THE MALE GERM CELL RESPONSE TO CYCLOPHOSPHAMIDE TREATMENT: A POTENTIAL ROLE FOR ZINC

By

Anne Marie Downey

Department of Pharmacology and Therapeutics
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Dedication

This thesis is dedicated to all my family, who have offered me their unwavering love and support.

To my parents for all their hard work and encouragement to do anything I dreamed.

To my mother, Helene Verreault, who reminded me that she had to write her thesis on a type writer

To my father, Michael Downey, who listened to me talk about my work even if he didn`t quite understand it.

To my Aunt Mo, for always having me in her prayers.

To my brother, Marc Downey, for his support.

Thank you for believing in me

ABSTRACT

Male factor infertility accounts for a large proportion of infertility cases and for the most part may in fact be related to lifestyle and/or exposure to toxic substances. Cyclophosphamide (CPA) is an alkylating agent commonly used in chemo- and immunosuppressive therapies and is a known germ cell toxicant. Men often become infertile after being treated with this drug. In rats, paternal CPA treatment leads to decreased sperm quality as well as defective early embryo development and negative progeny outcomes. The goal of the studies presented in this thesis was to evaluate the effect of chronic CPA treatment on the male germ cell transcriptome in order to gain a better understanding of how these cells respond to such an insult. In the first objective, we evaluated the effect of CPA treatment on the expression of microRNAs in isolated pachytene spermatocytes and round spermatids. We showed for the first time that a therapeutic drug such as cyclophosphamide alters the expression of miRNAs in male germ cells and that these may be involved in the germ cell response to toxic exposures. In the second objective we evaluated the effect of CPA treatment on genome wide gene expression in male germ cells. In addition to many transcripts involved in zinc binding being altered in both cell types following CPA treatment, we found that the expression of members of ZIP family of zinc transporters and zinc transport was increased in pachytene spermatocytes. This led us to believe that zinc may play an important role in the male germ cell response to CPA. In the third objective we examined whether zinc could protect male germ cells from CPA toxicity. We found that zinc supplementation decreased CPA induced oxidative stress and DNA damage in male germ cells. Collectively, the studies presented in this thesis contribute to our basic understanding of

male germ cell responses to toxic substances and suggest a potential role for zinc in protecting male germ cells against detrimental effects of CPA treatment.

RÉSUMÉ

Une large proportion des cas d'infertilité dans les couples peut être attribuée à un facteur mâle, souvent causé par le mode de vie ou l'exposition à des substances toxiques. Parmi ces substances, la cyclophosphamide (CPA), agent alkylant antinéoplastique et immunosuppresseur, est reconnue pour ses effets toxiques sur les gamètes mâles. De nombreux troubles de la fertilité sont en effet rapportés chez les hommes ayant été traités à la CPA. En outre, l'exposition de rats mâles à la CPA altère la qualité de leurs spermatozoïdes, entraînant des effets néfastes sur leur progéniture dès le début du développement embryonnaire. Le but de ces travaux de thèse était de mieux comprendre comment réagissent les cellules germinales mâles face à un traitement chronique à la CPA en faisant l'analyse de leur transcriptome. Notre premier objectif était d'évaluer l'effet de la CPA sur l'expression des microARNs dans les spermatocytes pachytènes et les spermatides rondes. Nous avons ainsi démontré pour la première fois qu'une exposition à une substance thérapeutique peut altérer l'expression des microARNs, des changements potentiellement associés à la réponse physiologique des cellules germinales aux substances toxiques. Dans un deuxième temps, nous avons évalué l'effet de la CPA sur l'expression des ARN messagers dans les spermatocytes pachytènes et spermatides rondes. Nous avons non seulement démontré que la CPA modifiait l'expression de transcrits impliqués dans les complexes de zinc, mais surtout que l'expression de transporteurs de zinc de la famille ZIP et le transport de zinc lui-même étaient accrus dans les spermatocytes pachytènes. Ces résultats établissent le rôle fondamental du zinc dans la réponse physiologique des cellules germinales à la CPA. Enfin, dans un troisième objectif, nous avons évalué le

potentiel du zinc dans la protection des cellules germinales contre les dommages causés par la CPA. Nous avons ainsi pu observer que, dans les cellules germinales mâles, une supplémentation en zinc produit une diminution des niveaux de stress oxydant et des dommages à l'ADN associés au traitement à la CPA. Considérés dans leur ensemble, ces résultats permettent une meilleure compréhension de la réponse physiologique des cellules germinales face aux substances toxiques, et mettent en lumière le potentiel du zinc dans la protection des cellules contre les effets nocifs de la CPA.

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LIST OF ABBREVIATIONS

$^1O_2$	Singlet oxygen
3'UTR	3' untranslated region
4-HNE	4-hydroxynonenal
4-OHCPA	4-hydroxycyclophosphamide
53BP1	P53 binding protein
8-oxodG	8-oxo-2'-deoxyguanosine
8-oxoG	7, 8-dihydro-8-oxo-guanosine
Aal	Aligned Spermatogonia A
ACT	activator of CREM in the testis
ADH	Alcohol dehydrogenase
AGO	Argonaut
ALDH	Aldehyde dehydrogenase
Apr	Paired ppermatogonia A
AR	Androgen receptor
ART	Assisted reproductive technology
As	Spermatogonia A stem cell
ATM	Ataxia telangatasia mutated
B	Spermatogoia B
BER	Base-excision repair
BSA	Bovine serum albumin
BTB	Blood testis Barrier
CAM	Complementary of alternative medicine
CAT	Catalase
CircRNA	Circular RNA
CMA3	Chromomycin A3
CoQ10	Coenzyme Q10
CPA	Cyclophosphamide
CREM	cAMP responsive element modulator
CYP450	Cytochrome P450
DAPI	4'-6'-diamidino-2-phenylindole
DDR	DNA damage response
DGCR8	DiGeorge syndrome critical region 8
DHA	Dehydroascorbic acid
diRNA	DSB-induced RNA
D-Loop	Displacement loop
Drosha	Drosha ribonuclease III

DSB	Double strand break
endo-siRNA	Endogenous small interfering RNA
ER	Endoplasmic reticulum
ES	Elongated spermatid
ETC	Electron transport chain
EU	5-ethynyl uridine
EXP5	Exportin 5
FACS	Fluorescent activated cell sorting
FSH	Follicle stimulating homone
FSHR	Follicle stimulating hormone receptor
GG-NER	Global genome-NER
GnRH	Gonadotropin releasing hormone
GnRHR	Gonadotropin releasing hormone receptor
GO	Gene ontology
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
H2AX	Histone variant 2AX
H_2O_2	Hydrogen peroxide
$HO_2^{\bullet}$	Hydroperoxyl
HPG axis	Hypothalamic-pituitary-gonadal axis
HR	Homologous recombination
ICP-OES	Inductively coupled plasma opitcal emission spectrometry
IDL	Insertion deletion loop
IL-2	Interleukin-2
In	Intermediate spermatognia
IR	Ionizing radiation
KO	Knock out
LA-ICP-MS	Laser alblation-inductively coupled plasma spectrometry
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
LncRNA	Long non-coding RNA
lncRNA	Long Non-coding RNA
LP	Long patch
m6A	N6-methyladenosine
MAR	Matrix attachement region
mBBr	Monobromobinane thiol labelling
MDA	Malondialdehyde
mDF	Modified Davidson's Fluid

miRISC	miRNA-induced silencing complex
miRNA	microRNA
miRNA	MicroRNA
MMR	Mismath repair
mRNA	Messenger RNA
NER	Nucleotide-excision repair
NHEJ	Non-homologous end joining
$O_2^{\bullet -}$	Superoxide
$O_2^{\bullet\bullet}$	Oxygen radical
O_3	Ozone
$OH^{\bullet}$	Hydroxyl
PCA	Principle component analysis
PCB	polychlorinated biphenyl
piRNA	PiwiRNA
piRNA	PiwiRNA
PNA	Peanut agglutinin
PRDX	Peroxiredoxin
pre-miRNA	precursor miRNA
pri-miRNA	primary microRNA
Prm1	Protamine 1
Prm2	Protamine 2
R-CHOP	rituximab, CPA, doxorubicin, vincristine and prednisone
RNA	
RNA Pol II	RNA polymerase II
RNP	Ribonucleioprotein granules
$RO^{\bullet}$	Alkoxyradicals
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
RPB	RNA binding proteins
RS	round spermatid
SAL/CPA	CPA treated
SAL/SAL	Saline control
SCSA	Sperm chromatin susceptibility assay
SDSA	Synthesis-dependent strand annealing
SEM	Standard error of the mean
SOD	Superoxide dismutase
SP	Short patch
SPC	Spermatocyte
SPG	Spermatogonia

SPZ	Spermatozoa
SSB	Single strand break
ssDNA	Single strand DNA
Sycp3	Synaptonemal complex
TC-NER	Transcription-coupled NER
TE	Transposable element
TP1	Transition protein 1
TP2	Transition protein 2
TPEN	N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine
TRD	Thioredoxin reductase
t-RNA	Transfer RNA
TRX	Thioredoxin
ts-RNA	Transfer RNA derived small RNA
UDS	Unscheduled DNA synthesis
XRFM	X-ray fluorescence microscopy
$Zn(OAc)_2$	Zinc acetate
ZN/CPA	Zinc supplemented
ZN/SAL	Zinc control
$ZnCl_2$	Zinc chloride
ZnPic	Zinc picolinate
$ZnSO_4$	Zinc sulfate
ZRE	zinc responsive element
ZRE	Zinc responsive element
γ-H2AX	Phosphorylated histone variant 2AX

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PREFACE

Format of the Thesis

This thesis is a manuscript based thesis composed of five chapters and conforms to the "*Guidelines for Thesis Preparation*" of the Faculty of Graduate Studies and Research of McGill University. References are included at the end of each chapter. The first chapter provides an introduction to the male reproductive system and background information on materiel relevant to this thesis including spermatogenesis, male germ cell transcriptome, defense and repair mechanisms and finally the concept of male mediated reproductive toxicology focusing on the chemotherapeutic and immunosuppressant drug cyclophosphamide. The chapter concludes with the rational and objectives of this thesis. Chapter 2 has been submitted for publication. Chapter 3 is a published manuscript and is available in *Biology of Reproduction*; 95(1):22, 1-12, 2016. Chapter 4 is in preparation for submission. Connecting texts are included in between chapters to ensure continuity. Chapter 5 is a general discussion of the findings presented in this thesis as well as their significance in the field of reproductive toxicology. Finally, a list of original contributions is provided.

Author contributions

All experiments and analyses presented in this thesis were completed by the candidate.

CHAPTER 1

INTRODUCTION

1.1 The Male Reproductive System Anatomy: and Physiology

The male reproductive system is composed of multiple components (Fig.1-1): 1) testes that are responsible for spermatogenesis and steroidogenesis; 2) the excurrent duct system (epididymides, vasa deferentia, ejaculatory ducts and urethra) responsible for the storage and transport of germ cells; 3) the accessory glands (prostate, seminal vesicles and bulbourethral glands) that are responsible for the production and secretion of constituents of the seminal fluid; and 4) the external genitalia from which sperm cells and seminal fluid exit the body. The purpose of these components is to develop, maintain and store the male germ cell – the spermatozoon; produce and to secrete the sex hormones (testosterone, estradiol) that drive spermatogenesis [1] and are responsible for secondary male sex characteristics [2]; and finally deliver the mature sperm cells to female reproductive tract.

1.1.1 The Testes

The testes are a pair of oval shaped organs located in the scrotal sac outside the abdominal cavity. They are composed of two internal compartments, the interstitium and seminiferous tubules, encapsulated within a layer of connective tissue, the tunica albuginea [3]. The steroid producing Leydig cells along with peritubule myoid cells, macrophages and blood vessels and lymphatics compose the interstitial space [4], while the Sertoli and the developing germ cells compose the seminiferous tubules [3]. The main functions of the testes are the production and secretion of male sex hormones (steroidogenesis) and the production of male germ cells (spermatogenesis). Although

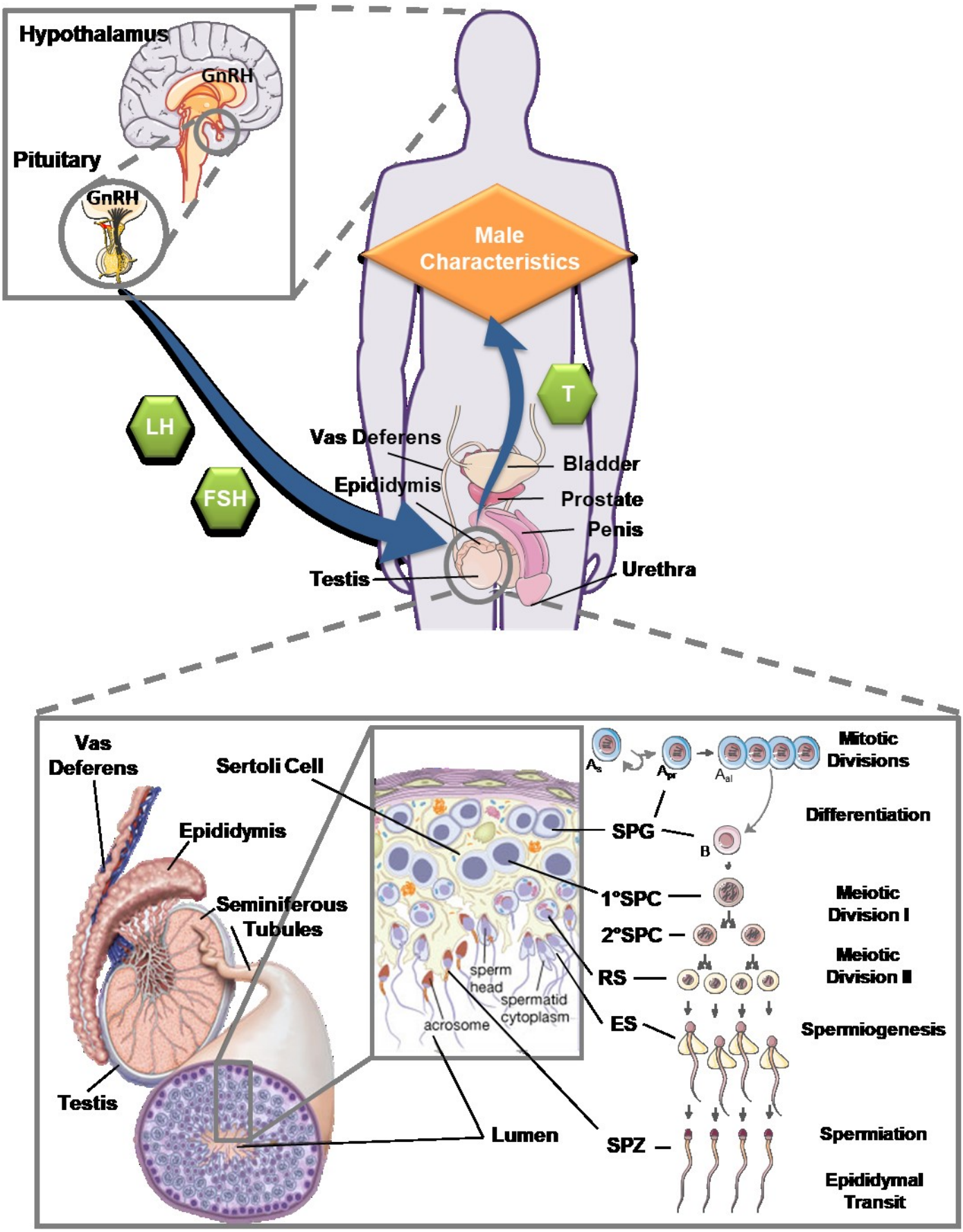

Hypothalamus
GnRH
Pituitary
GnRH
Male Characteristics
T
LH
FSH
Vas Deferens
Epididymis
Bladder
Prostate
Penis
Urethra
Testis
Vas Deferens
Epididymis
Seminiferous Tubules
Testis
Sertoli Cell
sperm head
spermatid
cytoplasm
acrosome
Lumen
SPZ
Mitotic Divisions
Differentiation
SPG
1°SPC
2°SPC
RS
ES
Spermiogenesis
Meiotic Division I
Meiotic Division II
Spermiation
Epididymal Transit

Figure 1-1. Schematic overview of the male reproductive system, organization of the seminiferous epithelium and spermatogenesis. Gonadotropin releasing hormone (GnRH) secreted from the hypothalamus cause release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary into the peripheral circulation. LH stimulates the production of testosterone in the testis which drives spermatogenesis and is responsible for male characteristics. The testis is composed of seminiferous tubules which contain layers of germ cells at different stages of development: the seminiferous epithelium. The process of spermatogenesis begins with the spermatogonial stem cells (SPG), at the base of the seminiferous epithelium, which undergo a series of mitotic divisions and differentiate into 1° spermatocytes (SPC). The 1° spermatocytes undergo the first meiotic division to become 2° spermatocytes. Round spermatids (RS) are produced from the second meiotic division, and go through spermigenesis, becoming elongated spermatids (ES) and then spermatozoa (SPZ). The spermatozoa are released into the lumen (spermiation) and continue to mature while traveling through the epididymis. Created in part using images from Servier Medical Art (www.servier.com), licensed under a Creative Commons Attribution 3.0 Unported License and adapted from Encylopedia Britannica, inc. (http://www.britannica.com/science/spermatogensis).

these 2 functions occur in separate areas of the testis, both steroidogenesis and spermatogenesis are regulated by signaling between the two compartments [5].

1.1.2 Steroidogenesis

The function and development of the male and female reproductive systems depend on the complex interplay between the hypothalamus, pituitary and the gonads, known as the hypothalamic-pituitary-gonadal (HPG) axis. In the male reproductive system, signals from the central nervous system are responsible for steroidogenesis which ultimately drives spermatogenesis (Figure 1-1). Gonadotropin releasing hormone (GnRH) is synthesized in the hypothalamus by GnRH neurons and secreted in pulses into the hypophyseal portal blood stream. The portal stream carries GnRH to the gonadrope cells located in the pituitary, where it binds the GnRH receptors (GnRHR), eliciting a downstream signalling cascade resulting in the synthesis and secretion of the gonatropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), into the peripheral circulation. In the testes, FSH acts directly on Sertoli cells via the FSH receptor (FSHR) to synthesize essential factors for germ cell development [6], while LH mediates its actions via the LH receptor (LHR) in Leydig cells resulting in steroid production.

