, it was reported

that hyperoside protects granulosa cells against H_2O_2-induced cell apoptosis and OS, revealing potential benefits on female reproductive capacity [198]. Marco Biagi *et al.* reported that the leaf extract of *Castanea sativa* Mill., particularly rich in hyperoside, showed scavenging properties against OS in human sperm and showed being capable to protect sperm membranes and acrosomes [199]. All these findings suggest that hyperoside may be used as a potential antioxidant in disorders associated with oxidative damage.

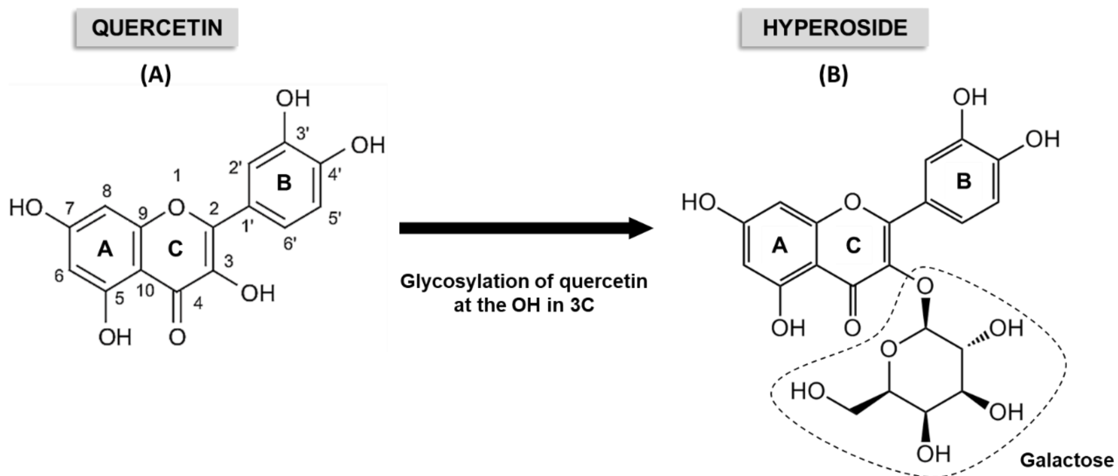

Figure 7: Chemical structures of quercetin and hyperoside (quercetin 3-*O*-β-D-galactoside). The synthesis of hyperoside occurs through the glycosylation of quercetin at the hydroxyl group in C3, with a galactoside group.

1.9. Assisted reproductive technology and oxidative stress

Currently, due to the increasing rates of infertility, assisted reproductive technology (ART) often becomes the only chance for couples affected by infertility to achieve a successful pregnancy. The birth of the first baby resulting from IVF occurred in 1978 [200] and since then, more than 8 million babies have been born from IVF worldwide [201]. However, despite the advances in technology, the success rates of ART procedures are still considered low. According to the European Society of Human Reproduction and Embryology (ESHRE) monitoring, the rate of successful pregnancy *per* embryo transfer is about 36% for both IVF and ICSI, and about 50% for egg donation, in < 35-year-old patients [201].

The successful outcome of ART is influenced by several factors, being the presence of ROS one of the most significant. It is recognized that an *in vitro* environment is unable to mimic *in vivo* conditions, so the risk of OS situations is most probable [202]. As mentioned above, ROS can be produced by the spermatozoa itself or by exogenous

sources. In the clinical setting, *in vitro* manipulation of male gametes enhances exposure to OS situations. The removal of seminal fluid is a common step in ART techniques. However, due to the content of antioxidants and enzymes with scavenging properties within the seminal fluid, its removal makes spermatozoa more vulnerable to ROS attack [203]. In addition, this step usually requires centrifugation, which is also related to ROS generation. Studies indicate that a long centrifugation time exposure is associated with a detrimental effect on sperm motility and viability [204]. Furthermore, sperm preparation for ART involves light exposure and variations in temperature, pH, and oxygen concentration [205]. During those procedures, spermatozoa are exposed to light, both from the microscope and from the environment, which in turn can trigger ROS production. There is evidence that stress caused by exposure to visible light could induce damage to cholesterol and unsaturated lipids contained in cell membranes [206]. The composition of culture media is another important factor, since the presence of metal ions, such as iron or copper, in the media may lead to increased ROS (through the Haber-Weiss and Fenton reactions) [207]. Additionally, it was also reported that variations in the incubator temperature, as well pH fluctuations in the culture media, negatively influence sperm quality [208].

In light of ART techniques, it is presumed that OS prompted by the *in vitro* environment, as well the composition of culture media surrounding gametes/embryos, during both IVF and ICSI, are the major causes of the high failure rate of ART [209]. IVF is a reproductive technique consisting of the interaction of male and female gametes in an *in vitro* culture media, leading to fertilization. This technique usually involves a long incubation of sperm with the oocyte and the respective cumulus cells, which together potentiate ROS generation in the media. In turn, ICSI is a technique based on injecting a single sperm into the oocyte cytoplasm (previously denuded of their cumulus cells) and has a very shorter incubation period [205]. A previous study has shown that prolonged exposure of oocytes to high concentrations of spermatozoa is prejudicial, reducing the quality of embryos and subsequent development, especially in cases of male factor infertility [210]. However, although ICSI has a shorter contact time between the sperm and oocyte, there is a risk of transporting a small quantity of medium containing ROS along with the spermatozoon into the oocyte, which can induce DNA damage [205].

Sperm cryopreservation is another important procedure used in ART that attends to different purposes, such as the preservation of male fertility before radiotherapy or chemotherapy [211]. However, the freezing-thawing processes are extremely associated with ROS-induced stress, being the overproduction of ROS one of the

major causes of sperm cryodamage [212, 213]. Several studies associated cryopreservation of human spermatozoa with motility loss, DNA damage and changes in plasma membrane integrity and structure [214-217].

In sum, exacerbated OS conditions have a severe impact on ART outcomes, resulting in lower fertilization, implantation, and pregnancy rates [218, 219]. As highlighted above, in the ART context, multiple sources of ROS contribute to reducing the quality of gametes and impairing embryo development, so it is essential to find alternatives to overcome this problem.

1.10. Antioxidant therapy in male infertility

Bearing in mind the many internal and external sources of ROS that contribute to the development of OS conditions in ART, it is essential to explore ways to minimize the negative effects of ROS on the success of ART outcomes. Over the past years, several strategies have been proposed to treat infertility problems associated with OS. Generally, in OS-induced infertility, the initial approach is focused on lifestyle changes, since smoking, excessive alcohol consumption and inadequate diet are intimately related to ROS overproduction. However, the advantages of the use of antioxidants as therapy have been highlighted in scientific research. The easy accessibility, low cost and low toxicity of antioxidants are some of the factors that support their use in male infertility treatment [126]. Antioxidant therapy in male infertility can be approached in two distinctive modes, either as oral supplementation of the subfertile couple before ART cycles or as *in vitro* supplementation of the sperm media during the ART protocols [205]. The existence of meta-analyses on the impact of antioxidant therapy on male infertility evidences a significant improvement in pregnancy and live birth rates in subfertile couples who resorted to ART [220, 221]. However, given the disparity of literature in this field, the effectiveness of antioxidant therapy to improve ART outcomes remains controversial and it is consensual that further investigations are required.

Considering the processing of male gametes in ART, several studies report the effects of *in vitro* supplementation of antioxidants in the sperm preparation medium, in order to counteract the detrimental ROS effects. These studies have clinical relevance because it is essential to optimize sperm motility before ART techniques such as intrauterine insemination and IVF. Fanaei and colleagues have shown that VC (600 µM) supplementation during teratozoospermic semen processing, protected spermatozoa against oxidative damage, improving motility, viability and DNA integrity [156]. The same author also investigated the effect of vitamin E supplementation (40 µM) in sperm

samples from normozoospermic patients. The results revealed that vitamin E was able to attenuate the harmful effects caused by OS, probably by the role that vitamin E plays in protecting PUFAs membrane and by preventing the propagation of the chain reaction of lipid peroxidation [222]. At the same line, Chi *et al.* investigated the supplementation of different concentrations of EDTA and catalase in a sperm-preparation medium. The results showed that the addition of EDTA (10 μM) improved sperm motility while combinations of both EDTA and catalase decreased the DNA fragmentation rate and reduced the levels of ROS in the sperm suspension [223]. Regarding studies of *in vitro* supplementation with flavonoids, it has been demonstrated that quercetin and rutin (quercetin-3-*O*-rutinoside) (used at 20 μM and 30 μM, respectively) were effective in protecting human sperm against induced-lipid peroxidation. The authors suggested that both flavonoids can be efficient ROS scavengers to be used in sperm culture media [224]. Besides, a recent study has demonstrated that *in vitro* supplementation of quercetin (10 μM) in leukocytospermic semen samples was able to improve sperm motility and decrease the H_2O_2 levels in sperm supernatant. In addition, after quercetin treatment, the authors observed an increase in levels of cytochrome B and NADH, suggesting that quercetin could improve the respiratory function of the sperm mitochondria [225]. On the other hand, in the past few years, the research addressed to supplementation of cryopreservation media with antioxidants has been increasing. Li *et al*. showed that VC (300 μM) or catalase (200 or 400 IU/mL) supplementation ameliorated sperm motility, inhibited DNA damage and protected the mitochondrial function. Along with these findings, the authors also reported a decrease in the levels of ROS, in frozen-thawed spermatozoa, evidencing the protective effect of VC or catalase supplementation in sperm media against cryodamage [226]. Similarly, other authors demonstrated that supplementation of the cryopreservation medium with quercetin (50 μM) induced a significant improvement in post-thaw sperm motility, vitality, and DNA integrity [227]. In fact, the reduction in the deleterious effects of ROS along with the improvement in post-thawing sperm quality, highlight the cryoprotective role of antioxidants.

Notwithstanding, while some authors have demonstrated positive effects of antioxidant supplementation in sperm media, others have reported opposite results. A study performed in normozoospermic and asthenozoospermic semen samples shown that sperm medium supplementation with VC (150–600 μM) and vitamin E (20–60 μM), either alone or combined, has a detrimental effect on sperm movement parameters, with the greatest impact observed with the highest concentrations of antioxidants [228]. In the same line, other studies involving sperm media supplementation with these

vitamins revealed that, although there was a significant decrease in ROS production, no beneficial effects on DNA integrity were disclosed [229]. Khanduja *et al.* observed that *in vitro* supplementation of quercetin (5-200 μM) caused a dose-dependent decrease in sperm motility and vitality, with an attendant increase in MDA levels. The authors proposed that motility loss may be associated with the decrease of Ca^{2+} ATPase activity, potentially triggered by quercetin [230].

Another controversial subject in antioxidant therapy is the prooxidant activity of some antioxidants. It has been reported that some antioxidants may act as prooxidants such as VC, vitamin E and some polyphenols. At least three factors can contribute to this condition, including the presence of metal ions, the concentration of the antioxidant, and its redox potential [231]. The prooxidant effect of VC usually occurs when metals ions (such as iron and copper) are present in the medium, inducing the Fenton and Haber-Weiss reactions and consequently the generation of the highly reactive •OH [232]. In turn, the prooxidant activity of vitamin E is attributed to the chain propagation of the tocopheroxyl radical formed in the reaction with ROS. When VC are in the medium it can reduce this radical to regenerate vitamin E, otherwise the tocopheroxyl radical can contribute to lipid peroxidation reactions and have a prejudicial effect on cells [233].

