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The epididymis as a target of endocrine disruption: effect on calcium homeostasis

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“Maybe I made a mistake yesterday, but yesterday’s me is still me. I am who I am today, with all my faults. Tomorrow I might be a tiny bit wiser, and that is me, too. These faults and mistakes are what I am, making up the brightest stars in the constellation of my life. I have come to love myself for who I was, who I am, and who I hope to become.”

– Kim Nam-joon

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Resumo

O epidídimo é uma estrutura fundamental para a fertilidade masculina. A regulação hormonal, principalmente na região do *caput*, estabelece a composição bioquímica (água, iões, moléculas orgânicas) do fluido do epidídimo, e é durante a exposição a esse ambiente único que os espermatozoides sofrem as transformações necessárias para se tornarem totalmente funcionais. Estudos em animais knockout para canais de cálcio (Ca^{2+}) demonstraram a importância da regulação dos níveis de Ca^{2+} no epidídimo para a função dos espermatozóides, uma vez que esses animais apresentaram motilidade e viabilidade espermática reduzidas, assim como, capacidade de fertilização prejudicada. Nas últimas décadas, tem surgido uma preocupação crescente relativamente ao efeito dos chamados desreguladores endócrinos (DEs) na fertilidade masculina. Sabe-se que os DEs podem perturbar a normal sinalização hormonal, desregulando a espermatogénese, no entanto, o efeito dos DEs na função do epidídimo é ainda amplamente desconhecido. Foi descrito que a vinclozolina (VNZ), um DE com atividade antiandrogénica, afeta a reprodução masculina. Assim, a presente tese investigou o efeito da VNZ na sobrevivência e função celular, homeostase do Ca^{2+} e defesas antioxidantes no epidídimo de rato. Foram realizadas culturas *ex vivo* de seções da região do *caput* na presença ou ausência de VNZ 500 μM. Posteriormente, foram avaliados os níveis de Ca^{2+}, a expressão do receptor sensível ao Ca^{2+} (CaSR), do receptor transiente de potencial membro da subfamília 6 (TRPV6), a atividade da glutationa peroxidase (GPX) e superóxido dismutase (SOD) e a atividade da caspase-3. Observou-se uma diminuição da expressão do CaSR e do TRPV6 na presença de VNZ, juntamente com um aumento das concentrações de Ca^{2+} extracelular. Além disso, a atividade das enzimas antioxidantes GPX e SOD e a atividade da caspase-3 diminuíram no grupo tratado com VNZ. Os presentes resultados demonstraram pela primeira vez as ações da VNZ na desregulação da homeostase do Ca^{2+} e redução das defesas antioxidantes no epidídimo, o que indica que a exposição a este DE pode afetar a qualidade do esperma e a fertilidade masculina.

Palavras-chave

Cálcio; Desreguladores endócrinos; Epidídimo; Vinclozolina

Abstract

The epididymis is a central player in male fertility. Hormone regulation, mainly in the *caput* region, establishes the biochemical composition (water, ions, organic molecules) of the epididymal fluid, and it is during the transit through the epididymis, and by exposure to this unique environment, that sperm undergo the transformations required to become fully functional. Studies in knockout animals for calcium (Ca^{2+}) channels demonstrated the importance of regulating epididymal Ca^{2+} levels for spermatozoa function, as these animals displayed impaired sperm motility, viability, and fertilization capacity. In recent decades, concern was raised about endocrine disruption and male fertility. Endocrine disruptors (EDs) can perturb the normal hormone signalling disrupting spermatogenesis. However, the effect of EDs in the epididymis function is largely unknown. Vinclozolin (VNZ), an endocrine disruptor (ED) with antiandrogenic activity, has been shown to affect male reproduction. The present thesis investigated the effect of VNZ on rat epididymal cells survival and function, Ca^{2+} homeostasis and antioxidant defences. *Caput* sections were cultured *ex vivo* in the presence or absence of VNZ 500 μM. Ca^{2+} levels, the expression of Ca^{2+}-sensitive receptor (CaSR) and Ca^{2+} transient receptor potential vanilloid subfamily member 6 (TRPV6), the activity of glutathione peroxidase (GPX) and superoxide dismutase (SOD) and the activity of caspase-3 were assessed. CaSR and TRPV6 expression decreased in the presence of VNZ, together with an increase in the concentrations of extracellular Ca^{2+}. Also, the activity of the antioxidant enzymes GPX and SOD and the activity of caspase-3 decreased in the VNZ-treated group. The obtained results demonstrated for the first time that VNZ dysregulates Ca^{2+} homeostasis and decreases the antioxidant defences in the epididymis, which indicates that the exposure to VNZ may affect sperm quality and male fertility.

Keywords

Calcium, Endocrine disruptors, Epididymis, Vinclozolin.

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List of Abbreviations

AQP	Aquaporins water channel
AR	Androgen receptor
ARKO	Androgen receptor knockout
BCA	Bicinchoninic Acid Protein
C	Control
Ca^{2+}	Calcium
CaSR	Calcium-sensing receptor
CO_2	Carbon dioxide
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
DTT	Dithiothreitoln
EAU	European Association of Urology
ECL	Enhanced chemiluminescence
EDs	Endocrine disruptors
ER	Estrogen receptors
ERKO	Estrogen receptors knockout
ERα	Estrogen receptor α
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized glutathione
H&E	Haematoxylin and eosin
H_2O_2	Hydrogen peroxide
HCO_3^-	Bicarbonate
KO	Knockout
Mg^{2+}	Magnesium
NIH	National Institutes of Health
O_2	Superoxide anion
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
pNA	p-nitro-aniline
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
sAC	Soluble adenylyl cyclase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SOD	Superoxide dismutase
TRPV6	Transient vanilloid receptor potential channel
TRPV6 KO	Transient vanilloid receptor potential channel knockout
VNZ	Vinclozolin
WB	Western Blot

I. Introduction

1.1 Epididymis: anatomical features and role in sperm maturation

Spermatozoa produced in the seminiferous tubules, the testis functional unit, leave the testis as non-functional gametes. It is only during the passage through the epididymis (figure 1) that spermatozoa acquire the ability to move progressively and the capacity to fertilize (1-6). The efferent ducts carry spermatozoa from the rete testis into the epididymis (figure 1), and play a crucial role in fluid homeostasis, reabsorbing more than 95% of the luminal fluid released from the testicular epithelium and increasing the concentration of sperm as they enter the epididymis (6, 7). The process of sperm concentration at this passage is crucial for male fertility and dependent on hormone actions as discussed below.

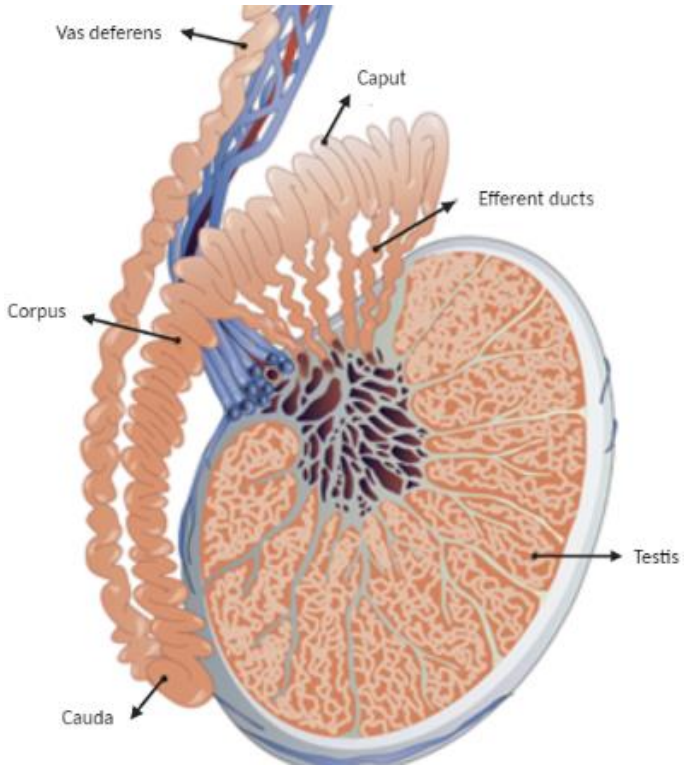

Figure 1. Schematic representation of the epididymis and its relationship with the testis in mammalians. The caput of epididymis receives testicular secretions by several efferent ducts.