The major steroid hormone produced in the testis is testosterone. In response to LH, the Leydig cells produce testosterone from cholesterol in a pulsatile manner [7]. The pathway for the synthesis of testosterone is depicted in Figure 1-2. The testis is responsible for 95% of circulating testosterone, that influences many target tissues such bone, muscle, fat, skin, as well as the cardiovascular and central nervous systems [8-

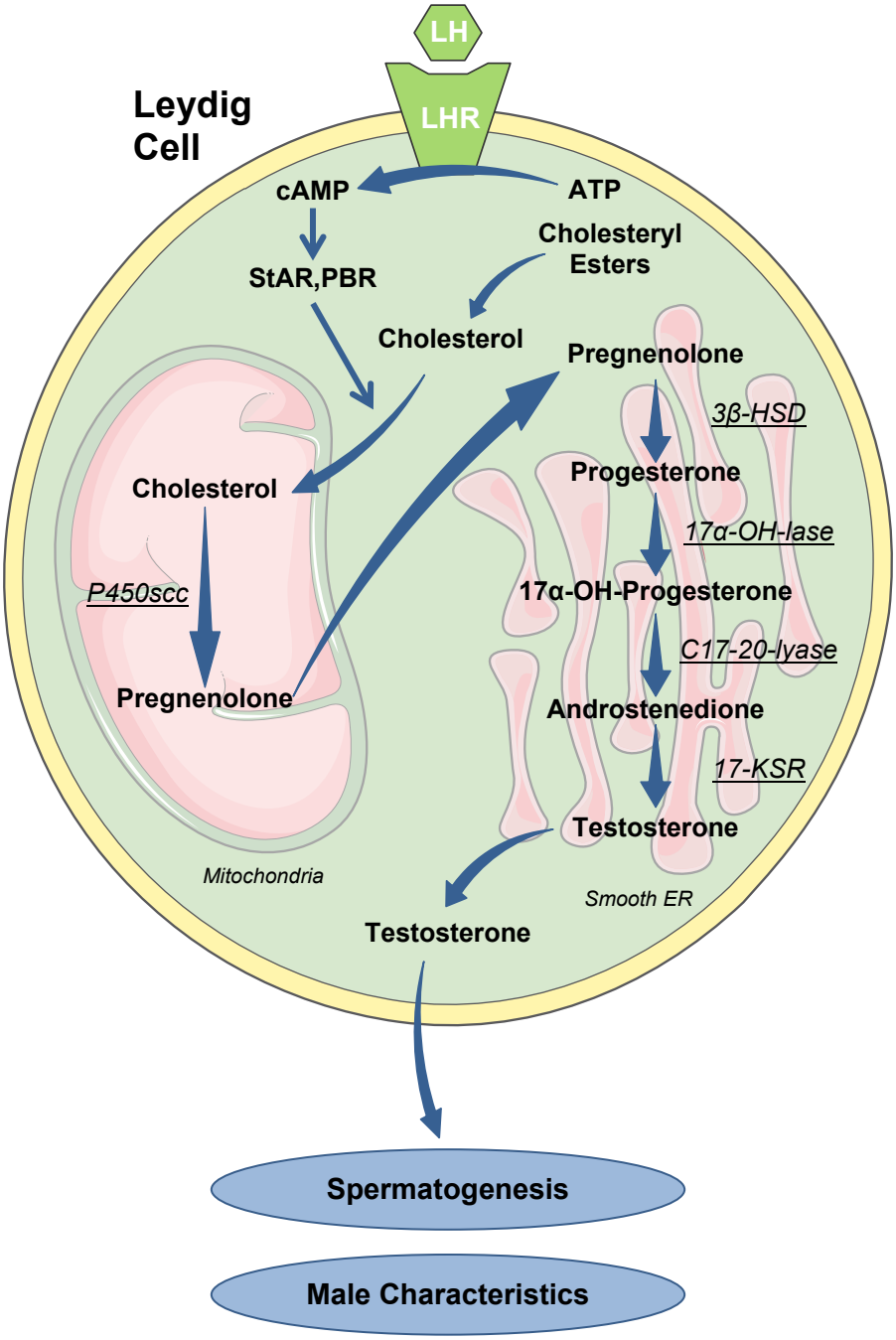

LH
LHR
Leydig Cell
cAMP
ATP
Cholesteryl Esters
StAR,PBR
Cholesterol
Pregnenolone
3β-HSD
Progesterone
17α-OH-lase
17α-OH-Progesterone
C17-20-lyase
Androstenedione
17-KSR
Testosterone
Cholesterol
P450scc
Pregnenolone
Mitochondria
Smooth ER
Testosterone
Spermatogenesis
Male Characteristics

Figure 1-2. Testosterone synthesis in Leydig cells. Luteinizing hormone (LH) binds the LH receptors (LHR) of Leydig cells initiating a cascade of molecular events including cholesterol transport into mitochondria, aided by steroidogenic acute regulatory protein (stAR) and translocator protein (TSPO), production of pregnenolone from cholesterol by P450scc, translocation of pregnenolone from the mitochondria to the smooth endoplasmic reticulum (SER) and conversion of prognenolone to testosterone via a series of reactions involving the enzymes 3β-HSD, 17α-OH-lase, C17-20-lyase and 17-KSR. Testosterone then freely diffuses across the cellular membrane. Created in part using images from Servier Medical Art (www.servier.com), licensed under a Creative Commons Attribution 3.0 Unported License and adapted from Zirkin and Chen (2000) [405].

12]. Testosterone is also the main driver of spermatogenesis [1]. Testosterone does not act directly on male germ cells, but rather exerts its effects via the androgen receptor (AR) in Sertoli cells [13-15].

1.1.3 Spermatogenesis

Spermatogenesis is a complex process during which, through a series of tightly regulated steps, a diploid spermatogonial stem cell is transformed into a highly specialized and compact haploid spermatozoon (Fig. 1.1). As the germ cells develop, they move from the basement membrane (basal compartment) towards the lumen (luminal compartment) of the seminiferous tubule. The entire process lasts approximately 35 days in mice, 52 days in rats and 72 days in humans [16, 17].

Germ cell development requires support from the Sertoli cells: irregularly shaped somatic cells located on the basement membrane of the seminiferous tubules with cytoplasmic extensions that reach towards the luminal space [18]. Tight junctions between Sertoli cells create the blood-testis-barrier (BTB) [19-21], that serves to provide an optimal environment for germ cell development and also protects the germ cells from toxic substances and the immune system [22, 23]. This barrier divides the seminiferous tubules into the basal and luminal compartments [20]. The Sertoli cells are in direct contact with the germ cells and provide support by secreting various nutrients and glycoproteins important for germ cell development (reviewed in [24, 25]). Sertoli cells can support a fixed number of germ cells, and thus maximum sperm output is dependent on Sertoli cell number [26].

Spermatogenesis can be divided into three separate phases: mitosis, meiosis and spermiogenesis (Fig. 1-1).

During the mitotic phase, a spermatogonial stem cells (As) located in the basal compartment divide to replicate themselves in order to replenish the stem cell population and to provide pairs of spermatogonia (Apr) that will begin spermatogenesis. The Apr go through a series of synchronous divisions leading to the formation of chains of aligned spermatogoia (Aal) . The Aal mature synchronously into more differentatiated spermatogonial cell types: $A_1 \rightarrow A_2 \rightarrow A_3 \rightarrow A_4 \rightarrow$ intermediate (In) $\rightarrow$ B [27-30]. The B spermatogonia differentiate into the pre-leptotene spermatocytes, cross the BTB into the adluminal compartment [31], begin DNA synthesis [32] and enter the meiotic phase of spermatogenesis.

During the meiotic phase of spermatogenesis, diploid spermatocytes go through two divisions, one reductional (meiosis I) and one equational (meiosis II) to become haploid cells. During meiosis I, the diploid primary spermatocytes (pre-leptotene) go through prophase I consisting of several stages based on the appearance of the chromosomes: 1) leptotene – the chromosomes, consisting of sister chromatids, condense into visible filamentous strands 2) zygotene – synapsis between homologous chromosomes begins 3) pachytene – synapsis is completed and recombination between homologous chromosomes occurs 4) diplotene – homologous chromosomes begin to unsynapse 5) diakinesis – separation of the chromosomes. This is followed by metaphase I, anaphase I and telophase I and the result is the production of two haploid secondary spermatocytes. These secondary spermatocytes then undergo meiosis II, that involves the separation of the two sister chromatids and results in the formation of

four haploid spermatids [5]. Although four spermatids are formed from a single primary spermatocyte, these cells remain connected by cytoplasmic bridges [33] allowing sharing of cellular products [34].

The resulting haploid spermatids next undergo a remarkable differentiation process to become the ultra-specialized spermatozoa, a process called spermiogenesis [5]. During this phase of spermatogenesis, extensive chromatin remodeling occurs: the majority of the histones are removed and replaced with transition proteins and finally protamines [35], leading to a highly compacted, elongated nucleus that is transcriptionally quiescent [36]. Important changes in cellular morphology also occur: the cytoplasm elongates and is reorganized to form a tail with a flagellum surrounded by mitochondria and the acrosome on the anterior half of the head [37, 38]. At the end of the differentiation process, very little cytoplasm is retained. The final event in spermiogenesis is spermiation, where the spermatozoa are released into the lumen to be transported through the efferent ducts to undergo maturation in the epididymis.

1.1.4 The sperm chromatin structure

Extensive chromatin remodelling takes place during spermiogenesis resulting in a nucleus that is roughly 10% the volume of a somatic cell nucleus and a remarkably compacted DNA structure. To achieve this, the chromatin goes from a classical histone bound nucleosome structure to a mostly protamine bound highly condensed toroid structure [39] (Fig. 1-3). Protamines are small, arginine and cysteine rich, basic proteins exclusively expressed in male germ cells [40, 41]. Mice and humans express 2 variants, Prm1 and Prm2, while rats only express Prm1 [42]. The positive charges of the

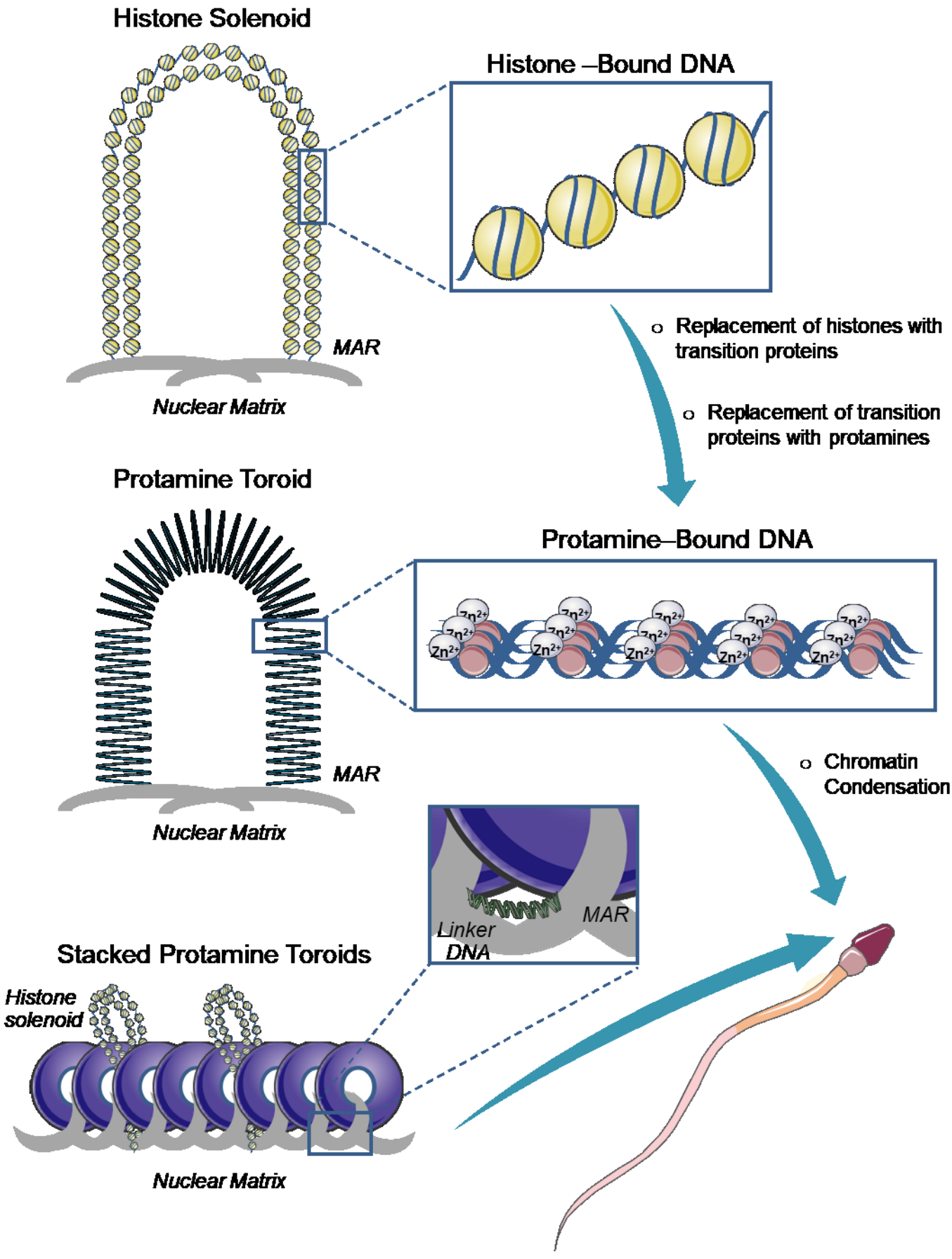

Histone Solenoid
MAR
Nuclear Matrix
Histone –Bound DNA
o Replacement of histones with transition proteins
o Replacement of transition proteins with protamines
Protamine Toroid
MAR
Nuclear Matrix
Protamine–Bound DNA
Zn2+
o Chromatin Condensation
MAR
Linker DNA
Stacked Protamine Toroids
Histone solenoid
Nuclear Matrix

Figure 1-3. Schematic overview of the sperm chromatin structure. During spermiogenesis, the chromatin transitions from a somatic histone-bound DNA solenoid structure to a mostly protamine-bound DNA structure. Compaction is aided and stabilized by the association of zinc ions with protamines. The protamine bound chromatin forms highly condensed toroids and that are stacked closely together and connected by short stretches of linker DNA. DNA is attached to the nuclear matrix at matrix attachment regions (MAR). Although the majority of DNA is protamine bound, a fraction of DNA remains histone-bound. Created in part using images from Servier Medical Art (www.servier.com), licensed under a Creative Commons Attribution 3.0 Unported License.

arginine residues neutralize the negative charge of the phosphodiester DNA backbone, reducing the electrostatic repulsion between chromatin and thus allowing formation of the toroids [39] [43]. The cysteine residues provide thiol groups that permit the formation of inter- and intramolecular cross-linking disulphide bonds between the protamines, further condensing the chromatin [44]. Zinc ions participate in these bonds by forming zinc bridges between the thiol groups of the cysteine residues and also possibly interacting with imidazole groups of histidine residues, stabilizing the DNA protamine structure in a similar way to zinc-finger DNA binding proteins [45-47]. This condensed packaging of sperm DNA renders the sperm transcriptionally quiescent and also protects the genetic material.

Approximately 15% of human and 1-2% of mouse sperm DNA remains packaged with histones in somatic cell like nucleosomes [48]. The locations of the retained nucleosomes seem to be non-random and appear to be enriched with developmentally critical genes, suggesting a role in early embryonic development [49, 50].

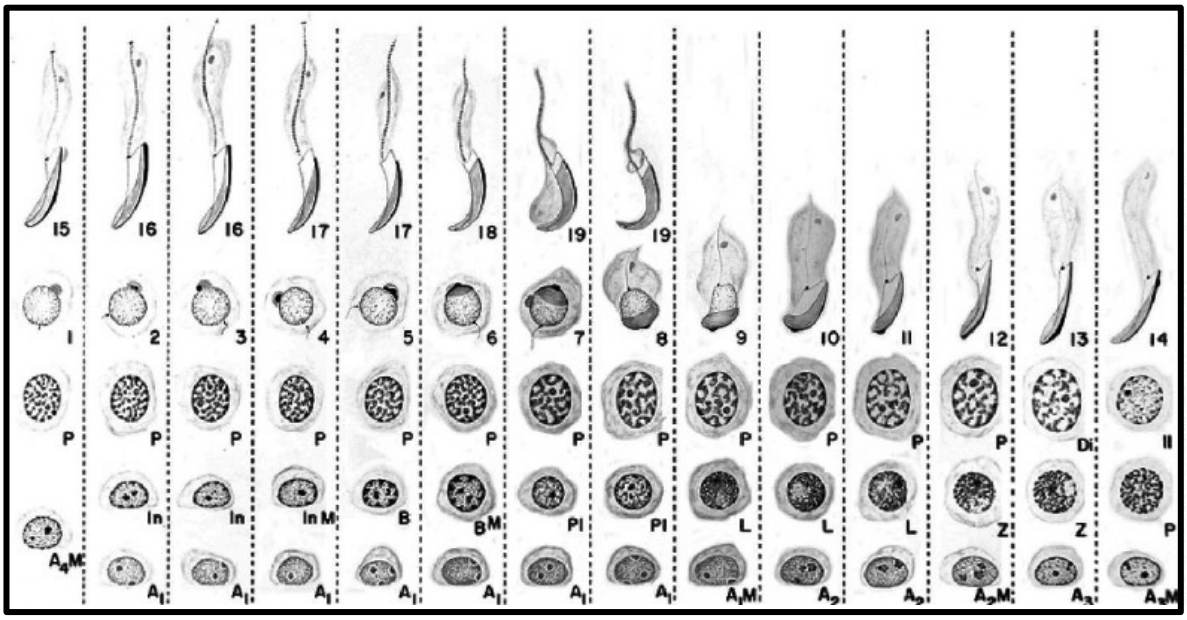
Finally, on a larger scale, sperm chromatin is also organized in the nucleus by a network of proteins and RNAs, also present in the nuclei of somatic cells, called the nuclear matrix [51]. The regions where the chromatin attach to the nuclear matrix are sequence specific and termed matrix attachment regions (MARs) [52]. Interestingly, these MARs may also correspond to sites of histone retention [53]. The proper structure of the sperm nuclear matrix is important for DNA replication in early embryo development [54, 55]. The structural features of the sperm chromatin serve to protect the DNA from damage and also perhaps poise specific regions for use in early embryonic development.