The heterogeneity of results along with the small numbers of patients recruited to the studies, contribute to the lack of consensus about antioxidant therapy. However, despite the conflicting reports, the generation of ROS during ART procedures cannot completely be avoided and there is strong evidence that OS affects the success rates of ART procedures. Given the aforementioned*, in vitro* use of antioxidants during ART procedures to counteract ROS appears to be a concept with high potential. However, further studies are required to investigate the appropriated antioxidants and their optimal dosages in the different techniques of ART. Moreover, it is important to continue the development and optimization of protocols of incubation/handling and cryopreservation of male gametes, to achieve better ART outcomes.

AIM

As OS continues to play an increasing role in contributing to reduced sperm quality and often to male infertility, it is essential to understand the molecular events associated with ROS-mediated damage in spermatozoa and find strategies to overcome this problem. The supplementation of sperm media with antioxidants is a current strategy used in ART. However, the therapeutic use of bioactive compounds with antioxidant properties requires an intensive investigation to unveil the effects and the mechanism of action of those compounds. Although some work has been done regarding the *in vitro* supplementation of sperm media with flavonoids, there is no literature concerning the effects of supplementation of hyperoside (a flavonoid glycoside) in human sperm media. Thus, this work aims to evaluate the impact of hyperoside supplementation on the protection of human sperm against H_2O_2-induced oxidative damage. It is also intended to increase knowledge about the antioxidant mechanism of this phytochemical, as well as its ability to prevent some of the deleterious effects caused by ROS. For this, the following objectives were established:

1. Perform a cytotoxic test with a range of HYP concentrations to ascertain potential cytotoxic effects in spermatozoa;
2. Evaluate sperm parameters (motility and vitality), OS biomarkers, DNA fragmentation levels and MMP in an induced OS condition and observe if HYP is able to protect human spermatozoa against oxidative damages;
3. Identify the metabolites in sperm media to better understand the sperm metabolic behaviour under the used experimental conditions.

Chapter II: Materials and methods

2. Materials and methods

2.1. Reagents

The following compounds and media were used in this experiment: Hyperoside (94,7% purity; powder) from HWI pharma services GmbH (Germany); L-Ascorbic acid (99% purity; powder) from Sigma-Aldrich (St. Louis, MO, USA); Hydrogen Peroxide (35%) from LABCHEM (Loures, Portugal); Ham's F-12 Nutrient Mixture from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Patient characterization

Human sperm samples were collected from *Centro de Genética da Reprodução Professor Alberto Barros* (Porto, Portugal), after approval by the Joint Ethics Committee CHUP/ICBAS (2021/CE/P002[P342/CETI/ICBAS), during November 2020 and June 2021. The semen samples were provided by male patients of reproductive age undergoing fertility assessment, after 2-4 days of abstinence. The samples were obtained by masturbation and placed in sterile tubes. Patients with inflammatory diseases, cancer, or other health conditions that may severely affect their fertility potential were discarded. Also, patients were asked to provide information on the consumption of tobacco, alcohol, and other substances that could interfere with the experiment. All patients included in this study signed an informed written consent.

Sperm samples were characterized according to the World Health Organization (WHO) guidelines for laboratory examination and processing of human sperm. Only samples having the following normal semen parameters, according to the WHO-criteria, were used in this project: sperm concentration ≥ 15 million/mL; total sperm motility ≥ 40%; and sperm vitality ≥ 58% [15].

2.3. Study design

The fresh semen samples were centrifuged at 500x*g* for 5 minutes at room temperature. The seminal fluid was discarded, and the pellet was washed with 1 mL of phosphate-buffered saline (PBS) solution. After another centrifugation at 500x*g* for 5 minutes, the supernatant was discarded, and the pellet was resuspended in Ham F12 medium.

To test if HYP had a cytotoxic effect in human spermatozoa, sperm samples of 6 normozoospermic patients (10 million spermatozoa/condition) were incubated with increasing concentrations of HYP (in µM: 0, 5, 50, 100, and 500), for 1h at 37º C, 5% CO_2. Sperm motility and sperm vitality were assessed before and after the experiment according to the WHO guideline [15]. The concentrations of 100 and 500 µM of HYP were selected for further experiments. Additionally, for further experiments, we decided to add VC to act as a positive control. This vitamin is a water-soluble antioxidant whose effects have already been described in human spermatozoa. VC concentration of 600 µM was chosen according to [143-147].

To test the sperm protective potential of HYP and VC, sperm samples of 20 normozoospermic patients were incubated for 1h at 37ºC, 5% CO_2, in the following conditions (10 million spermatozoa/condition):

1) Ham F12 medium;

2) Ham F12 medium + 600 µM VC;

3) Ham F12 medium + 100 µM HYP;

4) Ham F12 medium + 500 µM HYP;

5) Ham F12 medium + 300 µM H_2O_2;

6) Ham F12 medium + 600 µM VC + 300 µM H_2O_2;

7) Ham F12 medium + 100 µM HYP + 300 µM H_2O_2;

8) Ham F12 medium + 500 µM HYP + 300 µM H_2O_2.

In this study, H_2O_2 was used as an OS-inducer, as described in [234]. Figure 8 represents the experimental design.

Sperm motility and sperm vitality were assessed before and after the experiment according to the WHO guideline [15]. Afterwards, samples were centrifuged at 500x*g*, 37ºC and the sperm pellets and *post*-treatment media were stored at -80º C until used in further analysis.

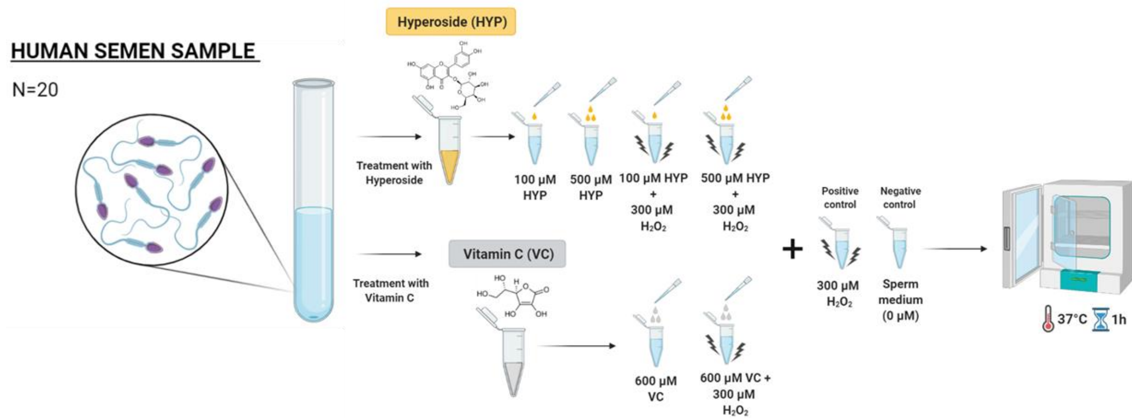

Figure 8: **Schematic representation of the experimental design.**

2.4. Sperm analysis

2.4.1. Sperm concentration

The sperm concentration of the samples was performed in a Neubauer chamber (hemocytometer). This apparatus is divided into 2 chambers with a microscopic pattern of 3 mm x 3 mm. Each counting area is divided into nine 1 mm x 1 mm grids and contains a large square in the centre divided into 25 medium squares. To sperm count, 5 µL of the sample was added to 45 µL of water (1:10). Then, 10 µL of the diluted sperm were placed under the coverslip, on each side of the hemocytometer. The concentration was evaluated under a microscope at 40x magnification, and the count of spermatozoa was made on the 5 medium squares of each side of the hemocytometer. The calculation was based on the following formula:

Concentration (sperm/mL) = *(Dilution Factor) x (Average sperm count of superior and inferior area) x (0.05 X* 10^6*)*

The concentration is expressed as the number of sperm *per* unit volume of ejaculate and should be ≥ 15 x 10^6 sperm/mL for being considered a normal sample [15].

2.4.2. Sperm total motility

Motility is one of the most important parameters as it reflects the ability of sperm to cross the entire female tract until reach and fertilize the oocyte. Motility was measured in the Makler chamber. This device contains a cover glass with a 1 mm^2 grid divided into 100 squares, each one of 0.1 x 0.1 mm. The depth of 10 µm allows the movement of sperm and the examination of mobile and immobile sperm in the same focal plane.

To measure motility, 5 µL of the homogenized sample was placed in the centre of the Makler chamber and immediately observed under a microscope with a 20x magnification. At least 15 squares of the chamber were counted. The total motility was calculated in percentage, and the minimum reference limit for a sample be considered normal is 40% [15]. The camera was kept on the heating plate, at 37ºC, during all the procedures.

2.4.3. Sperm vitality

Sperm vitality refers to the integrity of the sperm membrane and was performed based on the eosin-nigrosin staining. This staining technique uses the eosin to penetrate the sperm with damaged membranes, turning dead sperm dark pink, while the ones with the intact membrane remain colourless. The nigrosin is used to increase the contrast between the background and the sperm [15]. To prepare the eosin solution, 0.67 g of eosin Y and 0.9 g of sodium chloride were dissolved in 100 mL of distilled water under gentle heating. Then, 10 g of nigrosin were added to this solution. To perform the vitality test, 5 µL of sperm sample were mixed with 5 µL of eosin-nigrosin suspension. A smear was made from this mixture and allowed to air dry. Each slide was examined under a microscope, with a magnification of 100x. At least 200 spermatozoa were counted in each slide. The vitality was calculated as a percentage and according to WHO guidelines, it must be ≥ 58% to be considered normal [15].

2.5. Protein extraction and quantification

For protein extraction, sperm samples were centrifuged at 500x*g* for 5 minutes to isolate spermatozoa from media. The sperm pellet was then resuspended in a suitable volume of 1% SDS (1uL 1% SDS /10^6 sperm) and kept for 60 minutes at room temperature. Then, the samples were centrifuged at 14,000x*g* for 20 minutes, the supernatants were recovered, and the pellets were discarded.

Protein quantification was assessed using the bicinchoninic acid (BCA) assay. This method involves two reactions. The first consists of reducing Cu^{2+} to Cu^{+}, in alkaline conditions (Biuret reaction), and the amount of Cu^{+} is proportional to the amount of protein in the sample. The second reaction is based on the colourimetric detection of Cu^{+} ions by BCA, since the chelation of two BCA molecules reacts with a Cu^{+} ion and forms a purple-coloured product that absorbs light at 562 nm [235]. The absorbance capacity increases linearly with increasing protein concentrations in the range of 0.02-2

mg/mL. In this assay, 1 μL of each sample was diluted in 9 μL of PBS and subsequently loaded into the microplate. To each well, 200 μL of Working Reagent (WR) were added. Following these events, the microplate was incubated for 30 min at 37ºC (in the dark) and the absorbance was measured at 562 nm by a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA).

2.6. Oxidative stress marker evaluation

Three oxidative damage biomarkers were quantified through immunodetection to evaluate the lipid peroxidation, and protein nitration and carbonylation of the sperm samples. The procedure used for the quantification of each oxidative damage marker is described below.