The epididymis is a highly compartmentalized organ with three distinct regions, *caput*, *corpus* and *cauda*, which cooperate with different functions for sperm functionality. It is composed of septa formed by connective tissue oriented perpendicularly to the organ (8). These septa, in mice and rats, are well organized and divide the epididymis into 10 or 19 segments, respectively (9). In humans, the epididymis septa are not organized in defining

segments and show great variability from one specimen to another (8). It has been proposed that these septa provide not only support to the organ, but also a functional separation between the lobules allowing the selective expression of genes and proteins within individual lobules (10).

The epididymis has a luminal microenvironment that is regulated by the epididymis blood-barrier (11). The blood-epididymis barrier is formed by tight junctions between adjacent epithelial cells (figure 2) (11, 12) that are able to maintain an essential microenvironment for sperm, restricting the passage of solutes, ions, and macromolecules through the epididymal epithelium (13). Molecules such as carnitine and inositol can be concentrated 10 to 100 times in the lumen of the *caput* of the epididymis (14). This barrier carefully controls the microenvironment so that the sperm, during their passage through the epididymis, is bathed in an appropriate fluid milieu for each stage of maturation (14). The final composition of the epididymal fluid is achieved by the secretory and absorptive properties of epithelial cells (see below).

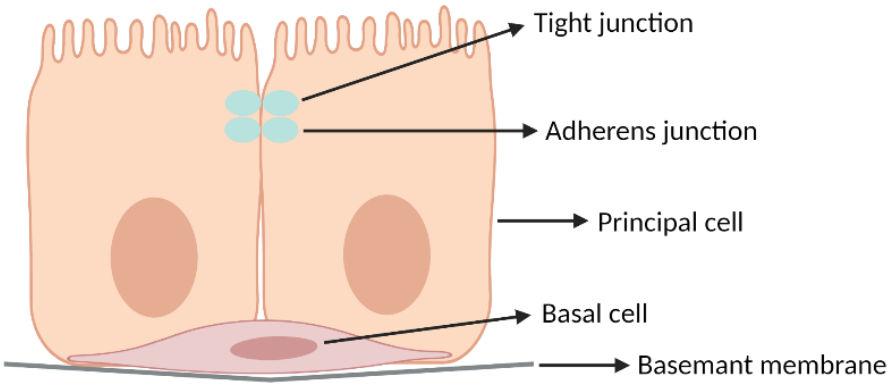

Figure 2. Organization of tight and adherens junctions. Tight junctional complexes are found between adjacent principal cells. Adapted from Dubé and Cyr, 2012.

The four main functions of the epididymis comprise transport of spermatozoa, elimination of defective gametes, storage and maturation of sperm (2). The *caput* region (figure 3) is the most active in terms of protein synthesis and secretion, as it presents a higher concentration of secreted proteins, around 70-80% of the total proteins in the epididymis lumen. The *corpus* and *cauda* region has a smaller amount of proteins (4-7). The *caput* and *corpus* regions are responsible for early and late sperm maturation, respectively, and the *cauda* is responsible for storing the functionally mature spermatozoa (13). As spermatozoa pass through this zone, they acquire the ability to acquire progressively motility and recognize an oocyte (4-7, 15), which is determined by the composition of the epididymal fluid. In mammals, the normal duration of the transit

through the epididymis *cauda* is in the range of 3 to 10 days, but spermatozoa can be stored in this segment for periods extending beyond 30 days (13).

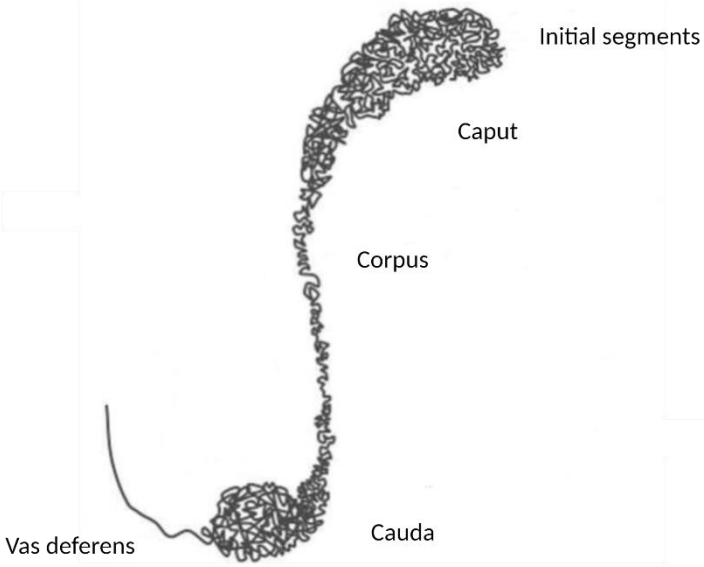

Figure 3. Schematic organization of the rat epididymis, showing the three regions of the epididymis – *caput*, *corpus*, and *cauda*, as well as the initial segments and vas deferens.

The epididymal lumen has a unique biochemical microenvironment established by the secretory and absorptive activities of the epididymis epithelial cells. The epididymis is made up of a pseudostratified epithelium containing several cell types, such as principal cells (65 to 80%), and also apical, basal, clear, narrow, and halo cells (figure 4). Principal cells are present along the duct, with structural differences, and are responsible for the secretion of proteins into the lumen (13, 16). These cells work together to maintain the ideal luminal microenvironment favouring sperm functionality.

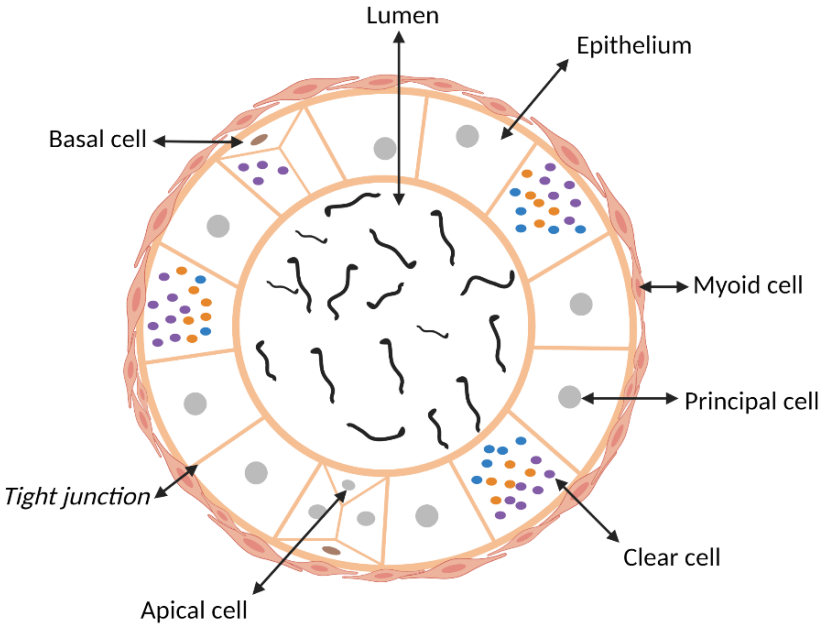

Figure 4. Schematic organization of the major cell types in the epididymis. The relative position and distribution of each of the main cell types are illustrated.