1.1.5 The seminiferous epithelium cycle and spermatogenesis staging

In the adult testis, germ cells at all stages of differentiation are present at any given time. However these different germ cells are arranged in a set of defined cellular associations that can be used to stage the seminiferous epithelium [38, 56] (Fig. 1-4). Cellular morphology and the changes in the appearance of the acrosome that characterize the different steps in spermatid development are used to identify the different cellular associations that define the stages. There are 6 stages in the human, 12 stages in the mouse and 14 stages in the rat seminiferous epithelial cycles [37, 57]. These different stages progress sequentially and repeatedly along the seminiferous tubules in order to assure the continuous production of spermatozoa, a phenomenon is referred to as the spermatogenic wave [58].

1.2 The Germ Cell Transcriptome

The testis displays the most complex transcriptome compared to other tissues including the brain, heart, liver and kidney [59]. The source of this complexity stems primarily from the pachytene spermatocytes and round spermatids. The intricate differentiation processes and need for the transcripts required for late steps of spermatogenesis to be transcribed and stored before nuclear condensation and transcriptional quiescence drives the high transcriptional activity of these cells. Additionally, the substantial chromatin remodelling that occurs during meiosis and spermiogenesis allows for a permissive chromatin state, leading to promiscuous



Stages of the Seminiferous Cycle of the Rat

Figure 1-4. Schematic representation of the seminiferous cycle of the rat. During spermatogenesis, a stem cell goes through a series of processes to become a spermatozoon. This figure depicts the defined germ cell associations that can be observed at any given time in the seminiferous tubules. These defined associations are used to stage the semiferous epithelium. The rat has 14 stages (columns). Adapted from Dym and Clermont (1970) [376]

transcription [59]. Protein coding mRNAs, the small non-coding as well as long non-RNAs have all been shown to play important roles in the regulation of spermatogenesis.

1.2.1 Messenger RNAs

Gene expression profiles from the testis and male germ cells differ significantly from other tissues and somatic cells and display many testis-enriched or -specific transcripts [60-62]. In fact, it is estimated that 4% of the mouse genome is dedicated to the expression of testis-specific/predominant genes [63]. While some of the transcripts are for different homologues or splice variants, many transcripts are for unique proteins with testis-specific functions. Although testis specific gene expression studies began over 20 years ago, there are still new transcripts being discovered today [64]. The different germ cell types possess a variety of enriched or testis-specific transcripts and display distinct gene expression profiles owing to the unique cellular differentiation processes they undergo and the unique structures they develop. Round spermatids display the most enriched or specific transcripts, followed by the pachytene spermatocytes, while spermatogonia display the least [60]. Well known examples of germ cell enriched or specific genes are synaptonemal complex 3 (Sycp3), part of the synaptonemal complex that is essential for meiotic recombination and the transition proteins (Tp1/2) and protamines (Prm1/2) which, although transcribed in the round spermatids, are only translated when required during later stages in development for chromatin remodelling [60, 65, 66].

The unique and highly coordinated steps of spermatogenesis require an equally unique and tightly regulated gene expression program mediated through transcriptional

and posttranscriptional mechanisms. Many differences in the regulation of transcription between germ cells at different stages of differentiation have been documented giving male germ cells a unique gene expression program. For instance, many factors and components of the transcriptional machinery are over-expressed in the testis compared to somatic tissues [67]. Furthermore, male germ cells express many testis specific transcription factors, chromatin associated factors and components of the transcriptional machinery that are often paralogs of those used by somatic cells [68-70]. Additionally, the mechanisms by which some factors are activated are different from those found in somatic cells, as is the case for CREM, which bypasses the requirement for phosphorylation in germ cells by acting with activator of CREM in testis (ACT) [71]. Finally, epigenetic mechanisms also allow for the unique germ cell transcriptome: the promoters of testis specific genes, while often highly methylated in somatic tissues, are hypo-methylated in germ cells [70].

Male germ cells also possess many mechanisms for post-transcriptional regulation of gene expression that occur at different steps of the RNA processing pathway. Alternative splicing is widespread in the testis [72] and can alter coding properties, stability, and spatial/temporal expression of transcripts [73-75]. Different splicing factors and splice variants seem particularly important for meiotic germ cells [75, 76].

Because transcripts needed for the later steps of spermiogenesis need to be transcribed and stored before transcriptional quiescence, mRNA stability and translational control are essential. Transcripts can be stabilized by the lengthening of the poly(A) tail [77, 78] and associated poly(A) binding proteins [78, 79]. However more

importantly, many RNA binding proteins (RBPs) are enriched or specifically expressed in the testis [80] that bind, store and protect mRNA transcripts in ribonucleioprotein (RNP) granules, such as the chromatoid body, until they are translated [81]. The chromatoid body, located in the cytoplasm of spermatids, is a germ cell specific RNA processing centre that contains numerous RBPs and mRNA processing machinery [82]. Many non-coding RNAs (discussed below) also locate to the chromatoid body, suggesting they too play a role in post-transcriptional control in male germ cells.

Although the mature spermatozoa are essentially devoid of cytoplasm, the compact structure contains a complement of RNAs [83, 84]. It remains unclear whether the retained RNAs are merely a footprint of spermatogenesis or are specifically retained and have functional roles that may influence embryo development. The complement of mRNAs retained in sperm from infertile men differs from that of fertile men, suggesting that sperm RNAs may play a role in fertility and serve as useful biomarkers [85-87]. Additionally, sperm mRNA transcripts can be altered by exposure to environmental toxicants [88] and paternal diet [89], possibly reflecting toxic insult to male germ cells during spermatogenesis or perhaps indicating a specific response in preparation for fertilization and embryogenesis in a stressful environment. Whether the mRNA transcripts found in the 1-5 fg of sperm RNA [90], compared to the 1ng of oocyte RNA [91], play a role in embryo development is still unknown. However, as discussed below, small non-coding RNAs retained in mature spermatozoa seem to play a role in transmitting paternal environmental experience to offspring.

1.2.2 Non-coding RNAs

Although most of the human genome is transcribed, only a small fraction of these transcripts are protein coding [92, 93]. Originally thought of as artifacts or junk [94, 95], there is ever increasing evidence that non-coding RNAs play crucial roles in cellular function via chromatin remodelling and post-transcriptional control mechanisms [96-98]. Furthermore, the importance of these transcripts in the development of male germ cells is apparent.

1.2.2.1 MicroRNAs

MicroRNAs are evolutionary conserved, single stranded non-coding RNAs 20-24nt in length. Most miRNAs are transcribed by RNA polymerase II (RNA Pol II) and initially form a distinctive hairpin loop structure [99].This pri-miRNA is then processed by Drosha and its cofactor DGCR8 into an approximately 70nt long pre-miRNA [100-102] and then shuttled out of the nucleus by EXP5 [103, 104]. In the cytoplasm, the pre-miRNA is cleaved by Dicer into approximately 22nt long double stranded RNA which is loaded onto an AGO protein forming the miRNA-induced silencing complex (miRISC). One of the 22nt strands will remain in miRISC as the mature miRNA while the other will be degraded. MicroRNAs can also be generated through other, non-canonical pathways [105].

The miRISC mediates post-transcriptional regulation of gene expression via base complementarity between the 5’ end seed region of the miRNA and the 3’UTR of target mRNAs [106-109], however there is also evidence for binding in coding regions [110-114] . The binding of the miRISC to its target leads to either mRNA degradation, translational repression or sequestration into cellular compartments (RNPs as

discussed above) [115-117]. The base complementarity between the miRNA and its target does not need to be perfect [118-120], therefore one miRNA can target multiple mRNAs and an mRNA can be targeted by multiple miRNAs. To add to this complexity, miRNAs have recently been implicated in regulating N6-methyladenosine (m6A) modifications on mRNA[121], a modification that influences RNA structure [122] and possibly stability and translation of transcripts [123, 124].

MicroRNAs are abundantly expressed in testes and some testis-specific or preferred miRNAs have been identified [125-127]. Additionally, profiling studies have demonstrated differential expression patterns throughout spermatogenesis [125, 127], indicating a role for miRNAs in germ cell development and differentiation. The importance of miRNA-mediated post-transcriptional control for the regulation of spermatogensis has been demonstrated in several genetically modified mouse models. The ablation of Dicer in spermatogonial progenitor cells [128], spermatogonia [129] and spermatids [130] and Drosha in spermatogonia [131] have revealed the requirement for miRNA biogenesis machinery and miRNAs for meiotic progression, spermatocyte survival and most strikingly spermatid development. Sertoli cell miRNAs are also important for spermatogenesis as Sertoli cells lacking Dicer fail to properly support germ cells [132, 133].

Individual miRNAs have also been shown to have important functions throughout spermatogenesis. Figure. 1-5 illustrates the known roles of individual miRNAs in male germ cells. Several miRNAs are important for spermatogonial stem cell maintenance, self-renewal and differentiation [134-139]. Despite the fact that meiotic and post-meiotic germ cells are the main source of miRNA production in the testis [125] and that lack of

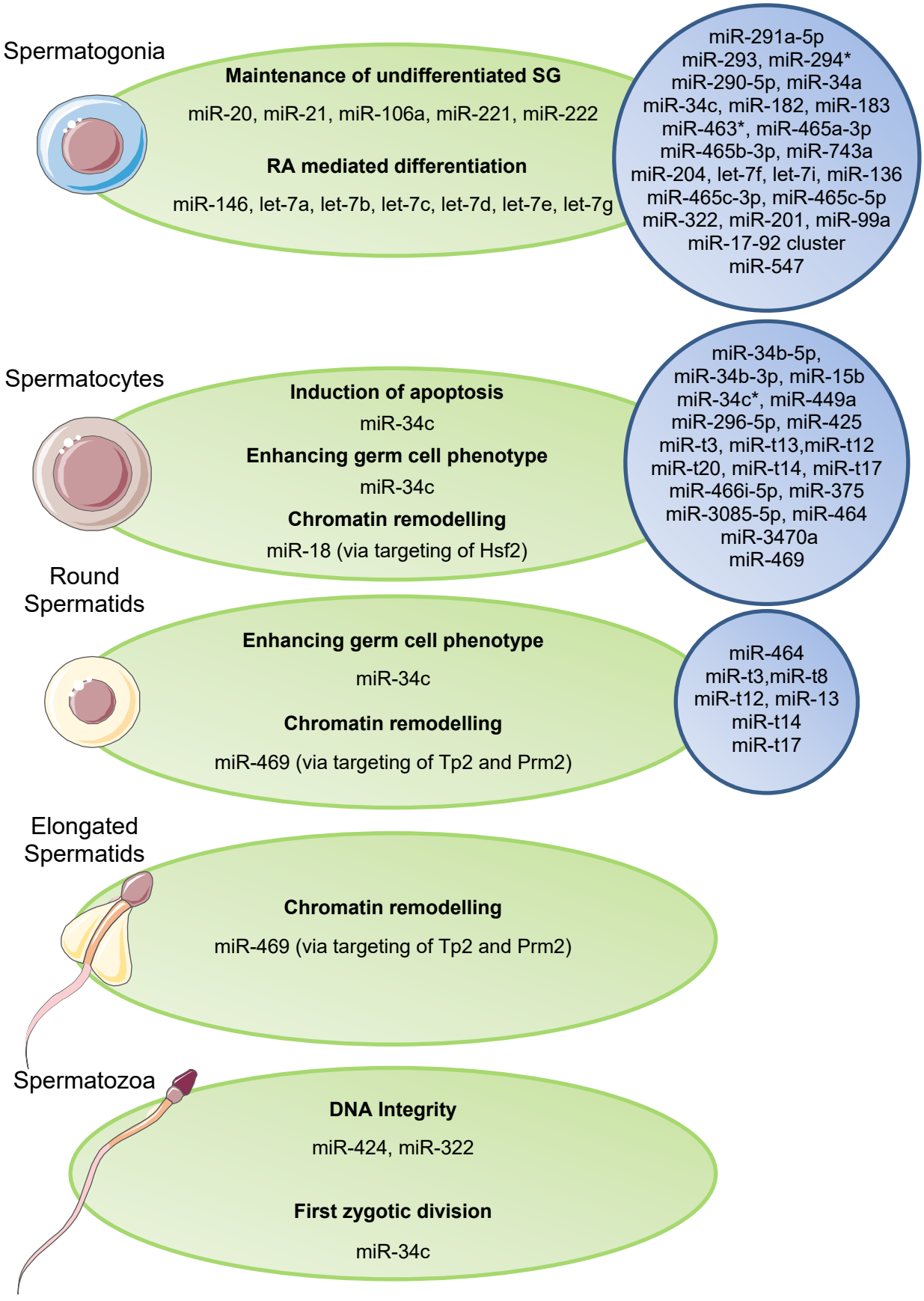

Spermatogonia
Maintenance of undifferentiated SG
miR-20, miR-21, miR-106a, miR-221, miR-222
RA mediated differentiation
miR-146, let-7a, let-7b, let-7c, let-7d, let-7e, let-7g
miR-291a-5p
miR-293, miR-294*
miR-290-5p, miR-34a
miR-34c, miR-182, miR-183
miR-463*, miR-465a-3p
miR-465b-3p, miR-743a
miR-204, let-7f, let-7i, miR-136
miR-465c-3p, miR-465c-5p
miR-322, miR-201, miR-99a
miR-17-92 cluster
miR-547
Spermatocytes
Induction of apoptosis
miR-34c
Enhancing germ cell phenotype
miR-34c
Chromatin remodelling
miR-18 (via targeting of Hsf2)
miR-34b-5p,
miR-34b-3p, miR-15b
miR-34c*, miR-449a
miR-296-5p, miR-425
miR-t3, miR-t13,miR-t12
miR-t20, miR-t14, miR-t17
miR-466i-5p, miR-375
miR-3085-5p, miR-464
miR-3470a
miR-469
Round Spermatids
Enhancing germ cell phenotype
miR-34c
Chromatin remodelling
miR-469 (via targeting of Tp2 and Prm2)
miR-464
miR-t3,miR-t8
miR-t12, miR-13
miR-t14
miR-t17
Elongated Spermatids
Chromatin remodelling
miR-469 (via targeting of Tp2 and Prm2)
Spermatozoa
DNA Integrity
miR-424, miR-322
First zygotic division
miR-34c

Figure 1-5. MicroRNAs in male germ cells. Roles for very few miRNAs have been found in different germ cell types and shown in green ellipse). MicroRNAs that have been identified as exclusively or highly expressed in the different cell types are shown in blue circles. Adapted from Kotaja (2014) [406] and Chen et al. (2017) [407]

miRNA biogenesis machinery leads to spermatogenic defects at these stages, very little is known about the functions of individual miRNAs in these cells. It would be logical for miRNAs to play a role in translational control of transcripts that are required in later stages of germ cell development once transcription has ceased. Indeed, miR-469 has been shown to target and block translation of transition protein 2 (Tp2) and protamine 2 (Prm2) transcripts in pachytene spermatocytes and round spermatids [140] while miR-122a cleaves Tp2 transcripts in post-meiotic cells [141]. These studies would suggest that miRNAs are involved in the regulation of chromatin remodelling via post-translational control mechanisms. Additionally, a handful of other miRNAs have also been found to play a role in meiotic entry [142-144] and apoptosis [145].

Although there are no known functions for miRNAs in the mature sperm, sperm born miRNAs have been shown to be transmitted to the oocyte at fertilization and play a role in early embryogenesis [146]. Interestingly, the miRNAs present in the nucleus of the sperm have been shown to transmit diet and stress phenotypes to offspring [89, 147-150]. Although there is still much unknown in the world of miRNAs, the evidence suggests the miRNAs that are produced in germ cells are not only important for spermatogenesis but also for embryo development.

1.2.2.2 PiwiRNAs

PiwiRNAs are 24-31 nt long transcripts that interact with the Piwi (Milli and Miwi proteins in mammals) family of AGO proteins. In mammals, they are expressed almost exclusively in the testis and can be classified, based on their expression patterns, as pre-pachytene piRNAs (Mili interacting) and pachytene piRNAs (Miwi interacting) [151-

155]. It has been proposed that the primary role of these small non-coding RNAs is the suppression of transposable elements (TE) [154, 156]. piRNAs may do this by 3 separate mechanisms: 1) by sequence complimentarity and TE degradation in a complex process called the ping pong loop [157, 158]; 2) the recruitment of factors to induce heterochromatin formation at targeted sequences [159, 160]; and 3) DNA methylation of TE promoters [161]. However recent data suggests that many of the pachytene piRNAs are derived from mRNA coding sequences [162] and may also be involved in inducing mRNA decay in elongating spermatids [163]. The importance of piRNAs in spermatogenesis can be seen in knockout mouse models. The loss of pre-pachytene piRNA activity by deletion of Mili leads to spermatogenic arrest between the zygotene and early pachytene stages of meiosis I [164], while with loss of pachytene piRNA acitivity by Miwi deletion leads to arrest at the early round spermatid stage [165]. Interestingly, piRNAs and their machinery are highly enriched in RNP granules such as the chromatoid body, suggesting they may play a role in RNA translational control [82].

1.2.2.3 *Long Non-Coding RNAs*

Long non-coding RNAs (LncRNA) are mRNA-like transcripts over 200nt long with no protein coding capacity. Their expression patterns are highly tissue-specific, and are they most abundant in the brain and testes [166]. Additionally, male germ cells display distinct stage specific lncRNA expression patterns, suggesting an important role in germ cell development [167, 168]. These non-coding RNAs are thought to regulate gene expression in multiple ways. They can act as competitors for DNA binding proteins, such as transcription factors and RNA binding proteins, either by interacting with the proteins or by binding complementary DNA or RNA sequences [169-173]. LncRNAs can

also recruit epigenetic modifiers to chromatin, thus playing a role in DNA methylation and histone modifications [174-176]. Finally lncRNAs can also be precursor RNA molecules and can be further processed to form smaller non-coding RNAs [177, 178]. A handful of studies have identified roles for individual lncRNAs in X-chromosome inactivation [179], spermatogonia [180-182] and meiosis [183]. However, functions of lncRNAs in the testes largely remain to be elucidated

1.2.2.4 Other non-coding RNAs

Many other non-coding RNAs have been identified in the testis. However these have been much less studied. These include endogenous small interfering RNAs, circular RNAs, and transfer RNA (tRNA) derived small RNAs.