2.6.1. Protein carbonylation

To determine the carbonyl groups, 5 μg of protein was diluted in a final volume of 20 μL of filtered PBS. For protein denaturation and carbonyl group exposure, 20 μL of 12% SDS was added to each sample. Subsequently, 40 μL of 20 mM 2,4-dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid (TFA) was added, and the reaction was allowed to occur for 30 min at room temperature, in the dark. To stop the reaction between carbonyl groups and DNPH, 30 μL of 2M Tris with 18% β-mercaptoethanol was added. Then, 2.4 μL of the derivatized samples were diluted in 107.8 μL of filtered PBS and transferred to a polyvinylidene difluoride (PVDF) membrane through a slot-blot system. The membranes were activated in methanol for 2 minutes and washed in deionized water for 5 minutes, before use. Membranes were blocked in 5% bovine serum albumin (BSA) in Tris-Buffered Saline, 0.1% Tween 20 (TBS-T), for 90 minutes. Membranes were washed 3 times, 5 minutes, with TBS-T prior to incubation with the primary antibody. For carbonylation quantification, membranes were incubated overnight, 4º C with the primary antibody anti-2,4-dinitrophenol (DNP) (D9656, Sigma-Aldrich, St. Louis, Missouri, USA), 1:5000 diluted in 1% BSA TBS-T. Membranes were washed 3 times, 5 minutes, with TBS-T before secondary antibody incubation. Afterwards, membranes were incubated with anti-rabbit antibody (A3687, Sigma-Aldrich, St. Louis, Missouri, USA) 1:5000 diluted in 1% BSA TBS-T for 90 min. Immuno-reactive proteins were detected through reaction with an ECF substrate (GE Healthcare, Buckinghamshire, UK), and analysed by ChemiDocTM MP Imaging system (Bio-Rad, California, USA).

2.6.2. Protein nitration and lipid peroxidation

To determine the protein nitration and the lipid peroxidation, 5 µg of protein was diluted in a final volume of 100 µL of filtered PBS. Protein samples were then transferred into a PVDF membrane through a slot-blot system. PVDF membranes were activated prior to use as described above. Membranes were blocked in 5% BSA TBT-T for 90 minutes, and washed 3 times, 5 minutes, with TBS-T before primary antibody incubation. For lipid peroxidation evaluation, the primary antibody used was anti-4-hydroxynonenal (4-HNE) (AB5605, EMD Millipore, Temecula, CA, USA), 1:1000 diluted in 1% BSA TBS-T. For protein nitration evaluation, the primary antibody used was anti-3-nitrotyrosine (NT) (9691S, Cell Signaling Technology, Danvers, MA, USA), 1:1000 diluted in 1% BSA TBS-T. Membranes were incubated with the corresponding primary antibodies overnight, at 4ºC. After 3 washes, 5 minutes, with TBS-T, membranes were incubated with the corresponding secondary antibody for 90 min, at room temperature. For lipid peroxidation evaluation, it was used the anti-goat antibody (A4187, Sigma-Aldrich, St. Louis, MO, USA), 1:5000 diluted in 1% BSA TBS-T. For protein nitration evaluation, it was used the anti-rabbit antibody (A3687, Sigma-Aldrich, St. Louis, MO, USA), 1:5000 diluted in 1% BSA TBS-T. Immuno-reactive proteins were detected through reaction with an ECF substrate (GE Healthcare, Buckinghamshire, UK), and analysed by ChemiDocTM MP Imaging system (Bio-Rad, California, USA). Total protein was assessed by Ponceau S staining.

2.7. Post-treatment media total antioxidant capacity

The total antioxidant capacity (TAC) of the post-treatment media samples was measured by ferric reducing antioxidant power (FRAP) assay as described in [236]. FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3{\cdot}6H_2O$ in the ratio of 10:1:1. Briefly, in a 96-well plate, 6 µL of post-treatment media samples were added to 180 µL of FRAP reagent. Triplicates were made for each sample. Distilled water (dH_2O) was used as negative control and VC (1000 µM) was used as an antioxidant standard. Both controls were treated in the same way as media samples. The absorbance was read at 593 nm, immediately after the addition of the FRAP reagent (0 min) and then after 40 min, by a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). Between measurements, the plate was kept in the dark, at room temperature. The antioxidant potential of the samples was determined against VC (1000 µM) standard and corrected using the absorbance value of dH_2O absorbance.

FRAP value (µmol antioxidant capacity/L), was calculated using the formula described in [236].

2.8. Sperm DNA fragmentation evaluation

The evaluation of DNA fragmentation was performed by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, using in-situ Cell Death Detection Kit (Fluorescein) from Roche Diagnostics GmbH (Mannheim, Germany). This assay is based on the identification of single- and double-stranded DNA breaks by using the terminal deoxynucleotidyl transferase (TdT) enzyme, which catalyses the addition of labelled deoxyuridine triphosphate (dUTPs) nucleotides to the 3'-hydroxyl terminal of the DNA breaks [237]. Spermatozoa (10 million) were collected and treated with HYP, and VC as previously described. Briefly, a smear of each sample was performed on previously adhesive slides. Subsequently, the slides were fixed with 4% paraformaldehyde, for 1h at room temperature and then rinsed with PBS. For cell permeabilization, slides were immersed in a solution of 0.1% sodium citrate containing 0.1% Triton X, for 2 min at 4ºC. After two washes with PBS for 5 min, 50 µL of the TUNEL reaction mix [5 µL of TdT and 45 µL of labelled dUTPs mixture] was added to each slide. A coverslip was placed to prevent evaporation. The slides were incubated in a humidified chamber at 37°C for 1h, in darkness. Then, the slides were washed four times with PBS (2 min each). Subsequently, coverslips were mounted using mounting medium VECTASHELD (30 Ingold Road, Burlingame, CA 94010, USA). The results were visualized in a Nikon Eclipse E400 microscope equipped with a Y-FL epi-fluorescence attachment and HB-10103AF Super high-pressure mercury lamp power supply (Nikon, Shinagawa, Tokyo, Japan), coupled with a Nikon NIS Elements Image Software. Al least 200 spermatozoa were counted on each slide. The total number of spermatozoa was quantified under blue fluorescence (corresponding to the DAPI staining of the nucleus). Spermatozoa presenting DNA fragmentation also present green fluorescence (corresponding to the dUTPs inserted in the DNA breaks by TdT). The results were expressed as a percentage of sperm with green fluorescence in the total stained with blue DAPI.

2.9. Mitochondrial membrane potential evaluation

The MMP of spermatozoa was measured by using the lipophilic probe 5-5′,6-6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (T3168, Invitrogen™, Carlsbad, CA, USA), which can selectively penetrate mitochondria. In

spermatozoa with damaged MMP, the JC-1 remains in the cytoplasm in a monomeric form; while in cells with normal MMP, the accumulation of JC-1 occurs in the membrane promoting the formation of JC-1 aggregates. Briefly, spermatozoa (1 million) were collected and treated with HYP, and VC as previously described. After, cells were washed with PBS (500x*g*, 5 min) and incubated with JC-1 (1 µg/mL in PBS) for 30 min at 37ºC. As a positive control, spermatozoa were incubated with a 20% DMSO solution to promote the increase of mitochondrial membrane potential. After the incubation period, cells were washed with PBS (500x*g*, 5 min) two times. Pellets were resuspended in 750 µL of PBS and transferred to 96-well plates (performed in triplicate). The fluorescence of the JC-1 monomers (ex 485/530 nm; excitation/emission) and JC-1 aggregates (535/590 nm; excitation/emission) was assessed by a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). The ratio between JC-1 aggregates/monomers was calculated and used as a mitochondrial membrane polarization marker.

2.10. Post-treatment media metabolite analysis

Metabolites present in the post-treatment media were assessed by proton nuclear magnetic resonance (^{1}H-NMR) and spectra analysis. 1D ^{1}H-NMR spectra were acquired on a 500 MHz Bruker Avance III HD spectrometer equipped with a 5-mm TXI probe, at 298 K. To each post-treatment medium (180 µL), 45 µL of sodium fumarate (2 mM) were added for a final sample volume of 225 µL. Sodium fumarate was used as an internal reference for metabolite quantification (multiplicity, chemical shift (in ppm)) in the media. The following metabolites were quantified: pyruvate (singlet, 2.37), acetate (singlet, 1.90), lactate (doublet, 1.32), alanine (doublet, 1.47), and malate (double doublet, 2.65). The spectra were manually phased, and baseline corrected. NUTS-Pro™ NMR software (Acorn NMR, Inc, Fremont, CA, USA) was used to integrate the chosen metabolite peaks. The variation (consumption or production) of the metabolites is expressed as nmol/10^6 sperm cells.

2.11. Statistical analysis

All data presented are expressed as whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Variations between groups regarding sperm total motility, sperm vitality, OS biomarkers, DNA fragmentation, MMP, and metabolite quantification were evaluated by one-way ANOVA followed by the correction for multiple comparisons by controlling the False Discovery Rate (FDR). The two-stage

step-up method of Benjamini, Krieger, and Yekutieli was the test used to assess multiple comparisons. Fold variation to the control group (0 μM) was calculated for OS biomarkers quantification, DNA fragmentation evaluation, and MMP assessment. The differences between the groups in the cytotoxic test and the TAC media assessment were performed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Values of $P < 0.05$ were considered statistically different. The statistical analysis of this work was performed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA).

Chapter III: Results and discussion

3. Results

3.1. Supplementation of sperm preservation media with hyperoside has no cytotoxic effects on human sperm

After treating human spermatozoa with increasing doses of HYP (in µM: 0, 5, 50, 100, and 500), for 1h at 37ºC, cytotoxicity was evaluated through the assessment of sperm motility and vitality. Figure 9 represents sperm total motility and sperm vitality, after preservation in media supplemented with HYP. Regarding sperm motility, we could not find any significant differences between the HYP treated groups (in µM: 5, 50, 100, and 500), and the control group (0 µM) (Figure 9A). Similarly, we could not find significant differences in spermatozoa vitality after preservation in media supplemented with HYP between the HYP treated groups and the control group (Figure 9B). No cytotoxic effects were found in sperm samples preserved in media supplemented with any of the tested HYP concentrations. The concentrations of 100 and 500 µM of HYP were selected for further testing.

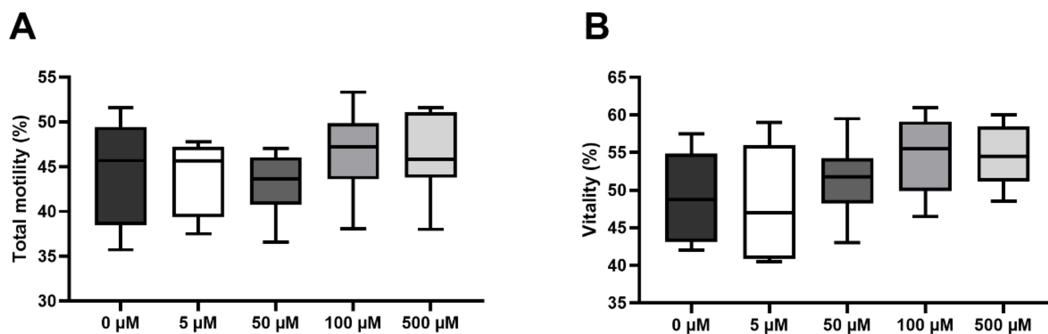

Figure 9: Evaluation of hyperoside (HYP) cytotoxicity for human spermatozoa. (A) represents the percentage of sperm total motility after supplementation with increasing doses of HYP (N=6). (B) represents the percentage of sperm vitality after supplementation with increasing doses of HYP (N=6). Results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). No statistical differences were found between groups (one-way ANOVA followed Tukey post hoc test for multiple comparisons).