The epididymal lumen is rich in inorganic ions and organic molecules that create the ionic, oxidative, and pH environment suitable for sperm maturation. Sperm migrate from a gradually lower ionic environment from the *caput* to the *cauda*, but in a progressively higher organic solute and protein environment (13).

Epididymal fluid has controlled concentrations of sodium, chloride, potassium, magnesium, bicarbonate (HCO_3^-), and Ca^{2+} ions (13, 17-19). However, Ca^{2+} concentrations in the epidydimal fluid are quite low comparing with those of others ions like sodium, potassium, among others (20). This ionic concentration pattern together with luminal acidification helps to keep sperm in a quiescent state as they mature (21, 22). The water transport along epididymis and acidification of epididymal fluid are critical events to ensure the appropriate sperm function. The increase in sperm concentration in the epididymis occurs due to the removal of water from the luminal fluid, which depends on through different transporters present in the membrane. Alterations in these transporters, such as the aquaporins water channel (AQP), resulted in impaired fertility (23-25).

The pH of the epididymis luminal remains at 6.5-6.8 keeping sperm in a quiescent state during maturation and storage (26). The acidification and reabsorption of HCO_3^- are controlled by the interaction of different cells types present in the pseudostratified epithelium of the epididymis, and have an essential role in maturation and obtainment of adequate sperm motility (16, 27-29). The importance of luminal acidification was

demonstrated in studies with male c-ros knockout mice that are infertile and exhibited an abnormally high epididymal luminal pH (21, 27).

It is also known that the epididymis has a role in the antioxidant defence, protecting sperm from oxidative damage, through the synthesis and secretion of specific proteins, and antioxidant compounds (13).

Physiological levels of reactive oxygen species (ROS) are important to preserve the integrity of the seminal fluid (30, 31), and are involved in the regulation of some sperm functions, playing positive effects on maturation. However, high levels of ROS can impair sperm viability, motility and fertility (32, 33). ROS induce chemical and structural changes that are harmful to sperm nuclear DNA and damage proteins and lipids in sperm plasma membrane negatively affecting the sperm function (13, 30, 34). This is particularly relevant as sperm are not capable of translating proteins and therefore need an exogenous antioxidant protection. The epididymis is able to express these antioxidant enzymes, playing a crucial role in maintaining appropriate ROS levels and protecting sperm from oxidative damage (13, 33). Among these enzymes are the glutathione peroxidase (GPX) and superoxide dismutase (SOD) (13), which play a crucial role in the epididymis' antioxidant defence (13).

SOD converts oxygen free radicals to hydrogen peroxide, starting a chain reaction whereas GPX catalyzes the reduction of hydroperoxides, such as hydrogen peroxide, by reducing glutathione (figure 5).

Therefore, elevated ROS levels can occur due to excessive ROS production or decreased antioxidant defences, such as diminished activity of SOD and GPX (32).

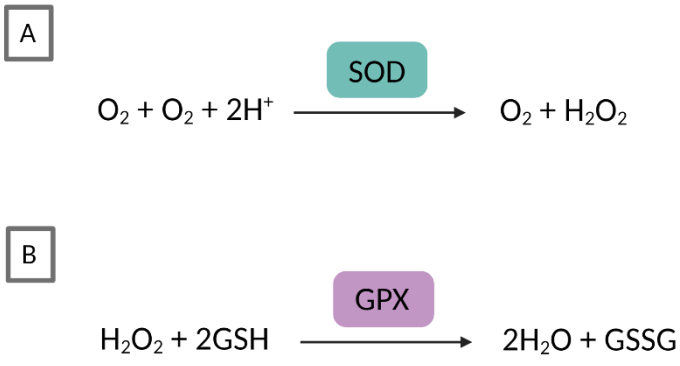

Figure 5. Reactions catalysed by the antioxidant enzymes SOD and GPX. **A.** The free radical superoxide anion (O_2) is supported by SOD which transforms it into hydrogen peroxide (H_2O_2). **B.** GPX plays the role of antioxidant enzymes by recycling H_2O_2 in the presence of two glutathione (GSH) molecules.

1.2 Hormonal regulation of the epididymis function

The absorptive and secretory activities of the epididymal epithelium are hormonally regulated, namely by androgens and estrogens. These steroid hormones affect a wide range of epididymal processes, like intermediary metabolism, transport mechanisms, and protein synthesis and secretion. Indeed, the synthesis of several secretory proteins is androgen-dependent (35). Steroid hormones reach the epididymis by way of the rete testis fluid and the bloodstream, being testosterone the principal androgen in rete testis fluid (35). However, there is a marked change in the androgenic constituents of luminal fluid after it enters the epididymis, with dihydrotestosterone (DHT) becoming the predominant androgen.

The action of androgens is mediated by the androgen receptor (AR) protein (36-40). The AR is differently expressed along the epididymis depending on the cell type, and being more expressed in the *caput* and *corpus* region (41). Androgens actions mediated by the AR are also required for the normal male sexual development (42). Studies of the genetic mutations in AR and the generation of AR knockout (ARKO) mice models demonstrated that the presence of functional AR is mandatory for the establishment of male phenotype (43). The absence of AR leads to a female-like appearance and body weight (43). Furthermore, it was identified that ARKO mice did not develop the initial segment of the epididymis and accumulate cell debris, proteinaceous material, and, at later ages, spermatozoa in their efferent ducts, which prevents the normal passage of spermatozoa from the testis into the epididymis, leading to infertility (44). Another study in transgenic male mice with AR deletion, reported the occurrence of an epididymal obstruction and consequently infertility in these animals , showing the direct role of androgens in regulating the epididymis (45).

Beyond androgens, also estrogens, vitamin D, melatonin, prolaction, progesterone, and growth factors have being associated with the regulation of epididymal function (46). Among these factors, it is noteworthy the action of estrogens in fluid reabsorption in the efferent ductules and proximal epididymis. Nuclear estrogen receptors (ER) α and β, which mediate the cellular responses of estrogens, are expressed in the efferent ductules and epididymis (47). They are responsible for regulating fluid reabsorption, essential for sperm concentration and thus improving sperm survival and maturation during passage through the epididymis (48). A study in the ERα gene knockout mice (ERKO) demonstrated that these animals are infertility, displaying inhibition of fluid reabsorption by the efferent ducts, reduced sperm concentrations, and abnormal sperm. As a result, the sperm enter the epididymis diluted, which results in infertility (49). These

findings definitely demonstrated the importance of fluid absorption for sperm maturation.

1.3 Ca^{2+} homeostasis in the epididymis

The process of sperm maturation occurring throughout the transit in the epididymis is a consequence of the specific absorption and secretion activity in the different segments of the epididymis. The *caput*, *corpus* and *cauda* regions differ in terms of protein secretion, water, and Ca^{2+}handling, which establishes this ion levels in the epididymal fluid. Moreover, it was shown a gradient of Ca^{2+} concentrations from *caput* (high) to distal regions (low) (13).

Also, the influence of intracellular signal transduction mechanisms in regulating the motility and metabolism of rat spermatozoa was examined in undiluted epididymal fluid from the *cauda* region (50). It was suggested that the motility of rat epididymal spermatozoa from the *cauda* is regulated by Ca^{2+} and the guanylate cyclase and adenylate cyclase pathways (50).

The main role of Ca^{2+} in sperm function has been associated with the capacitation process that occurs within the female reproductive tract and depends on the progressive activation of the cAMP-PKA-dependent signalling pathway mediating protein tyrosine phosphorylation (51, 52). Moreover, Ca^{2+} has been shown to be crucial for the initiation and maintenance of hyperactivated motility (53).

There is evidence that human spermatozoa express a cyclase with the properties of soluble adenylyl cyclase (sAC) and that Ca^{2+} can substitute bicarbonate ions ($HCO3^-$) in the stimulation of this enzyme, underscoring an important role for sAC and Ca^{2+} in the control of sperm function (54).