Endogenous small interfering RNAs (endo-siRNAs), are similar in size (21-23 nt) and function to miRNAs [184]. Both are first processed DICER and cause mRNA transcript degradation. Endo-siRNAs are also involved in heterochromatin formation [185]. However, while miRNAs function in the RISC with any of the 4 AGO protein family members, endo-siRNAs only interact with AGO2 [186]. Endo-siRNAs have been found abundantly in male germ cells [187]. However, deletion of AGO2 does not lead to any spermatogenic defects, suggesting that endo-siRNAs do not play a critical role in male germ cell development [188].

Circular RNAs (circRNAs are a newly identified class of small-non coding RNAs that form closed continuous loops and are derived from exons and introns of coding sequences [189, 190]. They are believed to function as miRNA sponges [191] and cis/trans regulators of transcription [192], and possibly also as translation templates

[193]. They can also interact with RBPs [190], suggesting possible functions in post-transcriptional control [190]. circRNAs are present in the testis and seminal fluid [194, 195]. Many circRNAs indentified in the testis are unique and derived from genes involved in spermatogenesis [195]. Specific roles for these small RNAs and whether or not they are critical for spermatogenesis remain unknown.

Transfer RNA (tRNA) derived small RNAs (tsRNAs) are 18-26nt small non-coding RNAs that result from specific cleavage of tRNAs and not simply their random degredation [196, 197]. Their functions and targets are not well known, but they may play a role in transposable element silencing [198, 199]. tsRNAs are relatively abundant in mature sperm where they have been shown to transfer dietary phenotypes to offspring [196, 198, 200]. tsRNAs are also present in male germ cells and testicular sperm but less abundantly than in mature sperm, suggesting that sperm born tsRNAs are accumulated during epididymal transit [198].

1.3 Germ cell defense and repair mechanisms

1.3.1 Oxidative Stress

1.3.1.1 Oxidative stress and damage

Oxidative stress is a cellular condition where levels of reactive oxygen species (ROS) become elevated, causing damage to lipids, proteins and DNA and has been implicated in several diseases. ROS are highly reactive, oxygen containing molecules that can be classified as radicals or non-radicals. The highly reactive radical species

contain at least one un-paired electron in their outer orbital and include superoxide ($O_2^{\bullet -}$), oxygen radical ($O_2^{\bullet\bullet}$), hydroxyl ($OH^{\bullet}$), hydroxyperoxyl ($HO_2^{\bullet}$) alkoxyradicals ($RO^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$). The non-radical ROS species are not radicals but lead to free radical formation and include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and ozone (O_3) [201].

ROS can be generated endogenously and exogenously (Fig. 1-6). ROS are formed naturally as by-products of metabolism. Under normal physiological conditions, they are required for various functions including the immune response [202], cell signalling pathways [203], response to mitogens [204], and sperm capacitation [205]. Mitochondrial enzymes, NADPH oxidases and xanthine oxidases [207] are important sources of $O_2^{\bullet -}$. Additionally, non-enzymatic reactions can create electrons that leak from the electron transport chain (ETC) and react with molecular oxygen to form $O_2^{\bullet -}$ [208]. This “primary” ROS can then be protonated to form hydroxyperoxyl ($\bullet HO_2$) which can easily pass through membranes and cause damage. Additionally, $O_2^{\bullet -}$can react with other molecules such H2O2 and NO to create “secondary” ROS that are highly damaging, the •OH and $ONOO^-$ radicals. The •OH radical can also be produced from H_2O_2 and iron via Fenton reactions [209]. Additional sources of endogenous ROS include, but are not limited to, auto-oxidation reactions [210], NADPH oxidases [211], xanthine oxidase [207], lipoxygenase [212], and cytochrome P450 [213].

Exogenous sources of ROS come from radiation, air pollution [214], cigarette smoke [215], alcohol, heavy/transition metals [216], acrylamide (chemical exposure or food) [217] and drugs [218]. Once

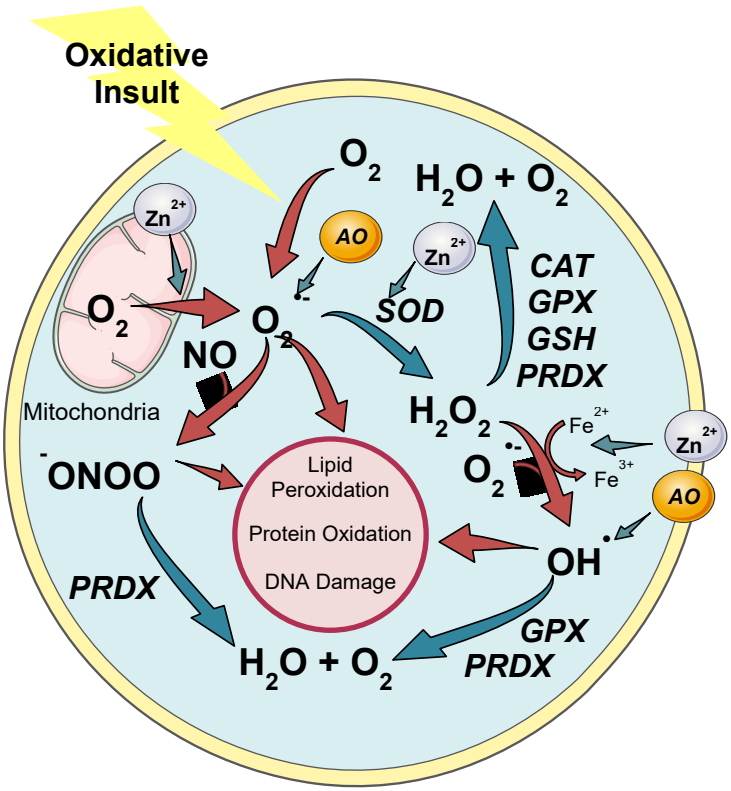


Figure 1-6. Reactive oxygen species, antioxidant and cellular damage. The $O_2^{\bullet-}$, •OH and $^{-}$ONOO radicals can be formed endogenously or from oxidative insult which can cause damage to biomolecules. Antioxidant enzymes neutralize these radicals. Superoxide dismutase (SOD) transforms O_2 into H_2O_2 which can be further broken down into H_2O and O_2 by catalase (CAT), gluatathione peroxidases (GPX), gluatathione (GSH) or peroxiredoxins (PRDX). The OH radical can be formed from H_2O_2 via reaction with O_2 or Fenton reaction and neutralized by either GPX or PRDXs. The $^{-}$ONOO radical, formed by the reaction of O_2 with NO, can be neutralized by PRDXs. Additionally, non-enzymatic antioxidants such as vitamins E and C can act as ROS scavangers and zinc can prevent endogenous production of ROS and fenton reactions and structurally stabilize SOD. Red arrows represent ROS production; Blue arrows represent ROS neutralization; AO = ROS scavengers.

inside the body, compounds are degraded and metabolized into free radicals that can then produce ROS and damage biomolecules.

High levels of ROS lead to damage of nucleic acids, proteins and lipids. The highly active OH reacts with the purine bases of DNA and RNA as well as the DNA backbone creating damaging DNA and RNA lesions such as the well characterized 8-oxo-2'-deoxyguanosine (8-oxodG) [219] and 7, 8-dihydro-8-oxo-guanosine (8-oxoG) [220]. These lesions, if left un-repaired lead to decreased DNA integrity, dysregulation of gene expression, mutagenesis, carcinogenesis and various pathological conditions. Although less reactive than •OH, $O_2^{\bullet -}$ and H_2O_2 can interact with membrane lipids, initiating lipid peroxidation, a cascade of oxidative reactions that lead to the formation of toxic DNA and protein damaging end products [221]. ROS can also attack the amino-acid backbone of proteins causing oxidation of side-chain residues, formation of carbonyls, peptide bond cleavage and protein-protein cross links, leading to loss of protein structure and function [222, 223]. Furthermore, elevated levels of $O_2^{\bullet -}$and H_2O_2 can reduce the activity of catalase and glutathione peroxidases, key players in the cellular defense mechanisms against ROS [224-226] [vasudevan 1990 - 224][Blum 1985 - 225][Day 2012 - 226] .

While cells have developed mechanisms to repair the damaged caused by ROS, cells that have reduced or have lost repair capabilities, such as spermatozoa, are at a greater risk of harmful effects of free radicals.

1.3.1.2 Defense against oxidative stress

Cellular mechanisms have evolved to protect against the formation of free radicals and prevent oxidative damage. This vast antioxidant defense system is made up enzymatic and non-enzymatic antioxidants.

1.3.1.2.1 Enzymatic antioxidants

In the testis, high levels of oxidative stress initiates a response involving NfKB mediated induction of several enzymatic enzymes [227].

Superoxide dismutase (SOD) is present mainly in 3 forms (SOD1, 2 and 3) that differ in structure and cellular location. However, all 3 serve the same purpose: catalyze the breakdown of $\bullet O_2^-$ into O_2 and H_2O_2 (Fig. 1-6). SOD1, also known as Cu/ZnSOD is found abundantly in the cytoplasm and also in the nucleus and mitochondria [228, 229]. The Cu ion provides the catalytic activity while the zinc ion provides structural stability to the enzyme [230]. A deficiency in either of these ions leads to loss of enzymatic function. SOD2 is also known as MnSOD because the catalytic function of the enzyme is executed by a Mn ion. It is predominantly found in the mitochondria [231]. Finally, SOD3, also known as ECSOD and SOD-Ex, is similar to SOD1 in structure but is located in the extracellular space [232].

All 3 SODs are expressed in the testis [233-235]. The activity of SOD1 is critical in male germ cells, as a knockout mouse model of the enzyme increased cell death and damage under increased stress conditions, such as heat and aging [236, 237]. Additional evidence for the importance of SOD activity is the remarkably high level of SOD2 and SOD3 expression in the testis [233, 235].

Catalase (CAT) catalyzes the degradation of H_2O_2 into H_2O and O_2, preventing the production of •OH via Fenton reaction. A group of 4 heme molecules bound to Fe is the catalytic centre of the enzyme. They are mostly located in peroxisomes, except in erythrocytes where it is also located in the cytoplasm [238]. Catalase is found in the testis, although it is less abundant than SOD1 and 2 [233]. Compared to SOD1, catalase may play a less important role in the testis , as deletion results in a less severe phenotype than SOD1 deletion, even under stress [237].This is due to the presence antioxidant enzymes capable of H_2O_2 detoxification. However, over-expression seems to have a protective function in male germ cells against age related increases in oxidative stress [239].

Glutathione peroxidase (Gpx) and glutathione reductase (GR). Glutatione peroxidases also catalyze the detoxification of H_2O_2 into H_2O by reduction with glutathione. Glutathione reductase regenerates the glutathione. There are several Gpxs and the activity of many of them is dependent on selenium [240]. Gpx4 is the most highly expressed in the testis and its deletion causes reduced fertility [241]. Additionally, decreased Gpx activity, increased levels of oxidative stress and germ cell loss are observed the testes of selenium deficient mice [242].

Peroxiredoxins (PRDX) also catalyze the detoxification of H_2O_2, but unlike CAT and GPX, their activity is metal ion independent. Thioredoxin (TRX) and thioredoxin reductase (TRD) are involved in regenerating the active form of PRDX [243]. A specific form of PRDX4 is present in the testis and its deletion leads to increased oxidative stress and testicular degeneration [244, 245]. Additionally PRDX6 has been shown to play an important role in sperm motility and protection against oxidative stress [246].

1.3.1.2.2 Non-enzymatic antioxidants

Zinc. Aside from being an essential cofactor for SOD1, zinc also plays additional roles as an antioxidant. Zinc is an important stabilizer of protein structures, including some that can influence levels of oxidative stress. The Cox4 subunit of cytochrome c oxidase of ETC requires zinc to properly function and loss of zinc binding alters the structure of Cox4 and disrupts the ETC, which can lead to the release of electrons [247]. Zinc has been shown to protect enzymatic activities in the presence of oxygen by protecting sulfhydryl groups from oxidation and formation of intramolecular disulfide bonds [248]. Zinc can also compete with redox active metals such Fe and Cu for binding sites on membranes and proteins, thus preventing Fenton reaction mediated production of •OH and lipid peroxidation [249, 250]. The anti-oxidant capabilities of Zn have been demonstrated in many tissues, including the testis, where Zn deficiency leads to increased oxidative stress. Furthermore, zinc has been shown to protect the testis against cadmium and lead induced oxidative damage [251, 252].

Vitamins E and C. Vitamin E (α-tocopherol) is a powerful lipophilic antioxidant and the most important inhibitor of lipid peroxidation. It is a powerful free radical scavenger because it can react with lipid peroxides (•ROO) faster that they can react with themselves [210]. Vitamin E is critical for spermatogenesis and has been shown to protect the testis against free radicals induced by polychlorinated biphenyl (PCBs) compounds [253], cadmium [254], formaldehyde and cyclophosphamide [255].

The water soluble vitamin C (ascorbic acid) acts as an antioxidant by reacting with O_2, HO_2, OH and RO_2 radicals to form a far less reactive ascorbyl radical, that is then further oxidized into dehydroascorbic acid (DHA). In this way it acts as a free radical scavenger [210]. It is also involved in the reduction of vitamin E, maintaining it in its active state. The enzyme dehydroascorbate reductase maintains vitamin C in its active state, dehydroascorbate, is highly expressed in the testis [256]. Vitamin C has been shown to protect the testis against oxidative stress induced by arsenic [257], PCBs [252], cadmium [253] and alcohol [258].

1.3.2 DNA damage

It has been estimated that in the mammalian genome over 10^5 DNA lesions are produced per cell per day as a result of replication errors and cellular metabolism. In addition, exogenous substances, including radiation, environmental chemicals and drugs, can also damage DNA.

1.3.2.1 DNA damage types

DNA can be damaged in various ways. One of the most common types of damage is single-strand breaks (SSBs); these are discontinuations in one of the two DNA strands. These types of lesions can lead to genetic instability, mutations and cell death. Furthermore SSBs can turn into highly toxic double strand breaks (DSBs) that are breaks in both DNA strands. If left unrepaired, DSBs lead to genomic instability, deregulation of cellular function and cell death.

Base mismatches arise during DNA replication or recombination after exposure to a damaging agent and can involve erroneous repair, insertions, deletions or

misincorporated bases. Base modifications are damaging chemical modifications to DNA bases. These can be caused by oxidation (ex: 8-oxodG), alkylation (ex: 6-O-methylguanine) and radiation (ex: pyrimidine dimers and 5-hydroxyuracil). Both base mismatch and modifications distort the structure of the DNA helix [259]. The damaged bases can also lead to the formation of DNA intra-strand and inter-strand crosslinks and DNA-protein crosslinks [260].

In response to the various types of damage, cells have developed the ability to repair DNA using several different mechanisms. The DNA damage response (DDR) is multilayered and consists of sensors, inducers and effectors.

1.3.2.2 DNA repair pathways

1.3.2.2.1 Nucleotide-excision repair

The nucleotide-excision repair (NER) pathway has the ability to repair mismatched base pairs and bulky adducts, oxidation and DNA intra-strand crosslinks. The versatility in substrates of NER lies in the many different proteins that assemble at the site of damage [259]. NER functions via a “cut and patch” mechanism where a section of single stranded DNA (ssDNA) (~24-32 nt in length) is excised and then replaced using the sequence of the remaining non-damaged strand as a template. NER can be subdivided into two sub-pathways: the global genome NER (GG-NER) which detects and repairs damage throughout the genome and the transcription-coupled NER (TC-NER), a more rapid pathway that removes damaged bases during transcription. In GG-NER, damaged DNA detection is accomplished by XPC/RAD23B proteins which recognize the thermodynamic destabilization of the DNA double helix and recruit the

TFIIH complex to unwind the DNA [261, 262]. In TC-NER, damaged DNA is recognized through stalling of RNA Pol II [263]. The repair mechanism in both sub-pathways is then the same. A second damage recognition factor, XPA, is recruited [264, 265] and then the endonucleases XPG and XPF/ERCC1 cleave the damaged DNA strand [266]. This is followed by synthesis and ligation of the new strand by DNA polymerases and DNA ligases [267, 268]. The NER pathway is cell cycle independent and thus active in both dividing and non-dividing cells.

1.3.2.2.2 Base-excision repair

In contrast to NER, the base-excision repair (BER) pathway removes the less bulky adducts that do not distort the double helix structure. The most well-known lesion repaired by this pathway is 8-oxodG, which is generated during oxidative stress [269]. Other modifications that can be repaired by this pathway include methylation and deamination [260]. Detection and recognition of the damaged base is accomplished by specific glycosylases that cleave the base from the deoxyribose. The excision of the base creates apurinic or apyrimidic sites which are then cleaved by the apurinc or apyrimidic nuclease APE1 [260]. The resulting ssDNA lesion is repaired via the short patch (SP) (1 nt) or long patch (LP) (2-20 nt) pathways that involves the replacement of the missing nucleotides by DNA Pol β and sealing of the remaining DNA nick by the XRCC-LIG3α complex or LIG1[270]. The BER pathway is also cell cycle independent.

1.3.2.2.3 Mismatch repair

The mismatch repair (MMR) pathway recognizes and repairs base-base mismatches and insertion deletion loops (IDLs) that arise during DNA replication and

homologous recombination [271]. In this pathway, the MSH1-6 recognizes the mismatched bases or IDLs in newly synthesized DNA. Discrimination between the nascent and original strands appears to be facilitated by the association of the MSH proteins to the DNA replication machinery [272]. Subsequently MLH1 and MLH3 are recruited and cleave the mismatched base. The resulting base is then excised by EXO1, followed by synthesis of the DNA segment by DNA polymerase and sealing of the nick by DNA ligase [260]. As this repair mechanism is coupled with DNA replication, it is mainly active in dividing cells.