3.2. Supplementation of sperm preservation media with hyperoside and vitamin C preserves sperm motility under oxidative stress conditions

To evaluate the cytoprotective effects of VC and HYP, human spermatozoa were supplemented with VC (600 μM) and HYP (100 and 500 μM), in the presence and absence of H_2O_2, for 1h at 37ºC. H_2O_2 (300 μM) was used as an OS-inducer [234]. Figure 10 represents spermatozoa total motility and spermatozoa vitality, after preservation in media supplemented with VC and HYP, in the presence and absence of H_2O_2. We found no differences regarding spermatozoa total motility after preservation in media supplemented with VC and HYP (100 and 500 μM) (52 ± 16%; 55 ± 15% and 51 ± 14%, respectively), in comparison with the control group (0 μM) (50 ± 15%), illustrating that supplementation of media with these antioxidants does not affect spermatozoa motility (Figure 10A). However, spermatozoa motility decreased in the group exposed to H_2O_2 (300 μM) (22 ± 10%) in comparison to the control group and the only-antioxidant groups, suggesting that spermatozoa are under stress (Figure 10A). The spermatozoa motility of the group preserved in the media supplemented with VC plus H_2O_2 (36 ± 15%) was higher than in the group treated with only H_2O_2. Nevertheless, the spermatozoa motility of this group was decreased in comparison to the control group and the only-antioxidant groups. Similarly, the motility of the groups preserved in media supplemented with HYP (100 and 500 μM) plus H_2O_2 (39 ± 12% and 35 ± 13%, respectively) was higher than the motility of the group treated only with H_2O_2. Still, the spermatozoa motility of those groups decreased when compared to the control group and the only-antioxidant groups. These results suggest a cytoprotective effect of the antioxidants VC and HYP under OS conditions.

Regarding spermatozoa vitality, none of the preservation media supplemented with VC (600 μM) and HYP (100 and 500 μM), with or without H_2O_2, presented a decrease in spermatozoa vitality after 1h treatment (Figure 10B). These results suggest that, although the concentration of 300 μM of H_2O_2 can induce a decrease in sperm motility, this treatment does not affect sperm vitality.

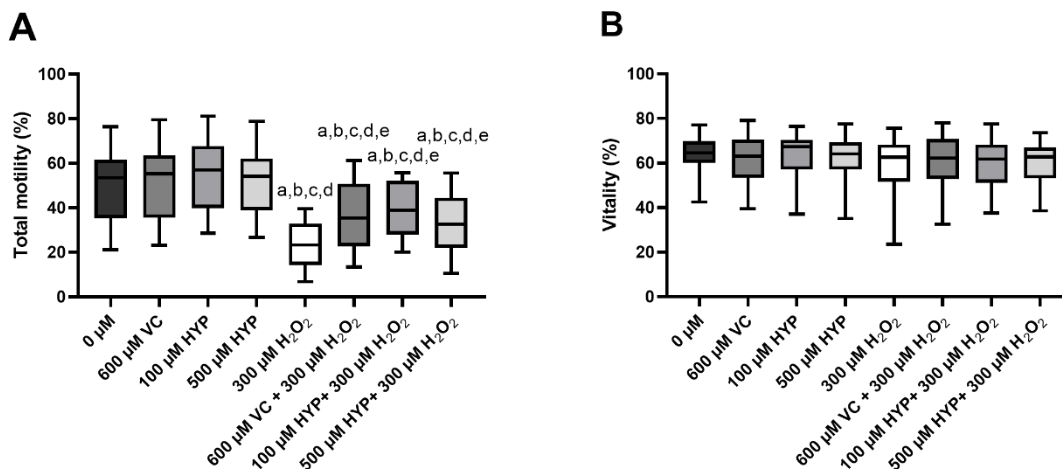

Figure 10: Evaluation of the cytoprotective effects of hyperoside (HYP) and vitamin C (VC) sperm media supplementation under oxidative stress conditions. (A) represents the variations in the percentage of total sperm motility (N=20) after 1h treatment with VC (600 µM), and HYP (100 and 500 µM) in the presence and absence of H_2O_2 (300 µM), herein used as an oxidative stress inducer. (B) represents the variations in the percentage of sperm vitality under the same conditions (N=20). Results are represented as whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p<0.05$) are indicated as:(a) relative to control; (b) relative to 600 µM VC group; (c) relative to 100 µM HYP group; (d) relative to 500 µM HYP group; (e) relative to 300 µM H_2O_2 group.

3.3. Supplementation of sperm preservation media with hyperoside increases the total antioxidant capacity of the media

After preserving spermatozoa with media supplemented with HYP (100 and 500 µM), in the presence and absence of H_2O_2, for 1h at 37ºC, the TAC of the media was measured by FRAP. Figure 11 represents the TAC levels of the media, herein measured by FRAP levels. VC was used as a standard for antioxidant capacity in the FRAP technique, and, because of that, TAC of the sperm preservation media supplemented with VC (600 µM) was not assessed. Nevertheless, VC (1000 µM) was used as a standard value of TAC (2 µmol antioxidant potential/L) [236]. The media containing 500 µM of HYP (in the presence and absence of H_2O_2) presented the highest TAC levels (4.78 ± 0.73 and 4.54 ± 0.58 µmol antioxidant potential/L, respectively). Also, the TAC value of the sperm preservation media supplemented with 500 µM of HYP was higher than the standard TAC level of VC (1000 µM). The media containing 100 µM of HYP (in the presence and absence of H_2O_2) also presented higher TAC levels (1.22 ± 0.31 and 1.15 ± 0.24 µmol antioxidant potential/L,

respectively) in comparison to the control (0.21 ± 0.06 µmol antioxidant potential/L) and only-H_2O_2 group (0.18 ± 0.06 µmol antioxidant potential/L). Although the TAC of these media was lower than the ones containing 500 µM of HYP. The medium containing only-H_2O_2 (300 µM) had the lowest TAC from all the tested media. However, no difference was found regarding the TAC from this group and the TAC from the control group (0 µM). Notwithstanding is important to note that the FRAP assay does not react with H_2O_2, which can justify the absence of differences concerning the groups with the presence of H_2O_2 [50]. The FRAP assay measures the ability of an antioxidant compound to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) and this reaction is linked to a colour change (absorbance at 593 nm) [47].

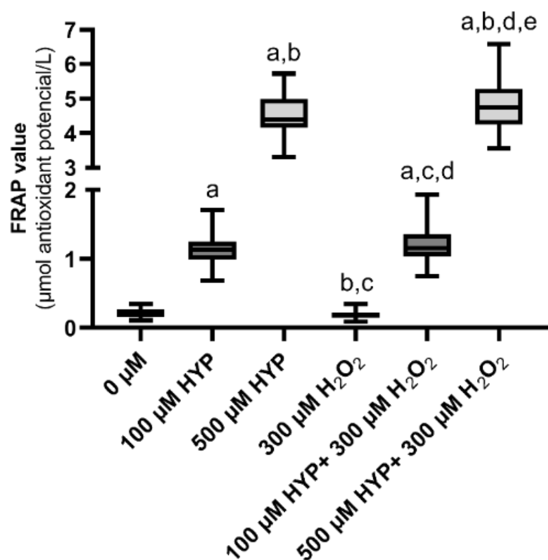

Figure 11: Effects of hyperoside (HYP) and vitamin C (VC) sperm media supplementation in the total antioxidant capacity (TAC) of media, under oxidative stress conditions (N=20). Results are represented as whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed Tukey post hoc test for multiple comparisons; $p<0.05$) are indicated as: (a) relative to control; (b) relative to 100 µM HYP group; (c) relative to 500 µM HYP group; (d) relative to 300 µM H_2O_2 group; (e) relative to 100 µM HYP + 300 µM H_2O_2 group.

3.4. Supplementation of sperm preservation media with hyperoside and vitamin C appears to protect human sperm against lipid peroxidation under oxidative stress conditions

Elevated levels of H_2O_2 are known to promote OS and cause several damages to spermatozoa biomolecules, such as lipids and proteins. OS biomarkers were quantified to evaluate the oxidative damage promoted by H_2O_2 in human sperm and to further test the cytoprotective effects of sperm preservation media supplementation with VC and

HYP. Figure 12 (panels A, B, and C) represent the levels of lipid peroxidation, protein carbonylation, and protein nitration found in human sperm after supplementation of sperm preservation media with VC (600 µM) and HYP (100 and 500 µM), in the presence and absence of H_2O_2, for 1h at 37ºC.

Lipid peroxidation was assessed by the quantification of 4-HNE levels (Figure 12A). Lipid peroxidation levels of the groups preserved in media supplemented with VC (600 µM) and HYP (100 and 500 µM) (1.25 ± 0.54; 1.04 ± 0.31 and 1.04 ± 0.45-fold, respectively) were similar to the levels of the control group (represented as a dashed line, treatment with no HYP, no VC, and no H_2O_2). Meanwhile, in the group treated with only H_2O_2 (300 µM) (1.71 ± 0.70-fold) the spermatozoa lipid peroxidation levels increased in comparison with the control group and those preserved in media supplemented with HYP (100 and 500 µM) groups. The peroxidation levels of spermatozoa preserved in media supplemented with VC (600 µM) and HYP (100 and 500 µM) plus H_2O_2 (1.39 ± 0.61; 1.23 ± 0.58 and 1.30 ± 0.57-fold, respectively), were not significantly different from the levels detected in spermatozoa from the group treated with only H_2O_2 (Figure 12A). Notwithstanding, spermatozoa peroxidation levels in those groups tend to be lower in comparison to the levels detected in spermatozoa subjected only to H_2O_2, suggesting that some cytoprotective action of VC and HYP may be occurring (to some extent).

The DNP quantification was used to assess protein carbonylation levels in spermatozoa (Figure 12B). No statistical differences were found regarding the carbonylation levels of spermatozoa preserved in media supplemented with VC (600 µM) and HYP (100 and 500 µM), in the presence and absence of H_2O_2, in comparison to the control group. Further, no statistical difference was found regarding spermatozoa carbonylation levels between the groups treated with H_2O_2 and the control group (Figure 12B). Similar results were found regarding spermatozoa protein nitration levels, evaluated by the quantification of NT (Figure 12C). No differences were found regarding the nitration levels of spermatozoa preserved in media supplemented VC (600 µM) and HYP (100 and 500 µM), in the presence and absence of H_2O_2, in comparison to the control group. Similarly, no statistical differences were found regarding spermatozoa nitration levels between the groups treated with H_2O_2 and the control group (Figure 12C).