Another study proposed that external Ca^{2+} binds an unidentified extracellular protein that is required for HCO3- to engage cAMP-mediated activation of sperm motility, by increasing flagellar beat frequency (55, 56).

Ca^{2+} influx and efflux across epididymal epithelium and the maintenance of intracellular Ca^{2+} concentrations are controlled by an orchestrated action of several proteins, Ca^{2+} pumps, Ca^{2+} channels and diverse Ca^{2+} -binding proteins, which determines the fine tuning of Ca^{2+} levels in the luminal environment. Studies in genetically modified animal models have been shown the huge importance of strictly maintaining epididymal Ca^{2+} concentrations, and identified target regulators in this process. It is the case of the

transient vanilloid receptor potential channel (TRPV6) and the calcium-sensing receptor (CaSR) that will be discussed in the next sections. Also, the importance of maintaining Ca^{2+} levels in the epididymal lumen was demonstrated in a transgenic rat model overexpressing the Ca^{2+} binding protein regucalcin. These animals showed decreased sperm counts and motility, concomitantly with the unbalanced Ca^{2+} concentrations in the epididymis lumen and a decreased height of the epididymal *caput* epithelial cells (20).

1.3.1 TRPV6

Ca^{2+} pumps either extrude Ca^{2+} across the plasma membrane to the extracellular environment or uptake Ca^{2+} into intracellular organelles. The TRPV6 plays an important role in maintaining blood Ca^{2+} levels in higher organisms. It has different characteristics from other members of the TRP superfamily of cation channels, as the: location of the apical membrane in epithelial tissues transporting Ca^{2+}, responsiveness to 1,25-dihydroxy vitamin D3, and high selectivity for Ca^{2+} over other cations (57, 58). TRPV6 is broadly expressed in the intestine, placenta, pancreas, prostate, salivary gland and sudoriparous, testis and epididymis (58).

It was demonstrated that the targeted disruption of the TRPV6 (TRPV6 KO) epithelial Ca^{2+} channel leads to a marked disturbance of Ca^{2+} homeostasis (59). Weissgerber et al. (60) disclosed the role of this Ca^{2+}-selective TRPV6 channel in regulating Ca^{2+} concentration in the lumen of the epididymis as well as sperm motility and survival. When the aspartate 541 residue is replaced by alanine (D541A) in the pore of the mouse TRPV6 channel, the epididymal epithelium is unable to absorb Ca^{2+}, causing an increase of Ca^{2+} in the epididymal lumen (61). Male transgenic mice harbouring the inactive form of TRPV6 showed decreased fertility, and the motility and viability of sperm isolated from the *cauda* region of epididymides were impaired (59, 62).

1.3.2 CaSR

The CaSR, is a Class C G-protein coupled receptor which senses extracellular levels of Ca^{2+} ions. CaSR plays a key role in Ca^{2+} homeostasis, monitoring the levels of Ca^{2+} in the body, controlling vastly other biological processes (63, 64).

It is a widely expressed receptor in the lung (65), epidermis (66), cardiovascular system (67), intestine (68), and pancreas (69), and is in charge of being a regulator for several processes as differentiation, proliferation and apoptosis (70). Furthermore, CaSR was identified in testicular tissue, sperm cells, and epididymis (71). The outstanding role of CaSR in Ca^{2+} homeostasis together with its expression in reproductive organs suggests a

role in the reproductive processes (71). Indeed, evidence for the functional involvement of CaSR in physiologic mammalian reproductive processes exists (72).

Changes in CaSR in the testis and epididymis are known to affect sperm motility (71). In addition, CaSR is also expressed in human sperm and is essential for detecting extracellular Ca^{2+} and Mg^{2+}. It was identified that patients with mutations in CaSR have sperm with lower influx of Ca^{2+} and that they are less likely to undergo acrosome reaction (64). Moreover, a patient with a heterozygous dominant loss-of-function mutation in CaSR (Trp818Stop in exon 7) causing hypocalciuric hypercalcemia type 1, showed low sperm counts, motility and morphology (64). Therefore, mutations, alterations or loss of function of the CaSR can impair sperm motility and function, and consequently affect male fertility.

1.4 Endrocrine disruptors

In 1991, the Wingspread Conference was officially introduced the term endocrine disruptors (EDs) (73). An ED is an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action (74). These compounds are natural or synthetic exogenous agents that are capable of interfering with the secretion, synthesis, transport, binding, action, or elimination of natural hormones that in turn are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour (74, 75). EDs have different structures and nature, which highly complicates predicting their mechanism of action, but over the years studies reported that some of these disruptors have an estrogenic, antiandrogen, thyroid hormone receptor agonist or antagonist activity (76).EDs are capable of being antagonists, preventing the endogenous hormone from binding to their receptors and, thus not emitting any signal. In opposition, other compounds can mimic the body's hormones, such as estrogens and androgens, causing overstimulation and interfering or blocking other hormones or receptors (77).

Concerning reproduction, alterations or disturbances normally occur through processes mediated by androgens and estrogens. Some reports have shown that exposure to some types of EDs, at different and specific periods of development, can hamper male reproductive function, decrease mean sperm counts, and cause a dysfunction in the formation of sex organs (78, 79).

At least 30 million men are infertile, and male infertility rates are highest in Europe, where 7.5% of men are infertile, as indicated in the European Association of Urology (EAU) guidelines for male infertility (80, 81). It has been suggested that life style, habits and the environmental exposure to damaging substances can be the reasons underlying

this trend with an increased number of infertile men. Therefore, it is essential to understand better the mechanism behind EDs and their effects on the male reproductive system.

1.4.1 Vinclozolin

Vinclozolin (VNZ, 3-(3,5-diclotophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione, CAS number 50471-44-8) is a fungicide that inhibits spore fermentation, usually used in fruit plantations (e.g. grapes) and vegetables to protect against disease. In Europe, it was widely used until 2007, being banned in some countries, such as Sweden, Denmark, Norway, and Finland (82, 83).

The effects of VNZ are mediated by two active metabolites, M1 and M2 (figure 8), which competitively bind to the AR, being able to antagonize the binding of natural androgens to this receptor and interfering with the expression of androgen-dependent genes (84). Therefore, VNZ is an ED that can impair the function and/or development of tissues that are sensitive to testosterone or other androgens, as is the case of male reproductive organs. Also, VNZ involvement in reproductive tract abnormalities has been suggested (83).

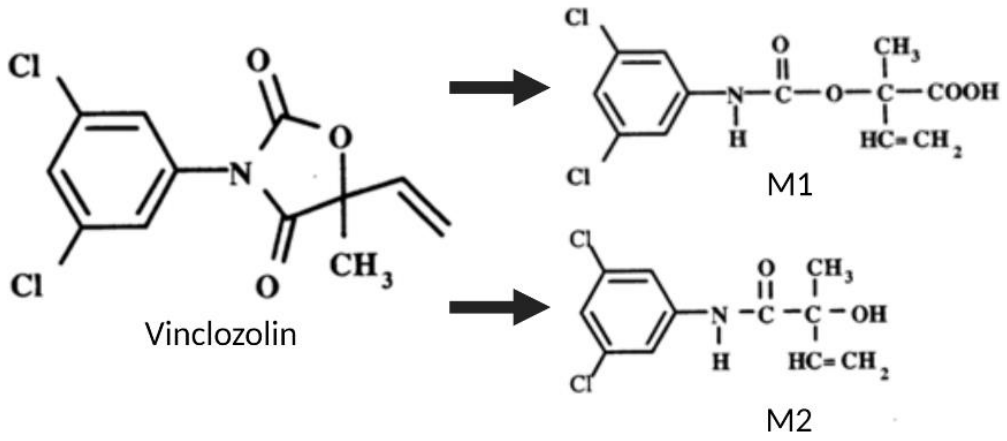

Figure 6. Representation of the chemical structure of vinclozolin (VNZ) and its active metabolites (M1 and M2).