1.3.2.2.4 Double-strand break repair

The first step in the response to a DSB is the recognition of the site of damage. Early after the formation of a DSB, histone variant H2AX becomes phophylated on serine 139 by ataxia telangatasia mutated (ATM) [273]. The phosphorylated form of H2AX, γ-H2AX, indicates the site of DSBs. The number and size of γ-H2AX foci correspond to the level of damage [274, 275]. γ-H2AX plays an essential role in the recruitment, accumulation and maintenance of DNA repair proteins to sites of damage and loss of H2AX phosphorylation results in impaired formation of irradiation induced foci [276-278]. One such protein that is recruited by γ-H2AX is P53 binding protein 1 (53BP1) [276-278]. 53BP1 plays an important role in DSB repair by recruiting additional repair factors to the site of DSB and involved in repair pathway selection [279, 280]. Loss of either γ-H2AX or 53BP1 results in increased sensitivity to radiation, growth retardation and reduced infertility. Additionally, while litters sired from 53PB1 knockout mice were smaller, male H2AX mutated mice were infertile [277] suggesting a role for DSB repair in male germ cells [2819]. Indeed, γ-H2AX foci are prominently visible on

the sex chromosomes in pachytene spermatocytes and may play a role in meiotic pairing, meiotic sex chromosome inactivation and the formation of the sex body [282].

Two different repair pathways are available to repair DDBs: homologous recombination (HR) or non-homologous end joining (NHEJ)

1.3.2.2.4.1 Homologous recombination

The homologous recombination (HR) pathway repairs DSBs in an error free manner employing sister chromatids. Facilitated by the γH2AX DNA damage signalling cascade, the DSB is first recognized by the MRN complex (comprised of MRE11, RAD50 and NBS1) [283] and recruitment of ATM and other mediators such as BRCA2 ensues. The DSB break is then resected to form ssDNA possibly through the activity of EXO1 [284]. RAD51 is recruited by BRAC2 to the ssDNA and forms nucleoprotein filaments [285]. The RAD51-ssDNA complex then invades the intact homologous DNA region on the sister chromatid. Once the complementary sequence is recognized, the DNA strand is elongated with help of RAD54 and DNA polymerase forming a displacement loop (D-loop) [286]. The resolution of the D-loop can then be completed by the synthesis-dependent strand annealing (SDSA) mechanism that avoids crossing over and reduces the potential genomic rearrangement. This is in contrast to the resolution of the D-loop in meiotic recombination, where cross over is achieved through the formation of a double Holliday junction [287].

1.3.2.2.4.2 Non-homologous end joining

The non-homologous end joining (NHEJ) repair pathway is used to repair DSBs when homologous chromosomes are not available, as is the case in haploid cells. NHEJ

rapidly repairs DSBs by directly joining broken DNA ends. For this reason it is inherently error prone, often resulting in lost genomic information at the DSB site. In the first step of NHEJ, the Ku70/80 heterodimer binds DSBs and recruits the active protein kinase complex DNA-PK, which stabilizes and aligns the DSB ends. The nuclease Artemis resects the DNA to provide the appropriate ends gap filling by DNA polymerase and ligation by LIG4. NHEJ is the predominant pathway of DSB repair in mammalian cells. There is competition between HR and NHEJ repair. Pathway selection depends on the cell cycle stage and appears to involve competition between BRCA1 and 53BP1 [280].

1.3.2.2.5 RNA directed DNA damage repair

Roles for RNA transcripts in the DDR have recently emerged. Recent studies have shown that DSBs induce the expression of a class of small RNAs (~21 nt), called DSB-induced RNA (diRNAs), that originate from the sequences in proximity to the damaged DNA [288, 289]. It is currently thought that diRNAs bound to AGO2 of the RISC, play a role in HR by recruiting RAD51 to the site of DSBs through complimentary sequence pairing [290].

Evidence is emerging that RNA transcripts can be used as templates to direct DSB, challenging the belief that HR occurs only between DNA. This DNA repair mechanism is present in viruses and has also been shown to function in yeast [291]. Various miRNAs have also been shown to influence the DDR by targeting genes involved in DNA repair, damage response and cell cycle checkpoint [92].

1.3.2.3 DNA damage repair in male germ cells

In male germ cells, DNA repair is required for meiotic recombination as well as the repair of damaged DNA that occurs during chromatin compaction. Many of the proteins involved in the different repair pathways are expressed in the different germ cell types, indicating that these pathways are active in the testis.

1.3.2.3.1 Spermatogonia

The proper repair of DNA damage in spermatogonia is critical because damage incurred by the stem cell population could lead to the generation of a large number of mutant gametes. The high level of mitotic activity in spermatogonia leads to an increased probability of DNA damage due to replicative errors. However because spermatogonia are actively dividing cells they can utilize both HR and NHEJ to repair damaged DNA. Although high levels of genes and proteins related to the BER as well as their activities have been shown in the testis [292], the extent to which BER is active in spermatogonia is unknown. However, the NER activity in spermatogonia, although lower than in somatic cells, is higher than in pachytene spermatocytes and round spermatids [293]. Additionally, the MMR related proteins MSH2 and PMS2 are highly expressed in spermatogonia compared to both spermatocytes and spermatids suggesting that MMR plays a role in the repair of replication related DNA damage [294]. Thus multiple repair pathways are present at the early stages of spermatogenesis.

1.3.2.3.2 Spermatocytes

The ability of spermatocytes to repair damaged DNA has been demonstrated using unscheduled DNA synthesis assays [295-297]. The HR pathway is highly active in early spermatocytes due to meiotic recombination [298]. Many homologues of RAD51

are highly expressed in the testis [299]. The localization of one such homologue, DMC1, to the synaptonemal complex is essential for meiosis as deletion of this protein leads to failure of synapsis [300]. Whereas HR is highly active in the early spermcatocytes, NHEJ seems to play a larger in DNA repair role in the late spermatocytes [301]. The BER related proteins MPG, UNG and APEX are expressed in spermatocytes. In addition, the activities of these proteins were higher than in somatic cells but did not differ from round spermatids [302]. The Ogg1 glycosylase, responsible for repair of 8-oxo-dG lesions, is most highly expressed in spermatocytes. NER is active in pachytene spermatocytes although less than in spermatogonia. Additionally, the NER related proteins Ercc1 and Xpf, that are also involved in HR, are highly expressed in spermatocytes. However, Ercc deletion does not cause meiotic defects and instead leads to increased DNA damage throughout the testis [303]. This suggests that Ercc1 and NER play a role in mediating DNA repair in germ cells. The MMR is critical for spermatocytes. Several MMR genes, Msh2, Mh3 Pms2 as well as the testis specific Msh4 and Msh5, are highly expressed in spermatocytes. Additionally the deletion of any of these genes leads to meiotic arrest with defects in homologous recombination [299]. Therefore although the MMR plays a role in repairing DNA damage caused during mitotic DNA replications in spermatogonia, its main role is repairing damage incurred during meiotic recombination.

1.3.2.3.3 Spermatids

The germ cells ability to repair damaged DNA declines drastically post-meiotically [295, 297, 304, 305]. DNA repair remains possible in round spermatids and activity of the NER and BER pathways is similar to that of spermatocytes [291, 300].

However the expression of proteins involved in MMR begin to decline at the round spermatid stage [294]. As would be expected of a haploid cell, HR is no longer active. Instead DSB are repaired through NHEJ. However this process is slow in round spermatids [301].

All the major DNA repair pathways appear to be less functional in late spermatids compared to early spermatids and other germ cell types [299, 306]. Additionally, components of the MMR and NER pathways that are present throughout spermatogenesis, decline after meiosis, reaching undetectable levels in elongating spermatids [299]. BER related proteins MPG and APEX are still expressed in elongating spermatids [302], but Ogg1 expression and excision of 8-oxo-dG lesions is reduced [307]. The highest levels of DNA damage are observed in sperm, when germ cells are exposed to toxic agents as late spermatids [305]. Some studies suggest that no DNA repair occurs in elongating spermatids [307, 308]. Unlike other germ cell types, elongating spermatids do not display unscheduled DNA synthesis after exposure to damaging agents [304]. This is mainly attributed to the transcriptional quiescence and highly condensed state of DNA by protamines that would hinder access of the DNA repair machinery to damaged sites. However the expression of γH2AX in elongating spermatids during chromatin condensations suggests that some level of DNA repair is possible via NHEJ, at least in the context of normal spermiogenesis [309]. Spermatids undergo transient DNA strand breaks in order to relax DNA structure in the final protamination steps of sperm chromatin remodelling [310, 311]. These DNA strand breaks, which occur mainly in intergenic regions [312], are repaired by the late steps of

spermiogenesis [310, 311]. The ability of elongating spermatids to repair DNA damage caused by exogenous sources has yet to be observed.

1.3.2.3.4 Spermatozoa

The shedding of the cytoplasm and highly condensed DNA leave mature spermatozoa devoid of any DNA repair mechanisms. However, the very nature of the compacted nucleus also provides a level of protection from exogenous sources of DNA damage.

Any damage that is carried in the spermatozoon will be transmitted to the oocyte at fertilization and can affect embryo development. While male germ cells lose their ability to repair DNA as they differentiate, the oocyte maintains DNA repair activity throughout oogenesis and provides the necessary machinery to respond to damaged spermatozoal DNA after fertilization. The maternal DNA damage response to DSBs can be seen as early as the prepronuclear stage of development in zygotes sired from rats chronically exposed to radiation or cylophosphamide [312, 313]. The efficiency of the maternal DDR, as assessed using knockput models, plays an important role in the transmission of aberrant genetic material to progeny [315]. Unfortunately, DNA damage that is acquired during germ cell development can escape the maternal repair machinery or be improperly repaired [316]. Therefore when it comes to fertility and progeny outcomes, it is necessary to consider paternal exposures.

1.4 Paternal exposures and male mediated reproductive toxicology

It is estimated that nearly 30% of infertility cases are solely due to male factor infertility [315]. While a small percentage of cases can be explained by genetic factors, many may in fact be related to lifestyle and/or exposure to toxic substances. Epidemiological studies have provided evidence that paternal exposures can adversely affect sperm quality and fertility. However, animal models have been crucial in providing direct evidence that paternal exposures can lead to adverse effects on male germ cell development and have consequences to progeny.

1.4.1 Environmental exposures

Chemicals in the environment have the potential to damage male germ cells. Epidemiological studies have shown that high levels of air pollution from coal combustion is associated with increased sperm DNA fragmentation [318]. After exposed of animals to industrial air pollution or its components, the formation of DNA adducts in spermatocytes, spermatids and spermatozoa, as well as DSBs and hypermethylation of sperm DNA have been demonstrated. An increase in germ line mutations has also been observed in animals exposed to industrial air pollution or one of its toxic component benzo(a)pyrene [319-321]. Embryos sired by animals exposed to benzo(a)pyrene display altered miRNA and mRNA transcripts involved in DNA damage response, transcription and chromatin modifications [322]. This suggests that paternal exposure to toxic substances found in air pollution can not only damage male germ cells but also affect embryo development.

Many other environmental exposures such as pesticides, insecticides and plasticizers act as endocrine disruptors that can have multigenerational effects that are passed on through the germline via DNA methylation.

1.4.2 Lifestyle exposures

Paternal lifestyle factors play an important role in germ cell quality. Factors such as occupational exposure to toxic substances (lead and pesticides) [323], cigarette smoking [324, 325] as well as diet and obesity [326] have all been associated with decreased sperm quality and fertility in humans.

In animal models, exposure to cigarette smoke causes increased oxidative stress [327] and germ cell death [328] in the testis. Sperm from exposed animals display increased DSBs, decreased chromatin quality and increased mutations. These cigarette smoke-induced defects result in reduced fertility as well as impaired embryo development [329-331]. In addition, exposure to side stream (second hand) smole also leads to DSB and heritable germ line mutations [330, 332]. The damaging effects of cigarette smoke on male germ cells are not surprising. However, in the last few years, evidence that stress, diet and exercise can influence sperm quality and progeny has also emerged.

In mice, paternal stress altered the DNA methylation [333, 334] and miRNA content in sperm [147, 148]. It is postulated that the changes in methylation and miRNA in the sperm are transmitted to embryo and reprogram neurodevelopment leading to altered stress response and metabolic function. Interestingly, two separate studies showed that the injection of spermatozoal miRNAs from stressed fathers into fertilized

eggs recapitulates the altered stress response phenotype in progeny [147, 149]. A high fat diet seems to have a plethora of effects on male germ cells. Mice fed a high fat diet had altered mRNA and miRNA expression in the testis [335] and increased levels of DNA damage and oxidative stress in mature sperm [336]. A paternal high fat diet results in impaired early embryo development [337]. It also leads to alter glucose metabolism and obesity in progeny [335]. The effects of a high fat diet on progeny may be mediated by the observed altered DNA methylation [338], histone distributions[339], miRNAs [89, 335, 338] and tRNAs in the sperm. As with paternal stress, the injection of miRNAs and of tRNAs into fertilized eggs was able to recapitulate the high fat diet phenotype [89, 200].

These studies not only suggest that germ cell quality can be affected by paternal lifestyle but also that epigenetics and RNAs play a role in transmitting damage to progeny.

1.4.3 Therapeutic drug exposures

The effect of paternal therapeutic drug exposures on sperm quality and fertility in humans has been well studied, especially in the case of chemotherapeutic drugs and radiation. The well documented time and doses make it easy to make clear associations between the drug treatment and reproductive effects. A large follow-up study of childhood cancer survivors found that individuals who were not sterile were only half as like to conceive than their siblings [340]. Patients treated with chemotherapy for either testicular cancer or Hodgkin lymphoma have significant damage in to their sperm, even

two years post-treatment [341-343]. However, variability is always high in human populations.

Cancer therapies are extremely cytotoxic and unfortunately unspecific. Because of the high activity and dynamic processes that occur during spermatogenesis, male germ cells are particularly sensitive to the effects of cytotoxic agents. Numerous animal studies have characterized the effects and mechanisms of toxicity of these agents on the male reproductive system as well as on progeny outcome. Ionizing radiation (IR) is regularly used in cancer therapy and one of the most well studied therapeutic germ cell toxicants. It causes DNA damage by the generation of free radicals. The accumulation of ionizing radiation-induced DNA damage leads to apoptosis. In the testis spermatogonia are most sensitive due to their high mitotic activity, followed by spermatocytes. Ionizing radiation often leads to azoospermia because of the complete loss of spermatogonial stem cells. Any remaining viable spermatogonia may still be damaged and go on to produce damaged spermatozoa. Low dose IR progeny outcome studies indicate that damage induced at all stages of spermatogenesis is capable of leading to increased fetal loss. However, meiotic cells were most sensitive to the induction of malformations [344]. Another well characterized cancer treatment is with the chemotherapeutic agent cyclophospamide

1.5 Paternal exposure to cyclophosphamide

1.5.1 Mechanism of action and pharmacokinetics of cyclophosphamide

Cyclophosphamide (CPA) is a commonly used cytotoxic alkylating agent. It is used to treat various cancers including malignant lymphomas, leukaemias, neuroblastoma, retinoblastoma and carcinomas of the ovary, breast, endometrium and lung [345]. It is often used in combination with other chemotherapeutic agents. CPA is also used in the treatment of various immune disorders including graft rejection, rheumatoid arthritis, autoimmune skin diseases, multiple sclerosis, systemic vasculitides and systemic lupus erythromatousus and to prepare for bone marrow transplants (reviewed in [345]). CPA can be administered orally or parenterally. The regimen for chemotherapy varies greatly but ranges from 2-6 mg/kg body weight (low dose) to over 6000 mg/m^2 body surface area (high dose). In immunosuppressive therapy, typical doses are 100 – 200 mg/day (~1.6-3.3 mg/kg body weight base on 60 kg), while for bone marrow transplant up to 240 mg/kg is administered over a 4 day period (reviewed in [345]).

CPA itself is a prodrug and is activated by cytochrome P450 (CYP450) in hepatic microsomes to form 4-hydroxycyclophosphamide (4-OHCPA) which exists in equilibrium with aldophosphamide (Fig. 1-7) [346, 347]. CYP isoenzymes CYP2A6, 2B6, 3A4, 3A5, 2C9, 2C18 and 2C19 have been shown to be involved in the activation of cyclophosphamide [345]. 4-OHCPA can readily diffuse through membranes [348]. It is non-toxic but very unstable. The cytotoxic drug is formed by spontaneous decomposition into phosphoramide mustard by β-elimination of acreoline [349]. Through the formation of aziridinyl ion intermediates, phosphoramide can then alkylate nucleophiles such as proteins and most importantly DNA at the N-7 position on guanine [350]. The resulting intra- and interstrand crosslinks and DNA protein crosslinks cause

DNA strand breaks, replication arrest and apoptosis [351-354]. The by-product of phosphoramide mustard formation, acreoline, is a highly reactive aldehyde. It can also covalently bind to DNA and proteins to form interstrand DNA crosslinks and DNA-protein crosslinks [355]. Additionally, acrolein increases levels of ROS and depletes levels of glutathione. Asides from its role in protection against oxidative stress, gluthathione can act as a scavenger of electrophiles and may protect cells against CPA by preventing the breakdown of 4-OHCPA into phosphoramide mustard. Thus acrolein depletion of gluthathione promotes the cytotoxicity of CPA [356].

The half-life of CPA in humans ranges between 5-9 hours [345]. The major pathway by which cyclophosphamide is detoxified and eliminated is the aldehyde dehygrogenase (ALDH) catalyzed formation of carboxyphosphamide from aldophosphamide [345, 357-360]. Other pathways are shown in Figure 1-7. Finally, CPA and its metabolites are eliminated through the urine [345].