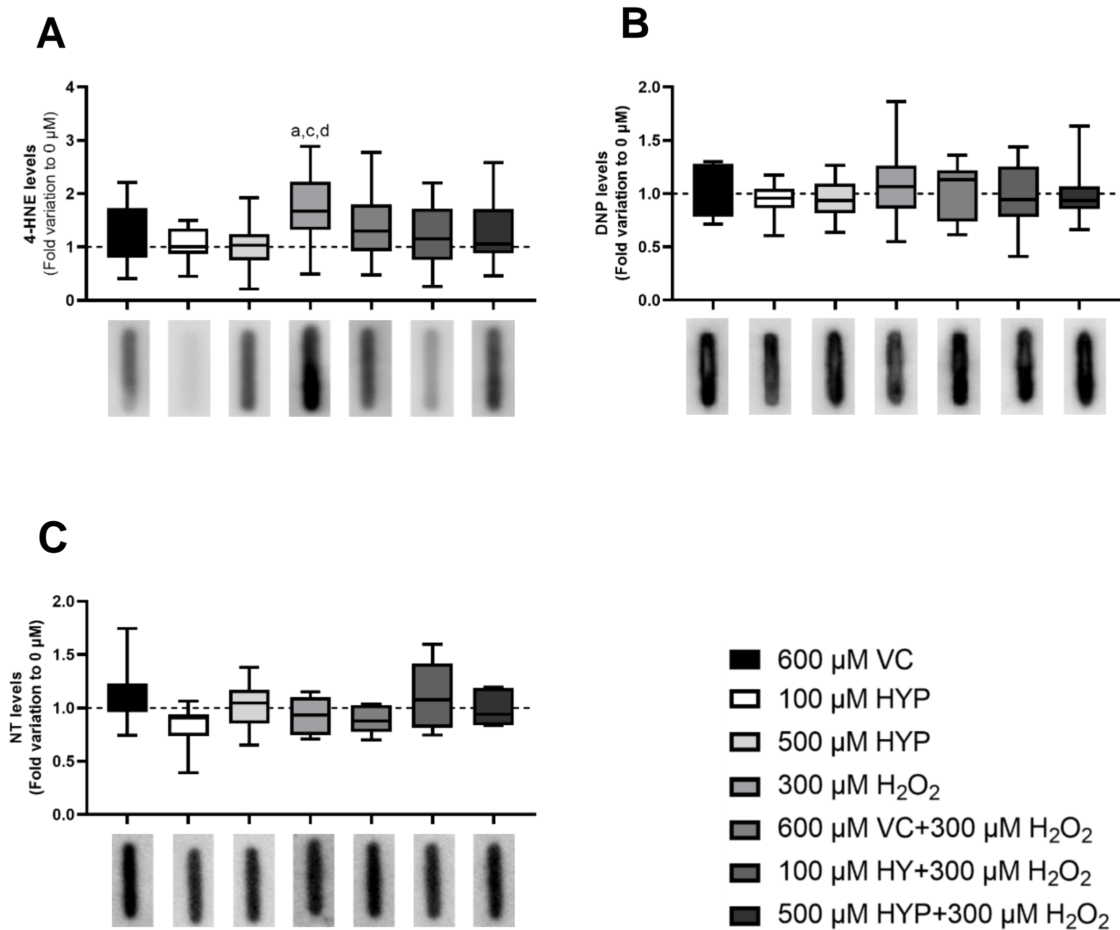

Figure 12: Effects of hyperoside (HYP) and vitamin C (VC) sperm media supplementation in oxidative stress biomarkers, under oxidative stress conditions. (A) represents the 4-HNE expression levels found in spermatozoa after 1h treatment in media supplemented with VC (600 µM), and HYP (100 and 500 µM) in the presence and absence of H_2O_2 (300 µM), herein used as an oxidative stress inducer (N=20). (B) represents DNP levels quantified after the reaction of DNPH with sperm protein carbonyl groups, after treatments (N=10). (C) represents the NT expression levels found in spermatozoa, after treatments (N=10). Fold variation was performed to the control group (Ham-F12 medium with no HYP, no VC, nor H_2O_2) represented as a dashed line. Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p<0.05$) are indicated as (a) relative to control; (c) relative to 100 µM HYP group; (d) relative to 500 µM HYP group. All results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values).

3.5. Supplementation of sperm preservation media with hyperoside and vitamin C prevents DNA fragmentation in human spermatozoa under oxidative stress conditions

One of the consequences of a prolonged OS situation is DNA damage [12]. In spermatozoa, DNA damage is known to severely decrease the chances of achieving a successful pregnancy and increase the risk of miscarriage. To evaluate if supplementation of sperm media with VC and HYP was able to prevent DNA

fragmentation induced by OS, we performed a TUNEL assay in the experimental conditions. Figure 13 represents the percentage of DNA fragmentation detected in human spermatozoa after supplementation of sperm preservation media with VC (600 µM) and HYP (100 and 500 µM), in the presence and absence of H_2O_2, for 1h at 37º C. Figure 14 represents the result of the TUNEL assay visualized by fluorescence. The spermatozoa nuclei (stained with DAPI) present a blue fluorescence and spermatozoa with DNA fragmentation also present a green fluorescence, corresponding to the dUTPs inserted in the DNA breaks. The merge of the green/blue channels allows the detection of the percentage of sperm DNA fragmentation for each condition.

Our results showed no differences regarding sperm DNA fragmentation between the control group (represented as a dashed line, treatment with no HYP, no VC, and no H_2O_2), the VC (600 µM) (0.88 ± 0.08-fold) and the HYP (100 µM) (0.91 ± 0.22-fold) supplemented groups. This indicates that supplementation with VC and HYP does not induce DNA fragmentation *per se.* Spermatozoa treated with only H_2O_2 (300 µM) presented the highest levels of DNA fragmentation by a 1.30 ± 0.19-fold variation to the control group. Furthermore, we found that spermatozoa supplemented with VC and HYP, in the presence of H_2O_2, (1.02 ± 0.23 and 1.02 ± 0.20-fold, respectively) exhibited lower levels of DNA fragmentation compared to the group treated only with H_2O_2. In addition, the DNA fragmentation levels of these groups did not differ from the control and antioxidants-only groups. These results suggest that a cytoprotective effect against DNA damage could be promoted by the supplementation of VC and HYP in sperm preservation media.

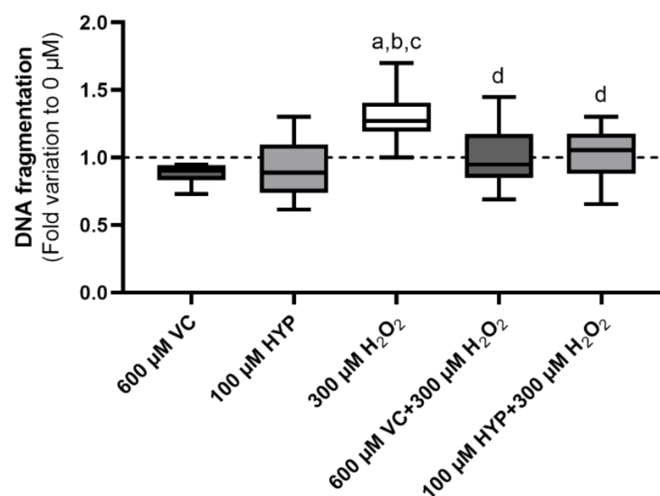

Figure 13: Effects of hyperoside (HYP) and vitamin C (VC) sperm media supplementation in spermatozoa DNA fragmentation, under oxidative stress conditions. The graph represents the variations on DNA fragmentation quantified in spermatozoa after 1h treatment in media supplemented with VC (600 µM), and HYP (100 µM) in the presence and absence of H_2O_2 (300 µM), herein used as an oxidative stress inducer (N=10). Fold variation was

performed to the control group (Ham-F12 medium with no HYP, no VC, nor H_2O_2) represented as a dashed line. Results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p<0.05$) are indicated as (a) relative to control; (b) relative to 600 μM VC group; (c) relative to 100 μM HYP group; (d) relative to 300 μM H_2O_2 group.

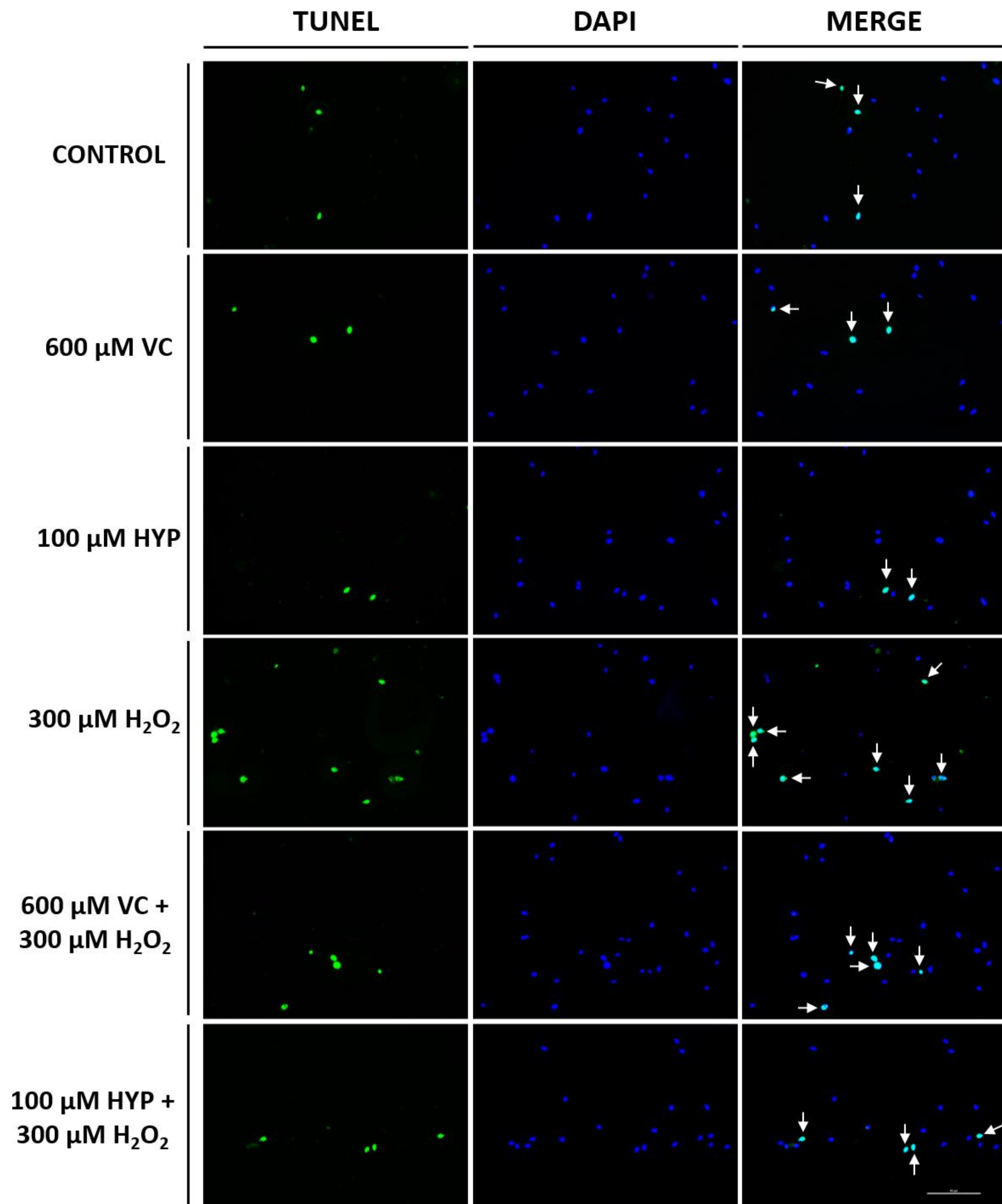

Figure 14: Results of immunofluorescence staining of the sperm DNA breaks by TUNEL (ex 480/535 nm; excitation/emission), and sperm nuclei by DAPI (ex 395/460nm; excitation/emission).