Exposure to VNZ affects the male reproductive system in different species during gestational morphogenesis and pubertal maturation (85-87). In a study in which pregnant female rats were exposed to VNZ at a dose of 100 to 200 mg/kg/day, an inhibition of the sexual differentiation of male offspring was observed (88). Moreover, an *in vitro* study with VNZ was performed in order to analyse its effect on the embryonic testicular cord formation, as well as the effects of transient *in utero* VNZ exposure on postnatal testis development and function. After treatment with 50-500 µM of VNZ,

gonads had significantly fewer cords and histological analysis demonstrated an abnormal formation of the testicular cords (85). In addition, transient *in utero* exposure to VNZ increased apoptotic germ cell numbers in the testis of pubertal and adult animals, In accordance, other studies have identified that exposure to VNZ can reduce sperm counts, daily sperm production (89-93) and sperm motility (85, 90) and malformations such as abnormally enlarged sperm head (92), cryptorchidism (89, 91) and hypospadias (89, 93).

There are several studies evidencing the effects of VNZ exposure, especially *in vivo*. However, few are found in what concerns the effect of this ED in epididymis function.

II. Aim of this dissertation

During the last decades, epidemiological studies have been reporting worrisome trends in the incidence of human infertility rates. Extensive detection of industrial chemicals in human serum and seminal plasma has led the scientific community to hypothesize that these compounds may act as EDs, leading to a vast array of physiological impairments. EDs are environmental compounds that perturb the endocrine function by altering the hormone metabolism, synthesis and mechanism of action. Vinclozolin, an antiandrogenic ED present in the environment as a result of human activities, has been shown to affect male reproduction. However, the effect of EDs in the epididymis function is largely unknown.

The epididymis is a central player in male fertility since spermatozoa leave the testis as non-functional gametes and it is during the passage through the different segments of the epididymis that they acquire the ability to move progressively and to become fully functional. This maturation process includes several biochemical and functional changes in the spermatozoa, which is dependent on androgen actions and the absorptive and secretory activity of epithelial cells that establish the unique ion and protein composition of epididymal fluid.

The importance of tightly controlled Ca^{2+} concentrations in the epididymal fluid in order to ensure proper sperm function was recently demonstrated.

The present work hypothesizes that the ED VNZ can disrupt the rat epididymis function by affecting epididymal cells survival and function, Ca^{2+} homeostasis and the antioxidant defences. For this purpose, *caput* epididymis sections were cultured *ex vivo* in the presence or absence of VNZ 500 μM, and the following parameters were evaluated:

- Ca^{2+} levels;
- Expression of CaSR and TRPV6;
- Activity of the antioxidant enzymes, GPX and SOD;
- Apoptosis by means of quantification of caspase-3-like activity.

III. Material and Methods

3.1 Animals and tissue collection

Wistar rats (*Rattus norvegicus*) were obtained from Charles River (Barcelona, Spain) and handled in compliance with the European Union rules for the care and use of laboratory animals (Directive 2010/63/EU), and with the guidelines established by the "Guide for the Care and Use of Laboratory Animals", published by the US National Institutes of Health (NIH Publication No. 85-23, revised on 1996). Rats were housed under a 12 h light:12 h darkness cycle, in a constant room temperature (20 ± 2 °C) with food and water available *ad libitum*. Four-month-old animals (n=6) were euthanized with carbon dioxide (CO_2) and the epididymides removed, trimmed free of fat and washed in cold phosphate-buffered saline (PBS).

3.2 Culture of *Caput* Epididymis

One epididymis from each animal (n=6) was orientated to identify the three functional segments, i.e. *caput, corpus* and *cauda,* and the *caput* region was placed in 6-well culture plates (VWR, Radnor, USA) containing 4 mL of pre-warmed RPMI (Sigma-Aldrich, United Kingdom) culture medium (control group). The contralateral *caput* epididymis was placed in RPMI containing 500 µM VNZ. After 48 hours of culture in an atmosphere of 5% CO2 at 37 ºC, the control and VNZ-exposed *caput* epididymides were recovered from the medium, snap frozen in liquid nitrogen, and stored at - 80 ºC until protein extraction. *Caput* epididymis samples from the control group were fixed in 4% paraformaldehyde (PFA, Acros Organics, New Jersey, USA) for histological processing and posterior haematoxylin and eosin (H&E) staining.

In addition, epididymis culture medium was collected and stored at -20ºC for the colorimetric quantification of Ca^{2+} levels.

3.3 Protein extraction

Control and VNZ-treated epididymis tissues were homogenized in RIPA buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0 and 1 mM EDTA) supplemented with 1% protease cocktail inhibitors (Sigma-Aldrich) and 10% phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich). 3 µL of RIPA per mg of tissue was added to each sample, then they were macerated with cotton swabs until the tissue was in small pieces. Samples were kept on ice for 40 min to 1 hour and mixed from time to time. Subsequently, samples were centrifuged at 14000 rpm, 4 ºC, for 20 min, and the supernatant was collected to fresh tubes and kept at - 80 ºC. Total protein

concentration was determined through the Bicinchoninic Acid Protein (BCA) method, using Bovine Serum Albumin as standard.

3.4 Western Blot (WB)

Total proteins were denatured at 100 ºC for 5 min using loading buffer (10% 2-Mercaptoethanol) and resolved on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Membranes were incubated overnight at 4 ºC with the mouse anti-CaSR (1: 1000, C0493, Sigma-Aldrich) and rabbit anti-TRPV6 (1: 500, SC-28763, Santa Cruz Biotechnology, California, USA) primary antibodies. Mouse-IgGκ BP-HRP (1: 10000, SC-516102, Santa Cruz Biotechnology) and goat anti-rabbit IgG-HRP (1: 10000, 1706515, Bio-Rad) were used as secondary antibodies. A mouse anti-β-actin (1: 10000, 5441, Sigma-Aldrich) antibody was used for protein loading control in all WB analyses. Membranes were developed with enhanced chemiluminescence (ECL) substrate (Bio-Rad) for 5 minutes and immune-reactive proteins were detected with the ChemiDoc™ MP Imaging System (Bio-Rad). Bands density was assessed according to standard methods using the Image Lab 5.1 software (Bio-Rad) and normalized by division with the respective β-actin band density.

3.5 Quantification of calcium

The commercial kit Calcium-*o*C (BSIS07-P, SpinReact, Girona, Spain) was used, to determine the Ca^{2+} levels present in the culture medium of control and VNZ-treated *caput* epididymis, following the manufacturer's protocol. The measurement of Ca^{2+} is based on the formation of a colour complex between Ca^{2+} and o-cresolphtalein in an alkaline medium. Briefly, 2 µl of culture medium (from both control and VNZ-treated samples) were mixed with the monoreagent containing the ethanolamine buffer and the chromogen, incubated for 10 min at 37 ºC, and the absorbance was read at 570 nm in xMark™ Microplate Spectrophotometer (Bio-Rad). Ca^{2+} concentration was determined against a standard provided in the kit, which was handled following the same procedures described.

3.6 Superoxide dismutase (SOD) assay

Superoxide dismutase activity in control and VNZ exposed samples was measured through a competitive inhibition assay (SOD Assay Kit, Sigma-Aldrich) using WST-1 substrate (tetrazolium salt) and xanthine oxidase following the manufacturer's instructions. Upon reduction with superoxide anion, WST-1 produces a water-soluble

formazan dye, which is linear with xanthine oxidase activity and inhibited by SOD. The assay was monitored by measuring the absorbance at 450 nm (the wavelength of the absorbance for the coloured product of the WST-1 reaction with superoxide) after a reaction time of 20 min at 37 °C using the xMark™ Microplate Spectrophotometer (Bio-Rad). Percentage of reaction inhibition indicates the SOD activity. Results were expressed as the activity ratio (percentage of inhibition) per mg of protein.