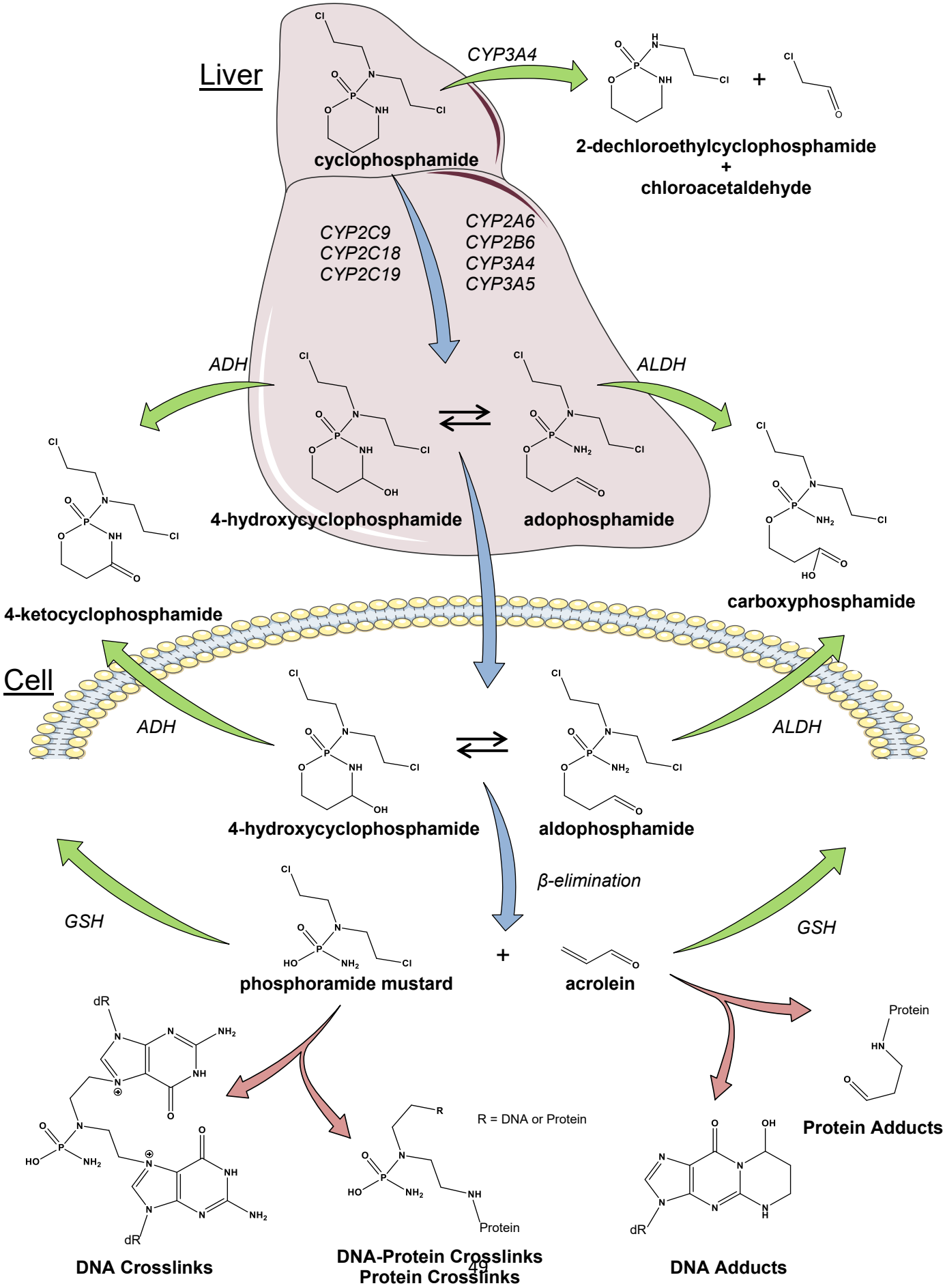

Liver
CYP3A4
cyclophosphamide
2-dechloroethylcyclophosphamide
+
chloroacetaldehyde
CYP2C9
CYP2C18
CYP2C19
CYP2A6
CYP2B6
CYP3A4
CYP3A5
ADH
ALDH
4-hydroxycyclophosphamide
adophosphamide
4-ketocyclophosphamide
carboxyphosphamide
Cell
ADH
ALDH
4-hydroxycyclophosphamide
aldophosphamide
β-elimination
GSH
GSH
phosphoramide mustard
+
acrolein
Protein
Protein Adducts
R = DNA or Protein
DNA Crosslinks
DNA-Protein Crosslinks
Protein Crosslinks
DNA Adducts

Figure 1-7. Metabolism and mechanism of action of cyclophosphamide. CPA is a prodrug that is activated and converted into 4-hydroxycyclophosphamide and aldophosphamide in the liver by cytochrome P450 enzymes. A portion is also directly inactivated. 4-hydroxycyclophosphamide and aldophosphamide exist in equilibrium and can be further metabolized and inactivateded by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) respectively. These 2 metabolites can diffuse into cells and undergo β-elimination to produce the metabolites that are responsible for the majority of CPA toxicity: phosphoramide mustard and acrolein. These metabolites can alkylate DNA and proteins causing cellular damage. Both phosphoramide mustard and acrolein can be inactivated by glutathione (GSH). Blue arrows = metabolism/activation of CPA; Green arrows = detoxification/inactivation of CPA; Red arrows = mechanism of action. dR = deoxyribose sugar. Adapted from de Jonge (2005) [345].

1.5.2 Effects of cyclophosphamide on male reproductive function

1.5.2.1 Human

Multiple studies have shown that men treated with CPA often become oligozoospermic or azoozpermic [361-363]. In some cases, all patients became azoospermic within 6 months of being treated and as early as 4 months. The penetrance and duration of these phenotypes is dependent on the dose. Cumulative doses over 19 g/m^2 body surface area lead to prolonged azoospermia, while doses below can still lead to azoospermia, but sperm formation usually resumes after treatment [364]. In combination therapy with doxorubicin, dacarbazine and vincristine, cumulative doses of CPA of over 7.5 g/m^2 were associated with long term azoospermia or oligozoospermia [365]. Recovery of spermatogenesis is possible; however, about half of men who were azoospermic at the end of cyclophosphamide treatment remained azoospermic for up to 5 years post-treatment [366]. CPA treatment in pre-pupertal boys can lead to long-term oligozoospermia and azoospermia. In two long-term follow-up studies, nearly half of the patients were either oligozoospermic or azoospermic a mean of 12 years post-treatment [367, 368]. The results of these studies indicate that in humans cyclophosphamide causes severe damage to spermatogonia. Despite low proliferative-rate of pre-pubertal spermatogonia, cyclphophamide treatment during childhood can lead to long-term spermatogenic defects.

1.5.2.2 Non-human reproductive system

Mice and rats have been used to study the effects and mechanisms of action of CPA on the male reproductive system. Chronic low dose CPA treatment does not affect

the reproductive organ weights, endocrine status, sperm number or fertility in male rats [369, 370]. However, more in depth studies show that CPA treatment affects the male germ cells, embryo development and progeny outcomes in a dose and stage dependent manner (Fig. 1-8) [369-371].

1.5.2.2.1 Male germ cells

CPA treatment causes increased damage to sperm DNA at high acute and chronic low doses [371, 372]. The timing of exposure and effects can be used to determine the susceptibility of the germ cell stages. In rats, a drug is targeting the active mitotic spermatogonia if effects are observed after 7-9 weeks, the meiotic germ cells after 5-6 weeks, spermiogenesis in early to mid-spermatids after 3-4 weeks, and maturation and epididymal transit of late spermatids and sperm after 1-2 weeks [17, 368] (Fig.1-8). The highest level of DNA strand breaks was observed 3 weeks after both high-dose and chronic low dose CPA treatment [371]. Additionally, decreased levels of protamination and chromatin condensation were observed [373]. This indicates that the most damaging effects of CPA occur in elongating spermatids at a key time in germ cell development when chromatin remodelling begins, transcription is shutting down and the expression of DNA repair genes is decreased. Consequently, active DNA repair in response to CPA insult, as assessed by the unscheduled DNA synthesis (UDS) assay, is impaired and damaged spermatozoa are produced [296].

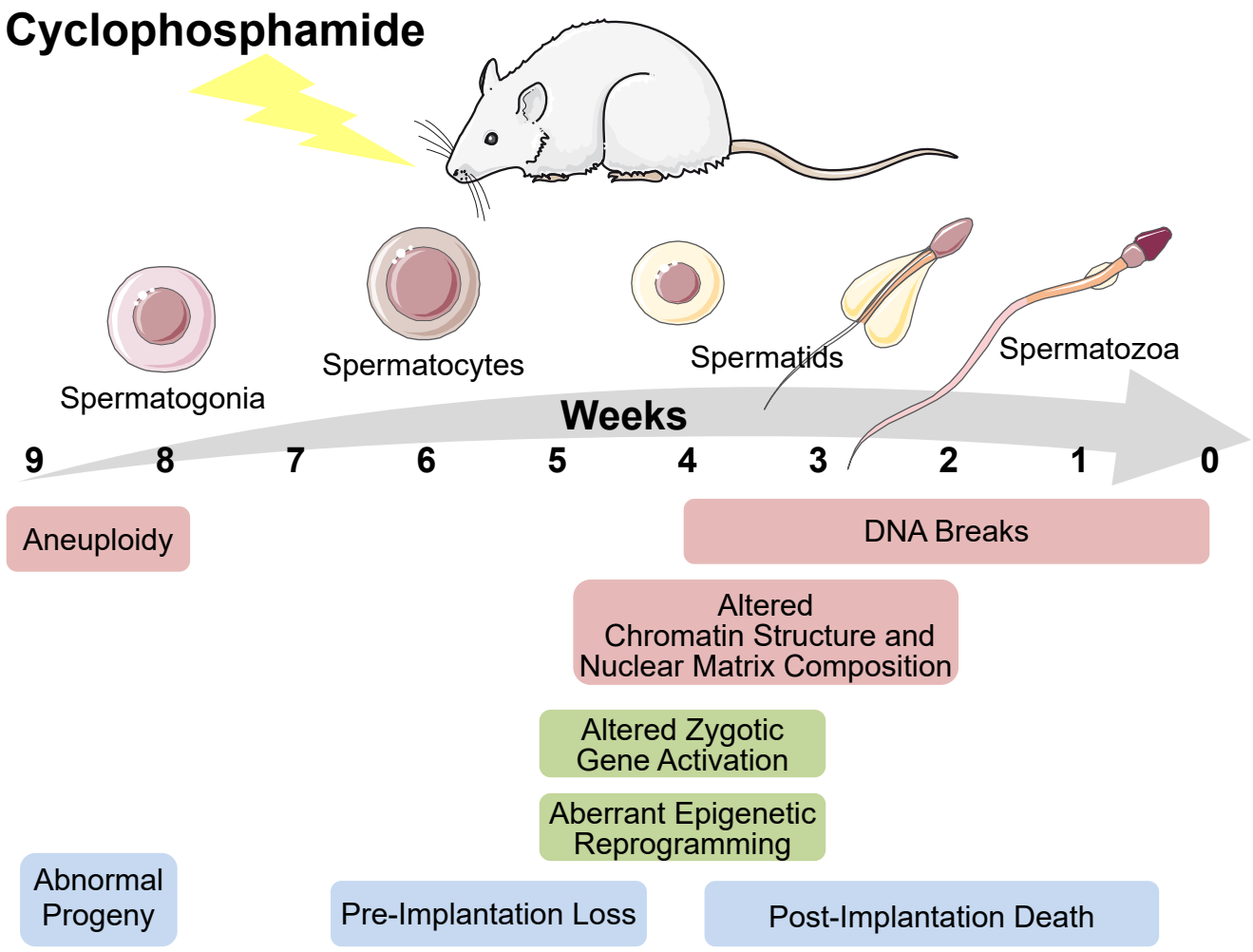


Figure 1-8. The specificity of the susceptibility of male germ cells to damage after treatment with chronic low dose of cyclophosphamide. The impact of chronic low-dose exposure of male rats to cyclophosphamide on the occurrence of germ cell damage (red), early embryo events (green) and progeny outcome (blue). The effect of CPA treatment is dependent on when, during spermatogenesis and spermatozoal maturation, the germ cells are first exposed to the drug. Figure was created in part using images from Servier Medical Art (www.servier.com), licensed under a Creative Commons Attribution 3.0 Unported License.

The number of spermatozoa with numerical chromosomal abnormalities (aneuploidy) after CPA treatment is increased after 9 weeks of chronic treatment but not 6 weeks. Cytogenetic studies in mice given a single acute dose of CPA pre-meiotically show damage to the synaptonemal complex and disrupted chromosomal disjunction leading to a significant increase in hyperploidy in metaphase II spermatocytes [374, 375]. These studies suggest that there is a critical window before the pachytene stage, likely leptotene or zygotene, where CPA treatment interferes with the chromosome segregation and recombination events that occur at the later stages of meiosis. Although the germ cells were exposed as spermatogonia, these cells possess extensive apoptotic programs to eliminate damaged cells and are most susceptible germ cell to radiation induced apoptosis [376-378]. Indeed, following an acute high dose of CPA, pronounced levels of apoptosis were seen in spermatogonia compared to other germ cell types [379]. Early spermatocytes are also susceptible to CPA induced chromosomal damage as evidenced by the formation of micronuclei [380]. Acute high doses of CPA induced high levels of γH2AX foci in spermatocytes and impaired meiotic progression in vitro. Despite the increased number of H2AX foci observed in spermatocytes, meiotic progression was not impaired after chronic low dose CPA treatment [381]. The lack of meiotic arrest despite induced damage raises concerns about the function of protective mechanisms, including cell cycle check points, in spermatocytes after chronic exposure to CPA.

In line with this, the evaluation of the expression of stress response genes in pachytene spermatocytes, round and elongating spermatids following acute high dose and chronic low dose CPA treatment suggests that germ cell protective mechanisms

become repressed over time [382, 383]. While acute CPA treatment resulted in an increase in the expression of genes involved in DNA repair and stress response (especially in round spermatids), chronic CPA treatment had a net down-regulating effect on the expression of genes involved in DNA repair, oxidative stress and cell cycle genes in both pachytene spermatocytes and round spermatids. The expression of genes involved in translational control were altered exclusively in round spermatids after chronic CPA treatment which could result in defective storage and regulation of transcripts necessary for the later steps of spermatogenesis. The observed changes in expression of sperm head and nuclear matrix proteins in mature sperm after CPA treatment may be a consequence of altered gene expression in round spermatids [373, 384].

Altogether, animal studies indicate that cyclophosphamide induces damage to the developing male germ cells at which point three different scenarios may occur: 1) the damage to the cell will lead to apoptosis; 2) the damage will be completely repaired; and 3) the damage will not be completely repaired and damaged spermatozoa with altered DNA, protein expression (and possibly RNAs as well) will be produced. The highest levels of damage appear to occur at the time in development when the germ cells lack the ability to repair DNA damage. However, chronic treatment with CPA also decreases the expression of DNA repair genes in the normally repair-competent spermatocytes and round spermatids, suggesting that damage incurred in these cells may not be fully repaired. This damage can then be transmitted to the oocyte at fertilization and have consequences to embryo development and progeny outcome.

1.5.3 Effects of paternal cyclophosphamide on fertilization, embryo development and progeny

Paternal CPA treatment has dose-dependent and time-specific effects on progeny outcome (Fig. 1-8). Increased post-implantation loss was first observed after 2 weeks of chronic low dose CPA treatment of male rats and hit a plateau at 4 weeks of treatment. Pre-implantation loss was highest after 5-6 weeks of CPA treatment. After 7-9 weeks of CPA treatment, an increase in malformed (hydrocephaly, edema and micrognathia) and growth retarded fetuses was observed [369, 370]. The timing indicates that post-implantation loss is at its highest when germ cells are first exposed during spermiogenesis, pre-implantation loss occurs when germ cells are first exposed as spermatocytes and the malformations result from exposure as spermatogonia [369, 370]. The effects of CPA treatment on pregnancy outcome were reversible, with levels of pre-implantation or post-implantation loss returning to normal within 4 weeks [385]. Furthermore, exposure of the post-meiotic germ cells to cyclophosphamide led to behavioural abnormalities, such as learning deficits, in progeny [386, 387]. Interestingly, the adverse outcomes, such as post-implantation loss, fetal malformations and behavioural effects, were heritable and also observed in progeny sired by the offspring of CPA treated male rats [388, 389].

Studies on early embryo development have been undertaken to determine the mechanisms by which paternal CPA treatment affects pregnancy and progeny outcomes. At the cellular level, embryos sired from CPA treated rats displayed reduced cell proliferation and numbers as early as gestational day 3 (4-8 cell stage embryos) [390] and a dying inner cell mass at the blastocyst stage (day 7 of gestation) [391].

Ultimately, the embryos lost the cell-cell contacts that are necessary for normal development, leading to the observed post-implantation death [392].

At a more molecular level, sperm chromatin decondensation and the formation of the paternal pro-nucleus were accelerated in embryos sired from CPA treated males [314]. This is consistent with the findings that CPA treatment causes DNA damage, decreased chromatin compaction and alterations to components of the sperm nuclear matrix [373]. Precocious RNA synthesis suggests that the accelerated formation of the male pronucleus causes a dysregulation the zygotic gene activation [393] . Additional evidence for an effect of paternal CPA treatment on the developmental clock is premature histone H4-K5 hypermethylation and DNA hypomethylation in the male pronucleus of zygotes from CPA damaged sperm [394]. The accelerated events of early embryo development may be the result of a heightened response to the damaged paternal genome and activation the maternal DNA repair machinery.

Increased DNA strand breaks were observed in one-cell embryos indicating that the sperm from CPA treated males were capable of fertilization despite damaged DNA [395]. The maternal response to the damaged paternal genome was activated as early as the pre-pronuclear stage as the formation of H2AX foci increased during sperm chromatin decondensation [314]. Expression of genes the NER, MMR and HR DNA repair pathways were increased and localization of DNA repair marker 53BP1 and PARP1 to damage paternal genome was increased [395, 396].

Thus paternal CPA treatment leads to altered early events of embryo development. Although, the maternal DNA damage response is active in CPA sired

embryos, it may not be sufficient to overcome the high levels of damage to paternal chromatin and result in early embryonic death or the survival of embryos with improperly repaired DNA and progeny that present with malformations and behavioural defects. Additionally, the altered epigenetic marks in the early embryo could represent a mechanism by which the effects of paternal CPA treatment are transmitted to multiple generations.

The findings of the studies on paternal exposures to toxic agents such as CPA and the consequences to the embryo highlight not only the need to better understand the underlying mechanisms, but also the need to develop strategies to protect male germ cells.