3.6. Supplementation of sperm preservation media with hyperoside and vitamin C cannot prevent alterations in the spermatozoa mitochondrial membrane potential under oxidative stress conditions

MMP is a strong indicator of mitochondrial functionality and can be used as a measure of electron transport chain (ETC) activity. Mitochondria are fundamental to producing cellular energy. Furthermore, in spermatozoa, several studies are reporting a positive correlation between MMP and motility. Herein, we use the JC-1 probe to evaluate if media supplementation with VC and HYP can ensure mitochondrial activity, under conditions of OS. Figure 15 represents the ratio of JC-1-aggregates/monomers in human spermatozoa, after 1h preservation in media supplemented with VC (600 μM) and HYP (100 μM), in the presence and absence of H_2O_2. Our results show no differences between the groups preserved in media supplemented with VC (600 μM) (0.97 ± 0.23-fold), HYP (100 μM) (0.92 ± 0.19-fold) and the control group (represented as a dashed line, treatment with no HYP, no VC, and no H_2O_2), meaning that no MMP alterations were promoted by the antioxidant treatment. However, a significant decrease in the MMP of spermatozoa was observed in the group treated with H_2O_2 (300 μM) (0.59 ± 0.22-fold) in comparison with the control group and with those preserved in media supplemented with antioxidants. Notwithstanding, no differences were found regarding spermatozoa MMP between the groups preserved in media supplemented with VC and HYP, in the presence of H_2O_2, (0.72 ± 0.20 and 0.64 ± 0.21-fold, respectively), and the group treated with H_2O_2-only. These results suggest that the supplementation of VC (600 μM) or HYP (100 μM) in sperm preservation media were not able to prevent the decrease in the sperm MMP promoted by H_2O_2.

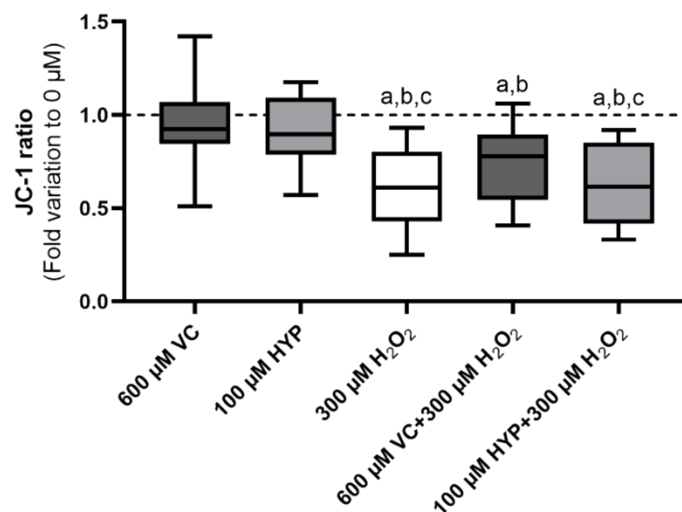

Figure 15: Effects of hyperoside (HYP) and vitamin C (VC) sperm media supplementation in the mitochondria functionality of spermatozoa, under oxidative stress conditions. The graph represents the variations in the MMP of spermatozoa after 1h treatment in media supplemented with VC (600 μM), and HYP (100 μM) in the presence and absence of H_2O_2 (300 μM), herein used as an oxidative stress inducer (N=15). Fold variation was performed to the control group (Ham-F12 medium with no HYP, no VC, nor H_2O_2) represented as a dashed line. Results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger and Yekutieli; $p<0.05$) are indicated as: (a) relative to control; (b) relative to 600 μM VC group; (c) relative to 100 μM HYP group.

3.7. Supplementation of sperm preservation media with hyperoside and vitamin C cannot prevent alterations in the metabolism of spermatozoa under oxidative stress conditions

Metabolism is a network of biochemical reactions that ensure cell function. The study of spermatozoa metabolism can enlighten us on what is going inside the cell. The effects of VC (600 μM) and HYP (100 and 500 μM) supplementation, in the presence and absence of H_2O_2, on spermatozoa metabolism were assessed by ^{1}H-NMR spectra analysis of the media. Figure 16 represents the major metabolites identified in the studied groups: pyruvate (Figure 16A); acetate (Figure 16B); lactate (Figure 16C); alanine (Figure 16D) and malate (Figure 16E).

Concerning pyruvate variation, no differences were observed in pyruvate variation in the media, between the control group (0.09 ± 1.58 nmol/10^6 cells) and the ones supplemented with VC (600 μM), and HYP (100 and 500 μM) (-0.23 ± 1.92; 0.06 ± 1.58 and 0.65 ± 1.48 nmol/10^6 cells, respectively) (Figure 16A). The group treated with only H_2O_2 presented the lowest levels of pyruvate (-2.65 ± 0.57 nmol/10^6 cells). The media supplemented with VC (600 μM), and HYP (100 and 500 μM), in the presence of H_2O_2, (-2.00 ± 0.83; -2.11 ± 0.81 and -2.08 ± 0.74 nmol/10^6 cells, respectively) presented similar pyruvate levels to the H_2O_2-only group, which were significantly lower in comparison to the control group and the antioxidants-only groups (Figure 16A).

Regarding acetate media levels, we found that media supplementation with VC (600 μM) (3.72 ± 1.09 nmol/10^6 cells) and HYP (500 μM) (3.49 ± 1.27 nmol/10^6 cells), presented an increase in acetate production by spermatozoa compared to the control group (2.65 ± 0.76 nmol/10^6 cells). However, no differences were found between the acetate levels of the HYP (100 μM) group (2.66 ± 0.56 nmol/10^6 cells) and the control group. On the other hand, we observed higher levels of acetate in the medium of spermatozoa treated with only H_2O_2 (7.11 ± 1.25 nmol/10^6 cells) (Figure 16B). The acetate levels in media supplemented with VC (600 μM) (6.80 ± 1.52 nmol/10^6 cells) and the HYP (500 μM) (7.04 ± 1.59 nmol/10^6 cells), in the presence of H_2O_2, were

similar to the acetate levels of the H_2O_2-only group. Curiously, acetate levels in the media of spermatozoa preserved with supplementation with HYP (100 μM) plus H_2O_2 (6.35 ± 1.51 nmol/10^6 cells) were lower than in the only- H_2O_2 group (Figure 16B).

Concerning lactate, no differences were found regarding the lactate levels of the control group (24.02 ± 12.43 nmol/10^6 cells), the ones supplemented with VC (600 μM), and HYP (100 and 500 μM) (22.36 ± 11.85; 26.11 ± 14.05 and 21.67 ± 10.10 nmol/10^6 cells, respectively). However, lactate production by spermatozoa was decreased in the group treated with only H_2O_2 (10.79 ± 7.23 nmol/10^6 cells) in comparison with control and the and antioxidant-only groups. Additionally, the lactate levels of the media supplemented with VC (600 μM) and the HYP (100 and 500 μM), in the presence of H_2O_2 (15.28 ± 7.07; 13.12 ± 7.17 and 13.14 ± 6.46 nmol/10^6 cells, respectively) were similar to the lactate levels of the H_2O_2-only group (Figure 16C).

Interestingly, in relation to alanine levels in sperm media, the spermatozoa preserved with HYP (100 and 500 μM) presented higher levels of alanine (0.93 ± 0.60 and 1.00 ± 0.77 nmol/10^6 cells, respectively) when compared with the control group (0.61 ± 0.65 nmol/10^6 cells), and the VC group (600 μM) (0.53 ± 0.44 nmol/10^6 cells). Notwithstanding, as seen in other metabolites, the media with only H_2O_2 supplementation (0.12 ± 0.48 nmol/10^6 cells), and the media supplemented with VC (600 μM) and HYP (100 and 500 μM), in the presence of H_2O_2 (0.13 ± 0.31; 0.20 ± 0.31 and 0.25 ± 0.34 nmol/10^6 cells, respectively) presented decreased alanine levels when compared to the control and antioxidants-only groups (Figure 16D).

Concerning malate variation, no differences were found in media malate levels between the control group (14.36 ± 17.04 nmol/10^6 cells) and the ones supplemented with VC (600 μM), and HYP (100 and 500 μM) (11.63 ± 11.39; 12.95 ± 11.56 and 20.00 ± 15.63 nmol/10^6 cells, respectively). The H_2O_2-only group presented the lowest malate levels (1.25 ± 2.76 nmol/10^6 cells) in comparison to the control and the antioxidant-only groups. Decreased malate levels were also observed in media supplemented with VC (600 μM) and HYP (100 and 500 μM), in the presence of H_2O_2, (2.71 ± 4.66; 7.21 ± 6.02 and 4.33 ± 5.46 nmol/10^6 cells, respectively) (Figure 16E).