3.7 Glutathione peroxidase (GPX) assay

GPX activity in control and VNZ-treated *caput* epididymis was determined using a commercial kit (703102, Cayman Chemical) according to the manufacturer's protocol. Briefly, GPX activity was measured by indirectly monitoring the glutathione reductase (GR) coupled reaction. Oxidized glutathione (GSSG), produced in the reduction of an organic hydroperoxide by GPX, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The absorbance was measured at 340 nm every minute and 5-time points were registered using the xMark™ Microplate Spectrophotometer (Bio-Rad). Under these conditions in which the GPX activity is rate limiting, the rate of decrease in absorbance is directly proportional to GPX activity in the sample. Results were expressed as U/L/ug of protein.

3.8 Caspase-3-like activity assay

Caspase-3 activity assay was performed as previously described (94, 95). Briefly, 25 µg of total protein extracted from the *caput* epididymis were incubated with reaction buffer (25 mM HEPES, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1 -propanesulfonate, 10% sucrose, and 10 mM dithiothreitoln (DTT), pH 7.5) and 100 µM of caspase-3 substrate (Ac-DEVD-pNA) for 2 hours at 37°C in a 96-well plate. Upon caspase cleavage, p-nitro-aniline (pNA) is released producing a yellow color, which is measured spectrophotometrically at 405 nm (xMark™ Microplate Spectrophotometer, Bio-Rad). The amount of generated product was calculated by extrapolation of the standard curve of free pNA.

3.9 Statistical analysis

The statistically significant differences between the control and VNZ-treated groups were assessed by the Unpaired Student's T-Test. Differences were considered significant when $p < 0.05$. Data analysis was performed using the GraphPad Prism v6.01 software (GraphPad Software, San Diego, California, USA). Experimental data are represented as mean ± SEM.

IV. Results

1. Morphological status of cultured *caput* epididymis

H&E staining was used to assess the cellular and morphological structure of cultured *caput* epididymis tissues. As showed in figure 7, structural epididymis architecture is maintained after 48 hours of culture.

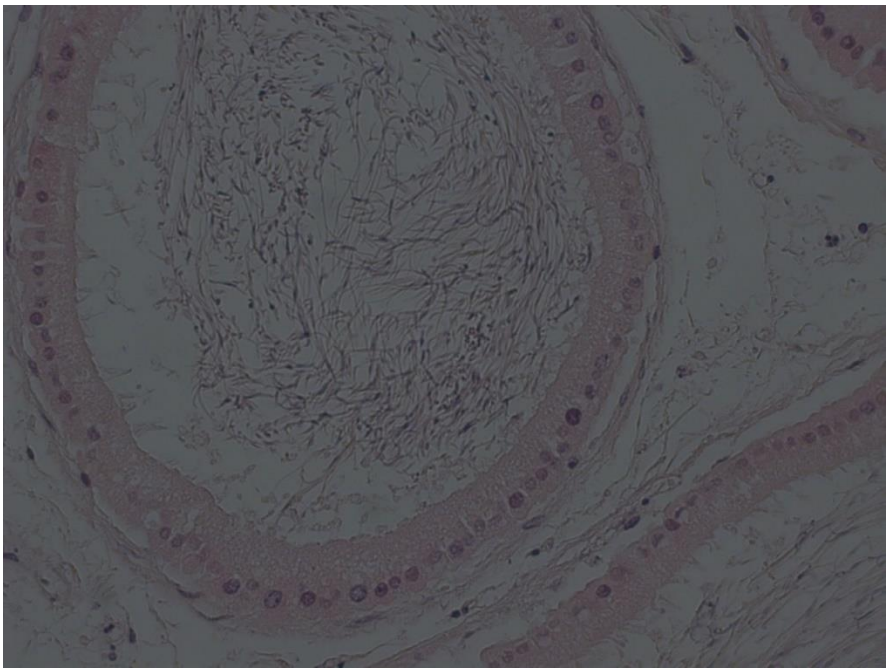

Figure 7. Representative photomicrograph of an H&E stained section of *caput* epididymis cultured for 48 hours (400x magnification; Zeiss).

2. Calcium homeostasis in the *caput* epididymis in the presence of VNZ

a. VNZ decreased CaSR and TRPV6 protein expression

The decreased concentration of Ca^{2+} along the epididymis generating a luminal gradient has been described (18). Moreover, the presence of Ca^{2+} modulators, such as CaSR and TRPV6 has been described in the epididymis (96, 97). The CaSR translates changes in extracellular Ca^{2+} concentrations into cellular reactions. Beyond its function in sperm capacitation a role in the regulation of sperm motility has been described (63, 64, 71, 72). There is also indications for the significant involvement of TRPV6 channels in sperm development and function. Although KO mice for TRPV6 showed intact spermatogenesis, these animals are infertile due to decreased sperm motility and viability (59, 61, 62). WB analysis showed that CaSR expression (figure 8. A) was reduced

by approximately an half in the *caput* epididymis treated with 500 μM VNZ for 48 hours in comparison with the control group (VNZ: 0.50 ± 0.04 vs. C: 1.00 ± 0.14; $p < 0.05$). A similar pattern was observed for the expression of TRPV6 protein (figure 8. B), which significantly decreased in the *caput* epididymis exposed to VNZ compared to control group (VNZ: 0.60 ± 0.06 vs. C: 1.00 ± 0.05; $p < 0.01$).

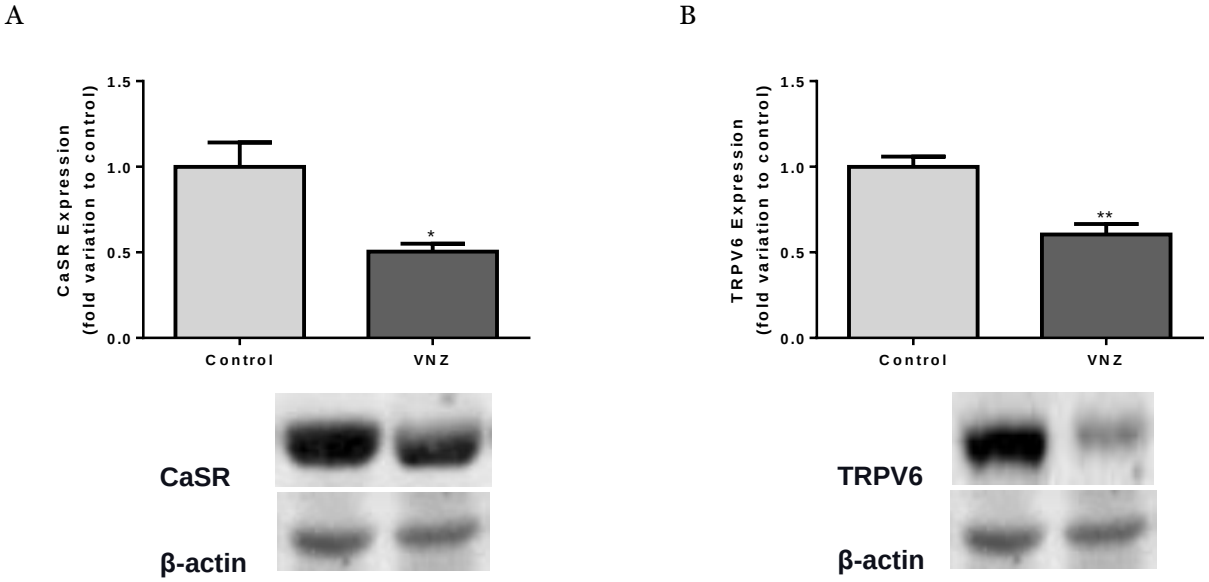

Figure 8. Protein expression of CaSR (**A**) and TRPV6 (**B**) in the caput of rat epididymis cultured for 48 h, in the presence or absence of 500 μM VNZ. Data are represented as mean ± S.E.M. (n=6 in each group). Results are expressed as fold-variation compared to the control group in the absence of VNZ after normalization of protein expression with β-actin. (*) Statistically significant difference when compared with the control group (* $p < 0.05$; ** $p < 0.01$). Representative immune-blots are shown in bottom panels.

b. VNZ increased extracellular Ca^{2+} levels in the *caput* epididymis

We also sought to determine the effect of VNZ on Ca^{2+} handling by the *caput*, which was analysed by quantifying this ion levels in the tissue culture medium by the end of culture period (48 h). Extracellular Ca^{2+} levels (figure 9) were strongly enhanced in response to VNZ- treatment (VNZ: 0.40 ± 0.01 vs C: 0.24 ± 0.02; $p < 0.001$), relative to the control group.