1.6 Protective strategies for male reproductive health

With medical advancements, more men of reproductive age being treated for cancer survive and wish to father children. Although regulatory agencies are now demanding more studies to better characterize the reproductive toxicities of drugs, the use of germ cell toxic substances remains unavoidable for the treatment of many diseases. Strategies to protect male germ cells and thus preserve fertility in men treated with potentially toxic agents are limited. The only current option available to men undergoing chemotherapy is the collection and cryopreservation of sperm samples before beginning therapy for later use with assisted reproductive technologies (ART) [397]. The use of a protective shield is also used in regimens that include radiation therapy. In the development of a treatment plan, fertility preservation is often overlooked

by oncologists and not discussed with patients [398-400] . Furthermore, the process of cryopreservation can introduce DNA damage and ART is costly and not without risks. Therefore the development of simple and effective germ cell protective strategies is needed.

1.7 Formulation of the project

It is clear the CPA is toxic to male germ cells. Although there has been extensive research on the effects of CPA on germ cells, we still do not know the full extent of how male germ cells respond to this toxic insult. The advancements in molecular biology methods and technologies have made it possible to evaluate the transcriptome. The purpose of the studies presented in this thesis is to evaluate the effect of chronic CPA treatment on the male germ cell transcriptome in order to gain a better understanding of how the meiotic and post meiotic germ cells respond to such an insult. The knowledge gained from these profiling experiments can then be put towards developing strategies to protect male germ cells and biomarkers to detect germ cell toxicity.

The objectives of this thesis are:

1) Evaluate the effect of cyclophosphamide treatment on miRNA profiles in pachytene spermatocytes and round spermatids

2) Evaluate the effect of cyclophosphamide treatment on genome wide gene expression profiles in pachytene spermatocytes and round spermatids

3) Determine whether male germ cells can be protected against CPA induced damage by zinc supplementation.

For these studies, adult male Sprague Dawley rats were gavaged with 6 mg/kg CPA daily, 6 days per week for 4 weeks. The Sprague-Dawley rat is the most widely used model in toxicological studies and was the animal model used for many of the studies described above. The chronic low dose of 6 mg/kg per day is clinically relevant and equivalent to the low doses used in chemotherapy maintenance and immunosuppressive therapies used in humans. We evaluated the pachytene spermatocytes and round spermatids after 4 weeks of CPA treatment. These cells were chosen because 1) they represent germ cells at different stages of: meiotic and post meiotic development; 2) chronic CPA treatment beginning at these two stages resulted in the highest levels of pre- and post-implantation loss in previous mating studies [369,370]; 3) they are the most transciptionally active cells in the testis; and 4) the ability to collect and isolate cells using the STA-PUT method.The elongating spermatids were omitted from these studies because with this collection method the isolated fractions are contaminated with residual bodies and thus transcripts evaluated would not be representative of what is actually present in the germ cells. The length of treatment ensures that the germ cells were exposed to CPA from the time they were spermatogonia. Although the spermatogonial stem cells are of interest, these cells possess extensive DNA repair and apoptotic pathways, as mentioned previously, and exposure to toxic substances at this stage in germ cell development usually results in proper repair of the damage or cell death [376-378].

We evaluated not only whole genome protein coding mRNA expression but miRNA expression because of their role in post-transcriptional control of gene expression, a control mechanism important for germ cell development. Although miRNA

expression has been shown to be altered by other toxic substance (such as ethanol, valproic acid and lead) in other tissues, it has never been evaluated in the context of male germ cells following exposure to a therapeutic drug.

The results from the second aim suggested that stress caused by chronic CPA treatment results in an increased zinc requirement. Zinc plays an important role in the defence against oxidative stress [230, 248-252], is important for DNA binding proteins, including some involved in DNA repair [401-404] and plays a role in sperm chromatin structure[46]. Thus, we examined whether zinc supplementation along with CPA treatment would help protect male germ cells against CPA insult.

1.8 References

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CHAPTER 2

ALTERED MICRORNA EXPRESSION IN PACHYTENE SPERMATOCYTES AND ROUND SPERMATIDS IN RESPONSE TO CHRONIC LOW DOSE CYCLOPHOSPHAMIDE TREATMENT

Anne Marie Downey[1], Barbara F. Hales[1], Bernard Robaire[1,2]

Departments of Pharmacology and Therapeutics[1] and Obstetrics and Gynecology[2],

McGill University, Montréal, Québec, Canada, H3G 1Y6

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ABSTRACT

MicroRNAs (miRNAs), a class of small non-coding RNAs, have emerged as major players in post-transcriptional gene regulation and are implicated in various cellular functions in normal and disease states. Although numerous miRNAs have been identified in the testis, their roles in regulating the highly specific events that occur in the different germ cell types throughout spermatogenesis remain largely unknown. Furthermore, whether male germ cell miRNA expression is altered in response to or as a consequence of exposure to a toxic agent is unknown. Here we examine miRNA expression profiles in pachytene spermatocytes and round spermatids obtained from control rats and from rats treated with a chronic low dose of cyclophosphamide, a male germ cell toxicant. We observed that pachytene spermatocytes and round spermatids display vastly different miRNA expression profiles, reflecting their different developmental stages and possibly influencing the cellular response to toxic insult. Chronic low dose cyclophosphamide treatment altered the miRNA profiles in both pachytene spermatocytes and round spermatids. Target prediction analyses revealed that miRNAs altered by cyclophosphamide treatment may be involved in the response to cellular stress and damage. However, many are also involved in processes that are crucial for proper germ cell development. This study suggests that pachytene spermatocytes and round spermatids display distinct miRNA profiles that can be altered by cyclophosphamide treatment. The observed changes may be part of a response and repair mechanism to cyclophosphamide-induced damage or a dysregulation that disrupts normal germ cell development.

INTRODUCTION

MicroRNAs are a family of small non-coding RNAs that are 21-24 nucleotides long. Their role in mRNA degradation and translational repression via the RNA-induced silencing complex (RISC) makes them important mediators of post-transcriptional gene regulation [1]. The large volume of research on miRNAs indicates that proper miRNA expression is crucial for normal cellular function, including proliferation, differentiation, metabolism and apoptosis. Dysregulation of miRNAs is associated with many diseases such as cancer, inflammation, neurodegeneration and cardiovascular, liver and autoimmune diseases [2]. Additionally, miRNA expression in various tissues, such as the liver, brain and kidney, is altered after exposure to toxic substances [3]. This would suggest that altered miRNAs may either mediate defense or repair mechanisms or contribute to cellular injury in response to a toxic insult.

The importance of miRNAs in spermatogenesis has been revealed by germ line specific knock outs (KO) of DICER and DROSHA, two endonucleases important for miRNA biogenesis. These KO animals present with severe defects in meiotic and post-meiotic germ cells [4-7]. Although numerous miRNAs have been identified in the testis, the functions of the majority of these remain unknown. However there is emerging evidence that some of these play roles in processes crucial for germ cell development. Several miRNAs have been shown to be important for spermatogonial maintenance, meiotic entry, apoptosis and the regulation of TP2 and PRM1 expression [8-17]. Additionally, miRNAs are present in mature sperm and are proposed to play a role in

early embryo development [18]. Considering the complexity of spermatogenesis and the male germ cell transcriptional program, a role for miRNAs in the regulation of key events is evident. Therefore any exposure that alters miRNA expression in male germ may affect the proper development of male germ cells.

Cyclophosphamide (CPA), an alkylating agent and known germ cell toxicant, is commonly used in cancer and immunosuppressive therapies. Previous studies have shown that CPA treatment causes an increase in DNA damage and oxidative stress in male germ cells and a decrease in sperm chromatin quality [19-24]. Paternal exposure of developing male germ cells to CPA leads to adverse progeny outcomes [25, 26]. The molecular mechanisms responsible for the toxic effects of CPA and how male germ cells respond to this insult are still not well understood. We have previously shown that mRNA expression in both pachytene spermatocytes and round spermatids was altered after CPA treatment. However whether CPA treatment, or any other toxic substance, can alter miRNA profiles in male germ cells has, to our knowledge, never been examined. We hypothesise that miRNA expression profiles in pachytene spermatocytes and round spermatids will be altered by chronic low dose CPA treatment. The altered miRNAs may serve to mediate defense mechanisms or lead to some of the detrimental effects we have previously seen after CPA treatment.

MATERIAL AND METHODS

Animals

Adult male Sprague-Dawley rats (350-400g; Charles River Canada, St-Constant, QC, Canada) were kept on a 12 hour light, 12 hour dark cycle and had access to food

and water ad libitum. After 1 week of acclimatization, rats were randomly assigned to 1 of 2 treatment groups and gavaged daily with saline (vehicle control) or CPA (CAS 6055-19-2; Sigma Chemical Co., St. Louis, MO) 6 mg/kg of body weight, 6 days per week for 4 weeks. This dose is a clinically relevant dose and has been used in previous animal studies examining the effect of CPA on the male germ cells [19, 21-24]. All animal care and handling were done in accordance with the guidelines outlined by the Canadian Council on Animal Care (McGill Animal Resources Centre protocol 2144).

Germ cell isolation and collection

At the end of 4 weeks of treatment, rats were euthanized by CO_2 asphyxiation and decapitation. Male germ cells were isolated and collected by velocity sedimentation (STA-PUT; Proscience, Don Mills, ON, Canada) as described by Bellve et al [27]. and modified by Aguilar-Mahecha et al [28]. Briefly, the testes were removed, decapsulated and seminiferous tubules were digested with collagenase (Sigma Chemical Co., St. Louis, MO) and further digested with trypsin (type 1; T8003; Sigma Chemical Co., St, Louis, MO) and DNAse I (DN-25, Sigma Chemical Co., St. Louis, MO). Seminiferous tubules were subsequently physically dissociated in the presence of DNAse I and filtered through a 70μm nylon mesh to obtain a mixed germ cell suspension. Cells were washed 3 times with RPMI 1640 medium (Life Technology, Grand Island, NJ) containing 0.5% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and filtered through a 55μm nylon mesh. A total of 5.6×10^8 cells was loaded into a velocity sedimentation cell separator apparatus and separated by unit gravity sedimentation with a 2%-4% BSA gradient in RPMI. Fractions containing pachytene spermatocytes and round spermatids were identified visually by phase-contrast microscopy. Fractions with

over 80% purity for pachytene spermatocytes and over 85% purity were pooled, pelleted and frozen at -80°C until further processing.

RNA extraction

Total RNA was extracted from isolated pachytene spermatocyte and round spermatid samples using TRIzol Reagent (Life Technologies, Carlsbad, CA) and cleaned using the RNeasy mini Kit columns (Qiagen, Mississauga, ON, Canada) according to the manufacturer's supplemental protocol to collect total RNA containing small RNAs. Total RNA concentrations were determined by spectrophotometry (Nanodrop 2000; Nanodrop Technologies, Wilmington, DE, USA). RNA quality and the presence of small RNAs was determined by electrophoresis (BioAnalyzer 2100 Expert; Agilent Technologies, Santa Clara, CA). Only samples with an RNA integrity number above 8 and containing small RNAs were used for microarray experiments.

miRNA Microarrays and Data Analysis

Total RNA from isolated pachytene spermatocytes and round spermatids was labeled and hybridized to rat miRNA microarrays (Sure Print Rat miRNA 8x15K microarray Release 16.0, Agilent Technologies) following the manufacturer's protocol. Raw data were quantile normalized and further analyzed using Genespring software version 12.0 (Agilent Technologies, Santa Clara, CA). A moderated t-test ($p<0.05$) was used to determine statistically significant differences in miRNA expression between samples from control and CPA treated animals. miRNA targets were predicted bioinformatically using two miRNA target databases, TargetScan and miRNA.org, via

Genespring Software. Only targets that were predicted by both databases were considered in the analysis.

RESULTS AND DISCUSSION

miRNA profiles differ between pachytene spermatocytes and round spermatids

This study, to our knowledge, is the first to look at miRNA profiles in purified rat spermatogenic cells. Using miRNA microarrays we assessed differences in miRNA expression in purified pachytene spermatocytes and round spermatids, two populations of male germ cells at different stages of differentiation. Principle component analysis (PCA) was used to reduce the dimensionality of miRNA expression sets and allow for visualization of relationships between groups (Fig. 2-1). PCA indicated clear differences in miRNA expression profiles between the two germ cell types. Samples of pachytene spermatocytes and round spermatids clustered separately along the primary (x) component. The two different cell types accounted for 65% of the variance in miRNA expression. Therefore, as shown previously [29, 30], and similar to gene expression, miRNA profiles in germ cells show phase specific expression patterns.

The arrays contained 677 probes, corresponding to 677 different miRNAs: 229 miRNAs (34%) were present in pachytene spermatocytes, while 218 miRNAs (32%) were present in round spermatids (Fig. 2-2a). Although there was considerable overlap among the miRNAs present in both pachytene spermatocytes and round spermatids, nearly half (46%) of these miRNAs were differentially expressed between the two cell types (Fig. 2-2b), and among nearly half of these the differential expression was greater than 2-fold. Additionally, 26 miRNAs were uniquely expressed in pachytene

spermatocytes and 15 miRNAs were uniquely expressed in round spermatids. In total, pachytene spermatocytes had 81 miRNAs displaying increased expression levels compared to round spermatids, while round spermatids had 53 miRNAs displaying increased expression compared to pachytene spermatocytes (Fig. 2-3a and b). These differentially expressed miRNAs include some that have been identified in the handful of prior studies profiling miRNAs in germ cells. Members of the miR-34 and miR-449 families were preferentially expressed in male germ cells and have previously been shown to be highly expressed in meiotic germ cells and post-meiotic germ cells [16, 31]. Loss of both these families (but not individually) leads to infertility, indicating that these two miRNA families have an important but redundant function [16, 32]. Members of both families target transcripts involved in the E2F-pRb pathway [17], a pathway whose suppression allows male germ cells to exit from the mitotic cell cycle and enter meiosis [16, 33]. We found that members of both these families were present in both cell types and displayed increased expression in pachytene spermatocytes compared to round spermatids.

The most differentially expressed miRNA between the 2 cell types was miR-146a, whose expression was increased 62 fold in round spermatids compared to pachytene spermatocytes. miR-146a is thought to play a role in the control of retinoic acid-induced spermatogonial differentiation as it is highly expressed in undifferentiated spermatogonia and is drastically down-regulated by retinoic acid [11]. The role that this miRNA may play in the differentiating haploid round spermatids is unclear. However, RARα, is predicted to bind the miR-146a promoter [34] and RARα KO mice display

defects in spermiation [35, 36]; thus increased miR-146a expression in round spermatids may play a role in proper spermatid development and release.

Differentially expressed miRNAs in pachytene spermatocytes and round spermatids are predicted to target multiple mRNAs

In mammalian cells, the base complementarity between a miRNA and the 3'UTR of its target does not need to be perfect, resulting in a single miRNA potentially targeting multiple transcripts and a single transcript also being targeted by multiple miRNAs [37-39]. Bioinformatic databases were used to predict biological targets of the differentially expressed miRNAs present in pachytene spermatocytes and round spermatids. The 81 miRNAs with increased expression in pachytene spermatocytes were predicted to target 500 mRNAs (Fig. 2-3a) while the 53 miRNAs with increased expression in round spermatids are predicted to target 432 mRNAs (Fig. 2-3b). Predicted targets were compared to differentially expressed genes between pachytene spermatocytes and round spermatids from previously published gene expression microarray data ([40];GEO dataset GSE79471). In pachytene spermatocytes, 297 predicted targets were differentially expressed compared to round spermatids. In round spermatids 239 predicted targets were differentially expressed compared to pachytene spermatocytes. In both cell types, these altered predicted targets represent just over 2% of the transcripts that are differentially expressed between pachytene spermatocytes and round spermatids. These data indicate that transcript cleavage/degradation by these differentially expressed miRNAs may play a small role in regulating differences in gene expression between the 2 cells types. Indeed several studies suggest that the primary action of miRNAs is to inhibit mRNA translation [41-44] and that translational repression

does not necessarily lead to RNA degradation [44-47]. Additionally, it has also been suggested that miRNAs are involved in regulating N^6-methyladenosine (m^6A) modifications on mRNA [48], a modification that can influence the RNA structure [49] and possibly expression levels, translation, nuclear retention, splicing and stability of the transcript [50-62]. The m6A modification has also been implicated in regulating transcript translation in oocytes during meiotic maturation [63]. Thus, although the differences in miRNA expression that we observed do not lead to degradation of their targets, these differentially expressed miRNAs may be influencing gene expression and cellular functions in alternate ways.

Targets of Differentially expressed miRNAs in pachytene spermatocytes and round spermatids are involved in important cellular processes

Although many targets of the differentially expressed miRNAs also display differential expression at the transcript level, the miRNAs may also be regulating gene expression at the translational level. We used Gene Ontology (GO) analysis of all the predicted targets to evaluate how differentially expressed miRNAs may influence cellular processes and germ cell development (Fig. 2-4a and b). While many of the differentially expressed miRNAs target genes that are involved in similar processes such as cell communication, the regulation of transcription, the response to stress and the regulation of cell death, some miRNAs also target processes specific to germ cell type. miRNAs that were up-regulated in pachytene spermatocytes have targets that are involved in meiotic processes such as meiotic nuclear division, DNA recombination and regulation of the cell cycle. Alternatively, targets from miRNAs that were increased in round spermatids play roles in chromatin organization processes such as histone

modifications and chromatin and RNA binding. Thus, male germ cells express specific miRNAs that are involved in cell type specific processes that are crucial for proper spermatogenesis. Indeed miRNAs have shown to be crucial for male germ cell development as the loss of miRNA biogenesis leads to severe defects or spermatogenenic arrest [4, 5, 7]. Which miRNAs are essential and whether some play redundant roles remains largely unknown.

Of great interest, we saw that the response to chemical is a very prominent term found in the target analysis of pachytene spermatocytes but appears less important in round spermatids. This suggests that the miRNAs expressed in pachytene spermatocytes may have a larger influence on this cell type's ability to respond to chemical insult compared to the round spermatids. The capacity of male germ cells to repair DNA damage decreases after meiosis as germ cells differentiate [64-67]. The level of DNA damage in spermatozoa is at its highest when male germ cells are first exposed to toxic agents as spermatids, a time when germ cells become transcriptionally quiescent [26, 68]. It is possible that miRNAs play a role in the differential response and DNA repair capacities of male germ cell types.

Chronic low-dose CPA treatment alters miRNA profiles in pachytene spermatocytes and round spermatids

We assessed the impact of chronic low-dose CPA treatment on miRNA expression in pachytene spermatocytes and round spermatids. To our knowledge, this is the first study to assess the effect of a drug on miRNA profiles in isolated germ cells. PCA was used to examine the relationships between samples (Fig. 2-5a and b).