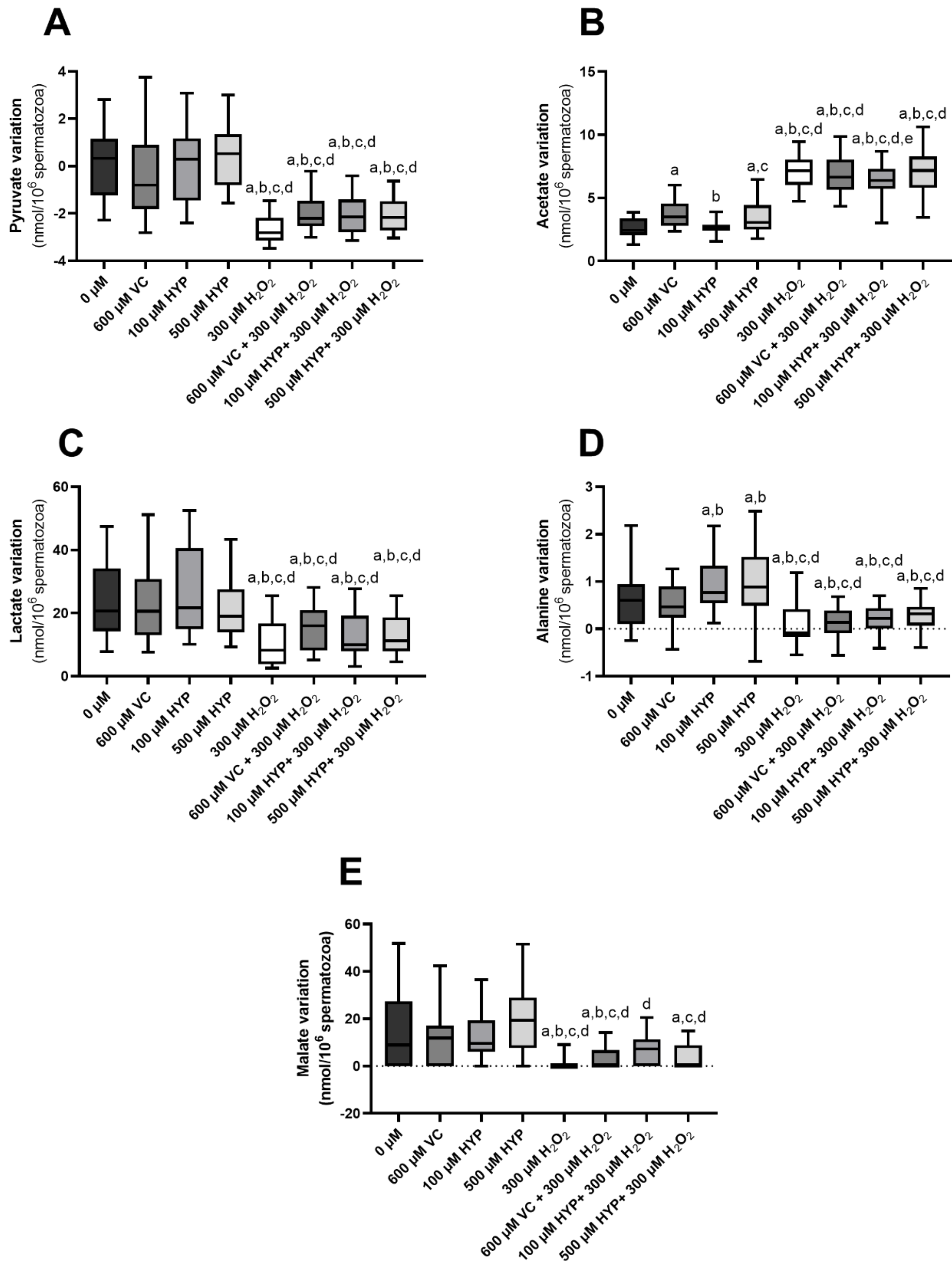

Figure 16: Effects of hyperoside (HYP) and vitamin C (VC) sperm media supplementation in the pyruvate (A), acetate (B), lactate (C), alanine (D), and malate (E) levels found in the media, under oxidative stress conditions. The graphs represent the variations of the metabolites found in media after 1h treatment in media supplemented with VC (600 µM), and HYP (100 and 500 µM) in the presence and absence of H_2O_2 (300 µM), herein used as an oxidative stress inducer (N=20). All results are represented as Tukey's whisker boxes (median, 25th to 75th percentiles ± minimum and maximum values). Significantly different results (one-way ANOVA followed by the correction for multiple comparisons by controlling FDR, two-stage step-up method of Benjamini, Krieger, and Yekutieli; $p<0.05$) are indicated as (a) relative to control (0 µM); (b) relative to 600 µM VC group; (c) relative to 100 µM HYP group; (d) relative to 500 µM HYP group; (e) relative to 300 µM H_2O_2 group.

4. Discussion

ROS are important elements of the complex signalling network used by cells for biological processes. In spermatozoa, ROS participate in several physiological events such as capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion [121, 238, 239]. These events are essential for sperm function and to ensure a successful pregnancy. Nevertheless, the overproduction of ROS can trigger pathological conditions such as lipid peroxidation [120], protein modifications [106], DNA fragmentation [240], among others. Several studies reported correlations between the high levels of ROS in the male reproductive tract and, loss of sperm motility [241], morphology [242], and loss of male fertility capacity [243]. In fact, ROS-mediated damage in spermatozoa contributes to around 30-80% of male infertility cases [29]. Infertile individuals often recur to ART to pursue parenthood; however, the *in vitro* manipulation of gametes and embryos in ART techniques results in higher exposure of these cells to OS conditions. Sperm processing techniques usually involves the removal of seminal plasma, centrifugation, light exposure, and temperature variations. These procedures usually contribute to the production of supraphysiological levels of ROS, resulting in a decrease in the chances of achieving a successful pregnancy [244]. To overcome this problem and confer protection to gametes and embryos against potential oxidative injuries, culture media are often supplemented with antioxidants.

In this project, we investigated the effects of HYP supplementation on sperm media, under an H_2O_2-induced OS condition. According to the WHO manual for semen analysis [15], after semen collection, samples should be treated as soon as possible, not surpassing the 1-hour time-point, to better preserve spermatozoa physiology [15]. Taking this into consideration, we established the timeframe of 1h to test the cytoprotective effects of HYP media supplementation on spermatozoa. As a positive control we used VC, which is a water-soluble antioxidant whose effects have already been described in human spermatozoa. The dosage of VC was chosen based on several studies [154, 156, 245]. The existing literature describes the seminal plasma VC levels to vary between 200-700 µM. We established the concentration of 600 µM of VC to act as the antioxidant control for our study. Considering the lack of literature about the effects of HYP on human spermatozoa, a cytotoxicity test was performed with a range of HYP concentrations (in µM: 0, 5, 50, 100, and 500). These concentrations were chosen considering the existent data regarding the flavonol-quercetin, the hyperoside aglycone [246, 247]. Similar to those works, we tested the cytotoxicity effects of HYP in spermatozoa through the assessment of sperm motility

and vitality. Since we could not detect any alterations regarding sperm motility and vitality after 1h treatment with increasing doses of HYP, the concentrations of 100 and 500 μM were chosen for further testing. This decision was based on the published works regarding the antioxidant effects of quercetin in sperm cells [246, 247].

In our study design, we used H_2O_2 as an OS-inducer. However, existing literature regarding the OS inducer potential of H_2O_2 (*in vitro*) in human spermatozoa is very divergent. Evdokimov and colleagues [248] showed that after 1h of incubation in the presence of 100 μM of H_2O_2, there was an increase in the progressive motility of spermatozoa, while other authors report a decrease of 36% in progressive motility after 1h with 120 μM of H_2O_2 [249]. Otherwise, it was proven that higher concentrations of H_2O_2 (600 μM) cause a drastic reduction in sperm motility and viability after 10 minutes of incubation [249, 250]. In order to promote OS in spermatozoa, but not induce a total immobilization (and death), we selected the concentration of 300 μM of H_2O_2 for our study. According to our results, this concentration of H_2O_2 was able to induce a severe decrease in sperm motility, while no alterations regarding sperm vitality were detected. Thereby the effects observed in sperm motility are not due to cell death, but a consequence of the effect of H_2O_2 on motility machinery. As in other studies, sperm motility was chosen as the major indicator of sperm function [246, 247].

Our results revealed that the incubation of spermatozoa in media supplemented with VC (600 μM) and HYP (100 and 500 μM) for 1h, did not induce deleterious effects in human spermatozoa. The sperm motility in antioxidant-only supplemented groups was similar to the control group. Also, there were no alterations in the expression of OS biomarkers between those groups. The levels of DNA fragmentation, as well as the MMP, were also similar between the control group and the antioxidant-only supplemented groups. To our knowledge, there is no information regarding the impact of HYP in spermatozoa physiological and metabolic biomarkers assessed in this work. However, some authors have already reported the impact of VC media supplementation in spermatozoa [226, 251]. Raad and colleagues supplemented sperm swim-up medium with increasing doses of VC (in μM: 0, 300, and 600). The swim-up technique is used for sperm differentiation and isolation, and it is based on the movement of pre-washed spermatozoa. Only spermatozoa with normal physiology can swim up into the overlying medium. Similar to our results, the authors could not find any alteration in sperm-quality parameters (morphology and motility), after the swim-up procedure with and without VC (300 and 600 μM). Interestingly, the concentration of 300 μM VC presented lower levels of abnormal chromatin integrity in comparison to the raw semen sample [251]. Herein, we could not find any alteration in the percentage of

DNA fragmentation between the control group and the VC-supplemented group after 1h treatment. However, we must have into consideration that Raad and colleagues only considered the spermatozoa from the overlying medium, after the swim-up technique. Abnormal spermatozoa are an endogenous source of ROS, and the fact that we used the total sperm sample could be the reason why no statistical differences were found between our control group and the antioxidant-supplemented groups [251].

As a haploid cell with a high motility activity, spermatozoa metabolism has been explored in the last decades. Energy production in spermatozoa can occur through two metabolic pathways, namely OXPHOS, which occurs in the mitochondria, and glycolysis, which occurs in the head and the flagellum [252]. Regarding those pathways, there are conflicting data about the main source of ATP in human spermatozoa. It is proposed that the ATP produced by OXPHOS contributes to human sperm motility, but it is not enough to sustain high motility [253]. Meanwhile, other researchers showed that an increased mitochondrial respiratory capacity is associated with increased sperm motility, supporting the crucial role played by OXPHOS in human sperm [254, 255]. During glycolysis, glucose enters the cell and is decomposed by a sequence of events originating ATP and pyruvate as by-products. Apart from glycolysis, pyruvate can also be generated by the oxidation of lactate or by the transamination of alanine, *via* alanine aminotransferase (ALT). Then, pyruvate can either be converted to acetyl coenzyme A, which can enter the tricarboxylic acid (TCA) cycle to generate ATP *via* OXPHOS or be converted to lactate *via* lactate dehydrogenase (LDH) in the cytosol. The latter reaction results in the regeneration of cytosolic NAD^+, which is essential for glycolysis [256]. NAD^+ and NADH cannot cross the inner mitochondrial membrane and for this reason are dependent on a system that transfers reducing equivalents to mitochondria, the malate-aspartate shuttle (MAS). In the cytosol, malate dehydrogenase (MDH) reduces oxaloacetate (OAA) to malate through oxidation of NADH to NAD^+, which then enters in mitochondria. Once in mitochondria, the reverse reaction is performed by MDH, mediating the regeneration of NADH [257, 258]. Thus, malate is a metabolite that plays a crucial role in mitochondrial respiration, being part of the TCA cycle and participating in the restoration of mitochondrial NADH levels [259, 260]. Herein, we analysed human spermatozoa metabolism through quantification of metabolites in the sperm preservation media by ^{1}H-NMR, and we were able to quantify the media levels of pyruvate, acetate, lactate, alanine, and malate. Of note, we could not find any data regarding the impact of HYP and VC media supplementation on the metabolism of spermatozoa. Our findings revealed no differences in the media levels of pyruvate, lactate, and malate between

the control and antioxidants-only groups; however, some interesting differences were found regarding the levels of alanine and acetate. The levels of alanine were higher in the media supplemented with HYP (100 and 500 μM), in comparison to the control and those supplemented with VC (600 μM). No differences between the control group and the media supplemented with VC were found regarding alanine levels. As mentioned, alanine is mostly used as a source of pyruvate, through the process of alanine transamination [256]. Since the pyruvate levels remain the same between the control and the antioxidant-only groups, HYP may induce the formation of alanine to act as a metabolite reservoir. The levels of acetate were higher when HYP (500 μM) and VC (600 μM) were present in the media compared to the control, though no difference was found between the control and the media supplemented with 100 μM HYP. Acetate and the related metabolism of acetyl-coenzyme A are known to play several roles in the cell, including lipid biosynthesis, protein acetylation and energy production [261]. A previous study conducted by our group, showed that Sertoli cells (SCs) produce and excrete large amounts of acetate into the extracellular medium [262]. In addition, a recent study performed in human spermatozoa also found considerable amounts of acetate in the sperm medium [263]. Herein, we hypothesized that the higher levels of acetate in media supplemented with HYP (500 μM) and VC (600 μM) (which are the groups with higher amount of antioxidant) can be associated with a higher metabolic rate of the spermatozoa. In those groups can be occurring a higher uptake of glucose which may result in a higher production and release of acetate, as the cellular intermediates of metabolic pathways are secreted when accumulate within the cell.