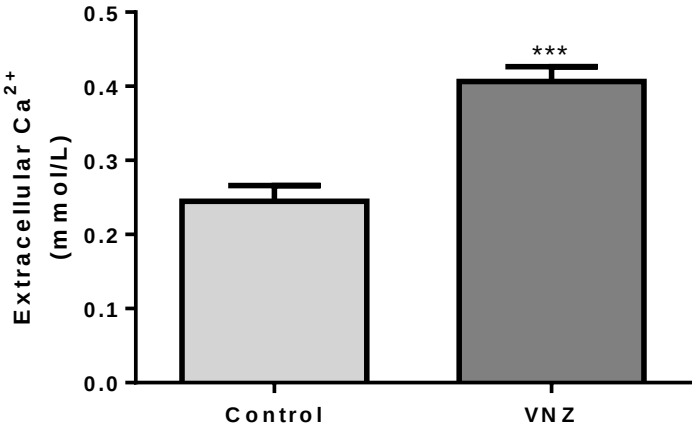

Figure 9. Calcium (Ca^{2+}) levels in the culture medium of rat *caput* epididymis in the presence or absence of VNZ (500 μM) upon culture for 48 h. Data are represented as mean ± SEM with n=6 in each group. *** Statistically significant difference when compared with the control group in the absence of VNZ (*** $p < 0.001$).

3. Antioxidant defences in the *caput* epididymis in the presence of VNZ

a. VNZ diminished the activity of SOD

It is widely known that oxidative stress adversely affects the spermatogenic process, also interfering with the proper sperm function (30, 34). It is also known that the epididymis plays a relevant role in protecting sperm from oxidative damage (98, 99). SOD is an enzyme that converts oxygen free radicals into hydrogen peroxide, starting a chain reaction, with an antioxidant defence role. Thus, we determined SOD activity as a measurement of the antioxidant status in response to VNZ exposure. SOD activity (figure 10) was significantly decreased in the *caput* epididymis treated with VNZ (500 μM) when compared to the control (VNZ: 5.94 ± 0.24 vs C: 7.23 ± 0.51; $p < 0.05$).

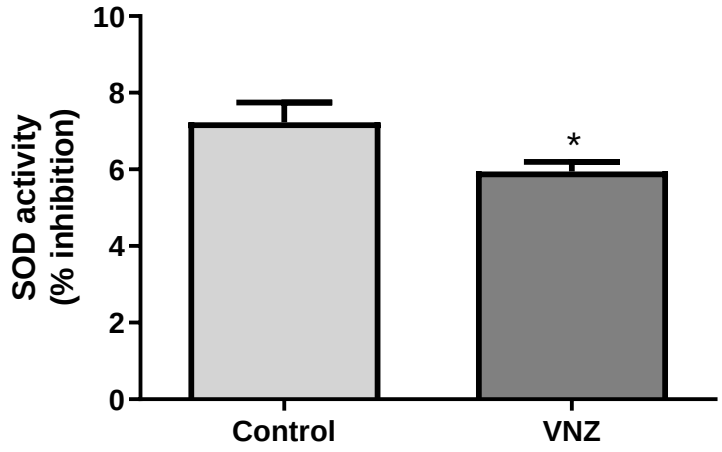

Figure 10. Superoxide dismutase (SOD) activity in the rat *caput* epididymis cultured for 48 h in the presence or absence of VNZ (500 μM). Data are represented as mean ± SEM (n=6 in each group). * Statistically significant difference when compared to the control group in the absence of VNZ (* $p < 0.05$).

b. VNZ diminished the activity of GPX

GPX is the enzyme responsible for catalyzing the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione, and as SOD has a crucial role in the antioxidant defence. GPX activity (figure 11) was significantly decreased in the *caput* epididymis treated with VNZ (500 μM) when compared to the control group (VNZ: 0.05 ± 0.01 vs C: 0.13 ± 0.02; $p < 0.05$).

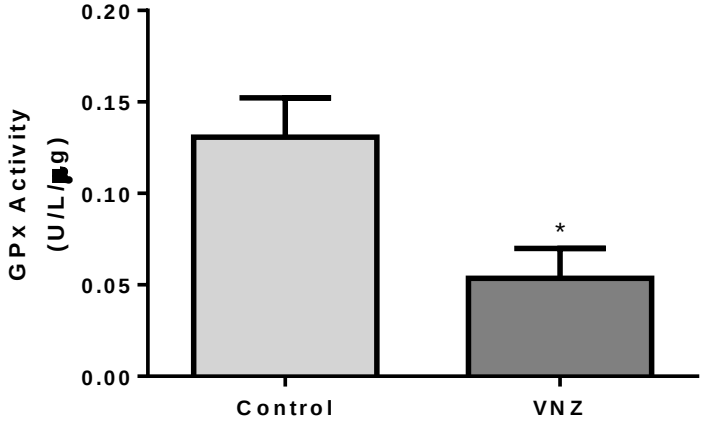

Figure 11. Glutathione peroxidase (GPX) activity in the rat *caput* epididymis cultured for 48 h in the presence or absence of VNZ (500 μM). Data are represented as mean ± SEM (n=6 in each group). * statistically significant difference when compared to the control group in the absence of VNZ (* $p < 0.05$).

4. Apoptosis status in the *caput* epididymis in the presence of VNZ

Activation of cell death pathways invariably leads to the stimulation of caspase activity, culminating in the activation of caspase-3 (100). Thus, we determined caspase-3 like enzymatic activity as a measurement of apoptosis in the experimental conditions. A decreased of almost 32% in caspase-3 activity was observed in cultured *caput* epidydimis in response to VNZ 500 μM stimulation for 48 hours ($p < 0.05$, figure 12).

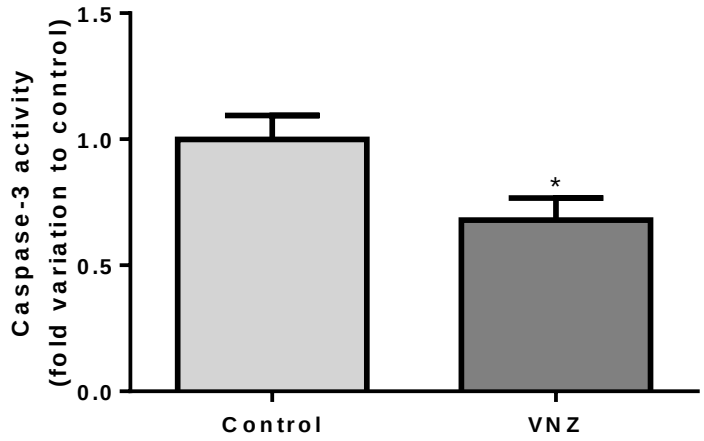

Figure 12. Caspase-3 like activity in the rat *caput* epididymis cultured for 48 h in the presence or absence of VNZ (500 μM). Data are represented as mean ± SEM (n=6 in each group). * statistically significant difference when compared to the control group in the absence of VNZ (* $p < 0.05$).