The scenario changed when we added H_2O_2 as an OS inducer. As mentioned, the incubation of spermatozoa with H_2O_2 severely decreased sperm motility, even though no alterations were found regarding sperm vitality. The group only supplemented with H_2O_2 presented the highest motility loss, the highest levels of lipid peroxidation and the highest levels of DNA fragmentation. H_2O_2 also prompt a decrease in the membrane mitochondrial potential. Notwithstanding, our data demonstrate that the presence of HYP and VC in the media were able to revert some of the oxidative damage induced by H_2O_2. The spermatozoa incubated with the antioxidants in the presence of H_2O_2 did also present a decrease in sperm motility, but this decline was not as accentuated as in the one subjected to H_2O_2-only. This pattern was also observed in sperm lipid peroxidation and DNA fragmentation levels. However, regarding mitochondria damage, the decrease of MMP in spermatozoa incubated with antioxidant plus H_2O_2 was not different from the decrease of MMP observed in the spermatozoa incubated only with H_2O_2.

The antioxidant cytoprotective effects of HYP have already been reported in several cellular systems. A previous study reported that HYP was able to attenuate apoptosis triggered by H_2O_2 and *t*-BuOOH in PC12 cells (at 160 µg/mL and 100 µg/mL, respectively). The authors demonstrated that hyperoside can permeate the cell membrane and inhibit ROS formation and the propagation of free radicals reactions by chelating transition metal ions [264]. Furthermore, HYP (5 µM) has been shown to possess cytoprotective properties against H_2O_2-induced OS and apoptosis in lung fibroblast cells. The HYP pre-treatment in those cells was able to reduce lipid peroxidation, protein carbonylation, and DNA damage. The latter study demonstrated that hyperoside acts as an antioxidant through the direct action on ROS radical scavenging and by enhancing catalase and glutathione peroxidase activities [197]. In human sperm cells, Biagi M and colleagues used a *C. sativa* leaf extract (rich in HYP) to study its protective properties against OS [199], though the particular effects of HYP were not directly explored. The authors incubated swim-up selected spermatozoa with several dilutions of *C. sativa* leaf extract in Biggers-Whitten-Whittingham (BWW) buffer (1:100, 1:200, and 1:500). H_2O_2 (100 µM) was used as an OS inducer. Similar to our results, the incubation of spermatozoa with the *C. sativa* leaf extract did not decrease sperm motility in comparison to the control group. Furthermore, the authors reported a rise in lipid peroxidation levels of spermatozoa incubated with H_2O_2. Nevertheless, when spermatozoa were incubated with the *C. sativa* leaf extract in the presence of H_2O_2, the lipid peroxidation levels remain similar to the control [199]. Our results are in line with this study since we reported that the presence of HYP (100 and 500 µM) was able to prevent (to some extent) the rise in lipid peroxidation associated with high OS induced by the presence of H_2O_2. A recent study (2021) investigated the effects of quercetin supplementation in freezing media of patients with asthenospermia. The study demonstrated that adding quercetin (50 µM) significantly reduced ROS production, DNA damage and MDA levels and significantly increases post-thaw motility and viability, in comparison to the control group [265]. These quercetin proprieties were also described in several animal sperm studies [266-268]. Our results regarding sperm media supplementation with HYP are in line with the mentioned studies. Our results show that the supplementation of sperm media with HYP was able to prevent some of the deleterious effects of H_2O_2 in sperm, which were represented by an increase in sperm motility, a decrease in DNA fragmentation levels and a tendency for a decrease in lipid peroxidation levels, when compared to the spermatozoa under the H_2O_2 effect.

In opposite to HYP, VC holds diversified literature concerning its antioxidant effects on human sperm. Mangoli E. and colleagues reported that the addition of VC (600 µM) in

both neat semen and prepared spermatozoa of normozoospermic samples improved the recovery rate of sperm parameters and DNA integrity following vitrification (a technique known to increase OS) [154]. A similar result was found by Fanaei and colleagues, which incubated swim-up spermatozoa of teratozoospermic patients with VC (600 µM). The teratozoospermia condition is often associated with high rates of ROS; however, the authors found that supplementation with VC was able to decrease DNA fragmentation levels, reduce lipid peroxidation levels, and improve sperm motility [156]. This radical scavenger and DNA protective effect of VC has already been described by several authors [156, 251, 269]. Our findings are in accordance with these studies since the sperm medium supplemented with VC (600 µM) was able to preserve sperm motility, decrease DNA fragmentation levels and show a tendency for decreased lipid peroxidation levels, when compared to the spermatozoa under the H_2O_2 effect.

Nevertheless, our results also suggest that HYP and VC cannot reverse the deleterious effects of H_2O_2 regarding sperm metabolism. Interestingly, we observed that incubation of spermatozoa with H_2O_2 promoted a decrease of pyruvate levels in the media, in comparison to the control and antioxidants-only groups. Pyruvate is a pivotal metabolite able to interfere with several metabolic pathways. To understand the metabolic roots that were being promoted by H_2O_2, we investigated how the levels of several other metabolites varied. It was previously reported that pyruvate is mainly converted to lactate in human sperm [270, 271]. The decrease in lactate production found in all groups with H_2O_2, even in the presence of VC and HYP, indicates that it is unlikely for spermatozoa to be producing energy by anaerobic respiration. Alanine production also decreased in the presence of H_2O_2, even when media were supplemented with VC and HYP. Since pyruvate consumption levels are higher in media with H_2O_2, spermatozoa might be using alanine as a source of pyruvate through the process of alanine transamination [256].

To further understand mitochondria function from the spermatozoa of these groups, we can associate the malate levels and the MMP results. As mentioned, all groups incubated with H_2O_2, even in the presence of antioxidants, presented a decrease in the MMP. Along with that, these groups also presented a decrease in the secretion of malate to the media. Malate is an important component of mitochondrial metabolism, [257, 258]. The lower MMP and the levels of malate found in spermatozoa treated with H_2O_2 could suggest a decline in mitochondrial activity, which can also justify the loss of motility in those groups. A previous study reported a strong correlation between MMP and forward motility, supporting the link between the functional status of mitochondria

and sperm quality [272]. In addition, another investigation found a positive association between increased ROS in spermatozoa and decreased MMP [273]. However, in this work, we could not find an association between sperm motility and MMP levels since the supplementation of antioxidants, in the presence of H_2O_2, were able to prevent the loss of motility but did not prevent the decrease in MMP. Herein, we hypothesize that antioxidants appear to be acting in other pathways of the motility machinery to prevent the loss of motility, in the presence of H_2O_2.

Interestingly, we found that the production of acetate was increased in all H_2O_2-treated groups. The overproduction of acetate is known to be promoted by situations of exacerbated OS [261]. In fact, the reaction of exogenous pyruvate with H_2O_2 is known to induce the formation of acetate, carbon dioxide, and water [274], a chemical reaction that will be further explored in this work. In addition, it is also reported that under severe OS situations, pyruvate can exert a protective effect on several cellular systems, acting as an antioxidant [274]. In normal conditions, pyruvate plays a little role in the clearance of H_2O_2, since its concentration and availability are regulated by metabolism. Several authors have explored the antioxidant role of pyruvate in the brain of mice since neurons are highly susceptible to OS [274, 275]. In human cells, Babich and colleagues studied the susceptibility of human fibroblast cells to H_2O_2 when treated in culture media with and without pyruvate, where a cytoprotective action of pyruvate was observed [276]. Having this in consideration, we propose that the decrease of pyruvate levels found in the media of spermatozoa treated with H_2O_2 is likely caused by its reaction with H_2O_2, forming acetate, carbon dioxide, and water in the process. Given the important role that pyruvate plays in cellular energy production, we hypothesize that the overflow of pyruvate to acetate can disrupt spermatozoa metabolism. Due to the unavailability of pyruvate, other metabolic pathways, such as anaerobic and aerobic respiration may be compromised. This can justify the loss of sperm motility found in the groups treated with H_2O_2, even in the presence of antioxidants. Nevertheless, the loss of motility, the DNA damage and the LPO levels were attenuated by antioxidants presence. Notwithstanding, the levels of acetate found in the group treated with HYP (100 μM) plus H_2O_2 were lower than the levels of acetate found in the spermatozoa treated with only H_2O_2. This could indicate that supplementation with 100 μM of HYP is more efficient in the protection of spermatozoa towards H_2O_2, most likely through a better neutralization of the available H_2O_2.

Our findings demonstrate that HYP was effective in protecting human spermatozoa against oxidative damage. HYP (100 μM) was shown to be more effective than VC (600 μM) and HYP (500 μM) since lower concentrations of HYP presented similar

protective effects in human spermatozoa. Previous studies reported that VC stability is influenced by pH, temperature, oxygen, and the presence of metal ions, such as copper and iron [277, 278]. VC decomposes rather quickly in an aqueous medium, and its oxidation is highly favoured in neutral or basic conditions, being the lowest rate of oxidation observed at pH 3 [279]. On the other hand, a recent study showed that HYP is stable at pH 4-8, remains unchanged at temperatures between 25-40°C and presents an excellent photostability [280]. In fact, flavonoids have the ability to protect plants against ultraviolet (UV) light damage. This is supported not only by the fact that flavonoids absorb UV radiation and may act as sunscreens, but also by observations that exposure to UV radiation induces higher levels of flavonoids in plants [281]. The photoprotective activity of HYP can be advantageous in an ART context, as it may confer a higher level of protection to spermatozoa. All the above mentioned HYP properties along with its high antioxidant capacity enhance the potential of this phytochemical to be used in human sperm preservation solutions.

Chapter IV:
Conclusion

5. Conclusion

Human spermatozoa are known for having high susceptibility to ROS attack and a very limited antioxidant defence. Despite small amounts of ROS being required for physiological sperm functions, its overproduction may trigger pathological conditions, thereby leading to male infertility. OS also plays a significant role in the outcome of ART. The *in vitro* supplementation of sperm medium with antioxidants might prevent and/or attenuate the impairment of sperm function induced by ART techniques. However, their application in clinical practice is still limited and require more research.

In this work, we provide evidence that the supplementation of sperm medium with HYP could be beneficial for the preservation of spermatozoa in ART protocols. This phytochemical demonstrated cytoprotective properties to human sperm under OS conditions, here represented by an increase in sperm motility, a decrease in DNA fragmentation, and a decreasing trend in lipid peroxidation levels. Our findings also reveal that the concentration of 100 µM HYP appears to be the most appropriate for sperm media supplementation since this lower concentration of HYP conferred the same level of spermatozoa protection as the 500 µM of HYP and 600 µM of VC. Furthermore, we found that supplementation of 100 µM HYP, in the presence of H_2O_2, led to lower levels of acetate in the medium. Herein, we hypothesized that this concentration may have been more efficient in neutralizing H_2O_2 since it reduced the pyruvate-derived overflow pathway for acetate, promoted by ROS. Notwithstanding, the deleterious effects promoted by H_2O_2 found in mitochondrial activity and sperm metabolism could not be reversed by the presence of HYP in the sperm medium. To our knowledge, this is the first study to explore the role of HYP as an antioxidant supplement of human sperm medium and the first to explore the metabolic consequences of the presence of HYP and VC in human sperm preservation media.

Nevertheless, further studies are required to better understand the antioxidant mechanism of HYP and to investigate its interactions with other cellular components, such as the antioxidant enzymes. Increasing the success rate and safety of ART will require continuous and extensive research to improve and develop appropriate techniques to overcome the multiple factors that affect reproductive outcomes. Overall, this work highlights the importance of investigating natural compounds with antioxidant properties and their potential for being used in clinical practice in ART protocols.

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