V. Discussion

Sperm maturation depends on the exposure to a panoply of signals in the epididymal lumen, which result from the secretory activity of epididymis epithelial cells (13, 16). It is in the lumen of the epididymis, that spermatozoa found the adequate biochemical composition (water, ions, organic molecules) necessary to become fully functional sperm (6, 13). Because of its crucial role in sperm transport and maturation, functional and structural abnormalities in the epididymis are translated in alterations in the composition of the epididymal fluid and can be associated with infertility or reduced fertility.

Ca^{2+} concentrations in the epididymis are low, but this ion was shown to be a pivotal player for sperm function. Knockout animals for Ca^{2+} channels (e.g. TRPV6) displayed impaired sperm motility and viability, and reduced fertilization capacity (20, 101, 102), which demonstrated that a fine tuning regulation of Ca^{2+} concentrations in the epididymal fluid is needed and may be affected by the altered activity of a single Ca^{2+} handling protein.

In the recent decades, a major concern has been placed on the role of EDs triggering male fertility. Several studies have been showing that the exposure to EDs can cause several dysfunctions, namely disrupted hormonal signalling, abnormalities, interruption of spermatogenesis, and reduced sperm motility and quality (78, 103). However, little is known about the effect of EDs on epididymal function.

VNZ is an ED with antiandrogenic activity, which has been shown to affect male fertility, with changes at testicular levels (83, 85). However, the molecular mechanisms through which it affects the reproductive function are poorly understood (83-85). In addition, the impact of VNZ on the epididymis and how it can affect epididymal Ca^{2+} homeostasis and oxidative status is poorly explored.

To start fulfilling this lack of knowledge, the present work investigated the effect of VNZ on rat epididymal cells survival and function, Ca^{2+} homeostasis and antioxidant defences, with a focus on the *caput* region, as it is the most susceptible to hormonal regulation. For this purpose, we firstly optimized *ex vivo* cultures of epididymal *caput* sections. Secondly, *caput* sections were cultured *ex vivo* in the presence or absence of VNZ 500 μM, and Ca^{2+} levels, the expression levels of Ca^{2+} modulators, oxidative stress, and apoptosis were analyzed.

Caput sections exposed to VNZ displayed decreased expression of CaSR and TRPV6, along with an increase in the extracellular Ca^{2+} concentrations. These findings suggest that VNZ can disrupts Ca^{2+} homeostasis in the epididymis. CaSR detects extracellular Ca^{2+} and activates several intracellular pathways. Studies in sperm from

rodents and cattle have shown that CaSR has a critical role in sperm motility and capacitation (96, 97). Also, in human sperm, loss-of-function mutations in CaSR lead to low sperm counts and motility, impairing sperm function (64). Altogether, the existent information supports the observed action of VNZ in deregulating Ca^{2+} concentrations, by affecting CaSR expression.

TRPV6 is expressed in epithelial cells and it is assumed that its function is related to Ca^{2+} reabsorption, being an essential component in the maintenance of Ca^{2+} homeostasis (60). Studies in a transgenic male mice carrying an inactive form of TRPV6 (61) or a mutation in the pore of TRPV6 channels (60), showed that these animals exhibited defects in sperm motility, viability, and abnormal Ca^{2+} concentrations in the epididymal luminal fluid, with consequently decreased fertility. Taking this premise into account, it is possible that VNZ could affect sperm function by lowering the expression of TRPV6, with an impact on Ca^{2+} absorption. This study first showed the action of VNZ in disrupting epididymis function. Further *in vivo* studies will demonstrate that the VNZ action in deregulating CaSR and TRPV6 expression can affect Ca^{2+} concentrations in the epididymis and sperm function.

Maintenance of adequate oxidative stress levels is another critical issue to ensure optimal sperm function. It is established that the epididymal epithelium is able to protect sperm against oxidative damage, maintaining balanced levels of ROS in the luminal millieu. Oxidative stress occurs when ROS production exceeds the body's natural antioxidant defences, culminating in cell damage (31). An increase in ROS can result in infertility, damaging the sperm membrane, decreasing sperm motility and fertilizing capacity, or altering sperm DNA (30, 31). In this context, the activity of the antioxidant enzymes GPX and SOD, lowering the possibilities of oxidative damage is crucial. GPX catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reducing glutathione (104, 105). SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide, reducing the levels of the superoxide anions (106). Thus, both enzymes play an essential role in protecting cells from oxidative stress. The results obtained in this dissertation showed a decreased activity of the antioxidant enzymes GPX and SOD in the group treated with VNZ. Accordingly, in an *in vitro* study exposing fish sperm to VNZ , oxidative stress was caused (107). Thus, it is reasonable to assume that sperm function in rats may be compromised upon exposure to VNZ.

Apoptotic cell death occurs depends on the activation of caspase-3, the endpoint in the apoptotic signalling pathway (108, 109). Recent studies in rat testes exposed to EDs resulted in increased caspase-3 activity, leading to impaired spermatogenesis (110). Moreover, a previous study in porcine ovaries exposed to VNZ also showed an

increased caspase-3 activity, being suggested that VNZ contributes to the amplification of apoptotic cell death (111). Curiously, herein VNZ treatment was shown to decrease the apoptosis of rat *caput* epididymis, as indicated by the decreased caspase-3 like activity observed. Complementary assays would be needed to ascertain the cell survival and apoptotic status of VNZ-treated epididymal cells, but it cannot be excluded that other important molecular processes that maintain cellular homeostasis are activated, for example, autophagy. In fact, Ca^{2+} disorders associated with endocrine disruption of the CaSR revealed it can be a potential way to activate autophagy (97). Further studies will be pivotal to understand these mechanisms better.

In sum, the results obtained in the present thesis showed that exposure to VNZ decreased Ca^{2+} absorption by the rat epididymis *caput* cells. This effect was underpinned by the increased extracellular Ca^{2+} levels, and diminished antioxidant activity.

VI. Conclusion and Future Perspectives

The present dissertation firstly showed that exposure to VNZ culminates in the dysregulation of Ca^{2+} homeostasis due to reduced expression of key Ca^{2+}regulators (CaSR and TRPV6), which was concomitant with the increased levels of extracellular Ca^{2+}. Furthermore, it was found that VNZ exposure decreased SOD and GPX enzymatic activity, which may lead to the impairment of the antioxidant defences in the epididymis.
The obtained findings highlight the impact that exposure to VNZ may have in disrupting epididymal function and impairing male fertility. Although preliminary, this thesis results clearly demonstrate the effect of EDs exposure in the epididymis and how it could affect male fertility.

In the future, the complete comprehension of the molecular mechanism involved in VNZ actions will be of uttermost importance. For example, we still do not know if VNZ affects the expression levels or activity of other Ca^{2+} modulators. Also, a deep analysis of, the oxidative status evaluating the expression of other antioxidant enzymes and measuring ROS production would be crucial. Moreover, *in vivo* studies are needed to demonstrate that the VNZ action in deregulating CaSR and TRPV6 expression affects Ca^{2+} concentrations in the epididymis with impact on sperm function.
Regucalcin is a Ca^{2+} binding protein studied by our research group, which has been shown to be involved in Ca^{2+} homeostasis in the testis and epididymis and was recognized as an androgen-target gene in the testis (20, 112, 113). Also, this protein has been associated with the control of cell proliferation, apoptosis, and oxidative stress. In this context, regucalcin would be an interesting player to analyse in response to VNZ treatment.

On the other hand, the analysis of molecular processes involved in the maintenance of cellular homeostasis like autophagy and necrosis should not be excluded in future studies.

Ultimately, as mentioned above, global holistic *in vivo* approaches will be pivotal to understand how VNZ affect male fertility by disrupting epididymis function. These expected outcomes may also have an impact in human assisted reproductive technologies and animal husbandry.

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