Pachytene spermatocyte and round spermatid samples from CPA treated and control animals separate along the z axis indicating differences in miRNA expression profiles after CPA treatment in both cell types.

The majority of the 229 miRNAs found expressed in untreated pachytene spermatocytes were still expressed after CPA treatment. However 4 miRNAs were no longer detectable after CPA treatment, while 10 miRNAs were expressed solely after CPA treatment (Fig. 2-6a and Table 2-1). Of the miRNAs common to both treatment groups, 7 were significantly up-regulated and 5 were significantly down regulated by CPA treatment (Fig. 2-6c and Table 2-1). In total, the expression of 26 miRNAs was altered by CPA treatment, corresponding to approximately 11% of miRNAs expressed. MicroRNA-483 expression was especially affected and was up-regulated over 7-fold compared to control. Although there is no known role for this miRNA in male germ cells, miR-483 has been shown to decrease sensitivity to cisplatin treatment in tongue squamous cell carcinoma via down regulation of FIS1 leading to decreased mitochondrial fission [69]. Chronic low dose CPA treatment does not result in germ cell apoptosis [70]. In contrast, CPA induces apoptosis with an acute high dose of 70 mg/kg. We hypothesize that up-regulation of miR-483 may be involved in the survival of pachytene spermatocytes after CPA treatment possibly through the same mechanism mentioned above.

Interestingly, CPA treatment altered the expression of miR-211*, miR-449a and miR-494 which have also been shown to be dysregulated in the hippocampus following lead exposure [71] and miR-30a which was also up-regulated in the hippocampus following cocaine administration [72]. In addition, miR-185 was also up-regulated in

blood cells after chronic exposure to benzene [73]. These miRNAs may be part of a general cellular response to toxic exposures.

Comparable to pachytene spermatocytes, the majority of the 218 miRNAs expressed in untreated round spermatids were still expressed after CPA treatment (Fig. 2-6b). Some miRNAs were unique to the treatment group: 9 miRNAs were only expressed in control samples and 4 miRNAs were only expressed after CPA treatment (Fig. 2-6b and Table 2-2). Additionally, of the miRNAs common to both treatment groups, 3 miRNAs were significantly up-regulated and 1 was significantly down-regulated by treatment (Fig. 2-6d and Table 2-2). Overall CPA altered the expression 17 miRNAs in round spermatids, approximately 8% of the total miRNAs expressed. The miRNAs altered by CPA treatment in round spermatids are listed in Table 2. MicroRNA-125b* expression was especially affected and was increased over 9-fold after CPA treatment. Although the miRNA from the opposite arm, miR-125b has been shown to promote cell proliferation and prevent cell death in various cancer cells [74], no known function of miR-125b* has been found.

The expression of miR-134 was increased in round spermatids following CPA treatment. Other DNA damaging agents (doxorubicin etoposide and ionizing radiation) have been shown to up-regulate the expression miR-134 in ovarian cancer cells [75]. Additionally, over-expression of miR-134 resulted in increased H2AX phosphorylation (a marker of DNA damage response) and improved the efficiency of DNA repair by non-homologous endjoining (NHEJ), an important DNA repair pathway for the haploid round spermatids. MiR-134 may be increased in round spermatids in response to CPA in order to repair damaged DNA. However, sperm show the highest levels of DNA damage

when they are first exposed to CPA as spermatids, indicating that the DNA repair mechanisms in these cells are not sufficient to overcome CPA insult.

Other substances have also been shown to alter some of the same miRNAs as the ones we have shown in round spermatids in this study in other tissues. MiR-192, which was no longer expressed after CPA treatment, is decreased in hepatocytes after toxic doses of acetaminophen [76] and increased in these same cells after exposure to silica nanoparticles [77]. The expression of miR-134 mentioned above, is also increased in the hippocampus after cocaine administration [72]. Thus, these miRNAs may be responsive to toxic insults and indicators of cellular toxicity.

Similar to our previous gene expression data [40], CPA treatment altered the expression of more miRNAs in pachytene spermatocytes than in round spermatids. The number of miRNAs with increased expression after CPA treatment was nearly double the number of decreased miRNAs in pachytene spermatocytes suggesting that CPA treatment may have a positive effect on miRNA expression in this cell type. These results diverge from previous data that suggests that CPA has a negative impact on mRNA expression in pachytene spermatocytes [40]. In contrast, in round spermatids the number of miRNAs with increased expression was slightly less compared to the number of miRNAs with decreased expression after CPA treatment. Suggesting, similar to our results from mRNA expression studies, that in round spermatids CPA treatment has a more uniform effect on miRNA expression.

Interestingly, 2 miRNAs are altered by CPA treatment in both cell types but in opposite directions. Both miR-196c and miR-340-3p are only detectable after CPA

treatment in pachytene spermatocytes whereas in round spermatids neither is expressed after CPA treatment. These results indicate that chronic low-dose CPA treatment affects miRNA profiles differently in pachytene spermatocytes and round spermatids. Neither of these miRNAs have known roles in spermatogenesis. However, miR-196c is downregulated in oxidative stress induced renal injury, indicating it may have a role in the cellular response to oxidative stress and toxic substances [78]. miR-340-3p has been shown to be down-regulated in whole testis after heat stress [79] and is potentially involved in regulating mTOR signaling by targeting MID1, a regulator of mTOR [80]. Thus altered miR-340-3p expression may play a role in differential germ cell response to stress and damage.

CPA altered miRNAs are predicted to target multiple mRNAs in pachytene spermatocytes and round spermatids

In pachytene spermatocytes, the 26 CPA altered miRNAs are predicted to target 608 mRNAs while the 17 CPA altered miRNAs in round spermatids are predicted to target 398 miRNAs (Fig. 2-7a and b). To determine whether the differentially expressed miRNAs may be altering gene expression at the transcript level, predicted targets were compared to previously published microarray data from CPA treated and control pachytene spermatocytes and round spermatids [40]. Differentially expressed predicted targets and their regulation in pachytene spermatocytes and round spermatids are listed in Tables 2-1 and 2-2 respectively. The majority of the differentially expressed mRNA targets of up-regulated miRNAs in pachytene spermatocytes and round spermatids were decreased by CPA treatment, fitting the general model of miRNAs decreasing the stability of target mRNAs. However, many of the differentially expressed targets of the

decreased miRNAs were also decreased by CPA treatment in both cell types, reflecting the complexity of regulation of gene expression by miRNAs. These results suggest that miRNAs may play a role in the stability of a subset of predicted target mRNAs in response to CPA treatment.

Multiple miRNAs can potentially target a single mRNA. This was observed in our dataset as many altered mRNAs were predicted to be regulated by 2 or more altered miRNAs. For example, Slc39a14 (ZIP14) is up-regulated after CPA treatment in pachytene spermatocytes and predicted to be a target of 4 altered miRNAs. Two of these (miR-195 and miR-497) are significantly down-regulated after CPA treatment. We previously showed that members of ZIP family of zinc transporters (including ZIP14) were up-regulated in response to CPA insult and lead to an increase in zinc uptake [40]. As zinc levels need to be tightly controlled, miRNAs may provide germ cell with a mechanism to finely tune the expression of zinc transporters and zinc transport in response to toxic substances.

Predicted targets of CPA altered miRNAs are involved in processes important for response to CPA treatment and proper germ cell development

GO analysis of the predicted targets was used to examine how differentially expressed miRNAs are involved in the germ cell response to CPA treatment (Fig. 2-8a and b). Targets of the CPA altered miRNAs in both cell types are involved in the response to stress, DNA damage and oxidative stress and the regulation of cell death, crucial processes for cell survival and countering the effects of CPA. The response to DNA damage is a more prominent term associated with the targets of altered miRNAs

from pachytene spermatocytes compared to the round spermatids. Additionally, targets are also involved in DNA recombination, a DNA repair pathway available to spermatocytes but not round spermatids, indicating that these altered miRNAs may be a pachytene spermatocyte specific response to repair DNA damage. Thus the differences in germ cell DNA repair capacity and the differences in susceptibility to damaging agents such as CPA may in part be modulated by miRNAs.

Many targets are also involved in the regulation of transcription, spermatogenesis and cell differentiation and communication, indicating that CPA altered miRNAs may also lead to the disruption of cellular function. We have previously shown that CPA treatment causes a net down-regulation of mRNA expression in pachytene spermatocytes [40], an observation that may be the result of CPA altered miRNAs that target genes involved in the regulation of transcription.

Interestingly, consistent with our previous study [40], the altered miRNAs in both cell types are predicted to target numerous transcripts involved in zinc binding, suggesting that zinc is involved in the germ cell response to CPA.

Processes essential to spermatogenesis may be affected by CPA via miRNA expression. Some targets of the altered miRNAs in pachytene spermatocytes are involved in DNA recombination and meiotic nuclear division. Indeed, we have previously shown that along with extensive DNA damage, acute CPA treatment impaired meiotic progression. In contrast, chronic CPA treatment did not impair meiotic progression despite increased levels of DNA damage [81]. Additionally, targets of altered miRNA were also involved in DNA damage checkpoint. Therefore it is possible that altered

miRNA expression may be involved in the survival of damaged germ cells and their progression through meiosis. As targets of altered miRNAs in round spermatids are involved in DNA and chromatin binding, the CPA altered miRNAs may affect chromatin remodelling that occurs later on in spermiogenesis. CPA treatment results in decreased DNA compaction and changes in sperm head and nuclear matrix proteins [22, 23], leading to decreased sperm chromatin quality.

In conclusion, we have shown that pachytene spermatocytes and round spermatids display distinct miRNA expression profiles that can be altered by chronic CPA treatment. The miRNAs may be altered as a cellular response to CPA induced damage or may be dysregulated leading to the negative effects previously observed in male germ cells after CPA treatment. Other toxic agents have also been shown to alter some of the same miRNAs in other tissues; however to our knowledge this is the first study to examine the effect of a toxic agent on meiotic and post-meiotic germ cells. Together these studies suggest that some miRNAs are associated with toxic exposure and may be useful as biomarkers. Finally, as roles for the majority of miRNAs in male germ cells have yet to be defined, further investigation of miRNAs in the context of both normal physiological function and in response to toxic agents is warranted.

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TABLES

Table 2-1. miRNAs and their mRNA targets significantly altered by CPA treatment in pachytene spermatocytes.

miRNA	Regulation of miRNA (CPA vs control)	Altered mRNA target	Regulation of mRNA (CPA vs control)
rno-miR-483	↑ 7.84	**Sulf1**	Control only
		Ace	↑ 1.52
		Aqp1	Control only
rno-miR-494	↑ 1.48	**Sulf1**	Control only
		Aqp1	Control only
		Slc18a1	Control only
rno-miR-211*	↑ 1.30		
rno-miR-328a*	↑ 1.19	**Slc39a5**	
rno-miR-188	↑ 1.18	**Neurod1**	Control only
rno-miR-150*	↑ 1.16		
rno-miR-30d	↑ 1.08	**Slc18a1**	Control only
		Neurod1	Control only
rno-miR-129	CPA only	**Cacng2**	Control only
		Slc39a14	↑ 1.57
rno-miR-138-1*	CPA only		
rno-miR-185	CPA only	**Clec10a**	Control only
		Slc39a14	↑ 1.57
rno-miR-196c	CPA only		
rno-miR-208*	CPA only		
rno-miR-22*	CPA only		
rno-miR-340-3p	CPA only		
rno-miR-3561-5p	CPA only		
rno-miR-671	CPA only	**Rgs7bp**	CPA only
rno-miR-678	CPA only		
rno-miR-30a*	↓ 1.10		
rno-miR-362*	↓ 1.13		
rno-miR-195	↓ 1.26	**Cd200**	Control only
		Sema4f	Control only
		Capn6	Control only
		Slc39a14	↑ 1.57
rno-miR-497	↓ 1.21	**Cd200**	Control only
		Sema4f	Control only
		Capn6	Control only
		Slc39a14	↑ 1.57
rno-miR-449a	↓ 1.34	**Capn6**	Control only
rno-miR-146a	Control only		
rno-miR-194	Control only	**Abcb4**	Control only
rno-miR-3544	Control only		
rno-miR-505	Control only	**Cxcl3**	Control only

Table 2-2. miRNAs and their mRNA targets significantly altered by CPA treatment in round spermatids.

miRNA	Regulation of miRNA (CPA vs control)	Altered mRNA target	Regulation of mRNA (CPA vs control)
rno-miR-125b*	↑ 9.20		
rno-miR-134	↑ 2.00	**Il27**	CPA only
rno-miR-3573-3p	↑ 1.18		
rno-miR-138	CPA only	**Wfdc2**	CPA only
rno-miR-181b	CPA only	**Gucy1b3**	Control only
		Cept1	Control only
rno-miR-186	CPA only	**Arl6ip6**	Control only
		Gucy1b3	Control only
		Cept1	Control only
		Aldh6a1	CPA only
rno-miR-3564	CPA only		
rno-miR-1949	↓ 1.40	**Cyp4x1**	↑ 1.88
		Gpr179	CPA only
		Fsd2	CPA only
		Pramef12	Control only
rno-miR-106b*	Control only		
rno-miR-1188-3p	Control only		
rno-miR-192	Control only		
rno-miR-196c	Control only		
rno-miR-32*	Control only		
rno-miR-340-3p	Control only		
rno-miR-547*	Control only		
rno-miR-582*	Control only		
rno-miR-92b	Control only		

FIGURE LEGENDS

Figure 2-1. Principle component analysis (PCA) showing differential miRNA expression profiles in isolated pachytene spermatocytes and round spermatids. (n=5)

Figure 2-2. MiRNA expression in pachytene spermatocytes and round spermatids. Venn diagram showing the numbers of miRNAs that are unique to pachytene spermatocytes and round spermatids and those that are common between the cell types (a). The differential expression of miRNAs common to both cell types is shown in (b). Fold change is based on expression levels in pachytene spermatocytes vs round spermatids. (n=5) $p<0.05$

Figure 2-3. Target prediction of diffrentially expressed miRNAs and differentially expressed mRNA targets between pachytene spermatocytes (a) and round spermatids (b).

Figure 2-4. GO analysis of target of miRNAs that are differentially expressed in pachytene spermatocytes (a) and round spermatids (b). The relative size of the GO term corresponds to the relative number of targets that fit that term.

Figure 2-5. Principle component analysis (PCA) showing distribution of CPA-treated and control pachytene spermatocytes (a) and round spermatid (b) samples. (n=5)

Figure 2-6. Changes in miRNA expression after CPA treatment. Venn diagram of miRNAs in both control and CPA-treated samples and those that are exclusively expressed in one treatment group in pachytene spermatocytes (a) and round spermatids (b). Number of miRNAs that are significantly altered after CPA treatment in pachytene spermatocytes (c) and round spermatids (d). (n=5) $p<0.05$

Figure 2-7. Target prediction of CPA altered miRNAs and mRNA targets that are altered after CPA treatment in pachytene spermatocytes (a) and round spermatids (b).

Figure 2-8. GO analysis of predicted targets of miRNAs that altered after CPA treatment in pachytene spermatocytes (a) and round spermatids (b). The relative size of the GO term corresponds to the relative number of targets that fit that term.

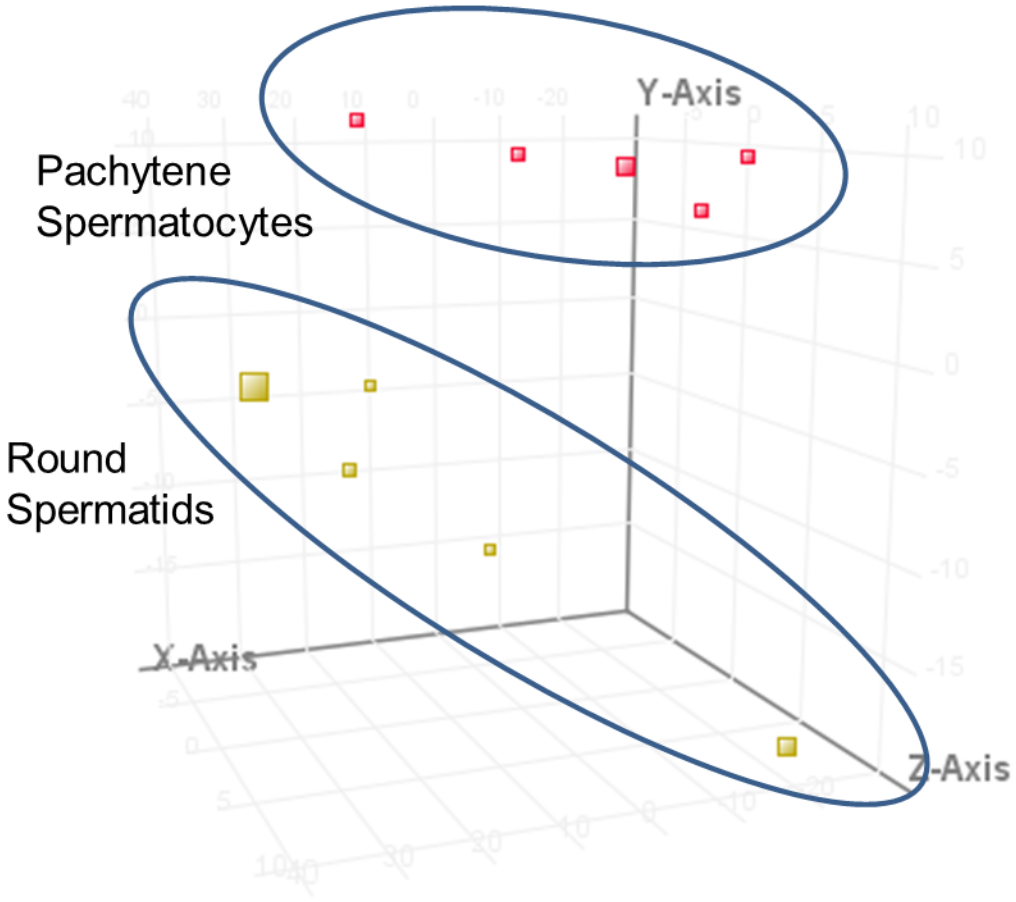

Figure 2-1. Principle component analysis (PCA) showing differential miRNA expression profiles in isolated pachytene spermatocytes and round spermatids. (n=5)

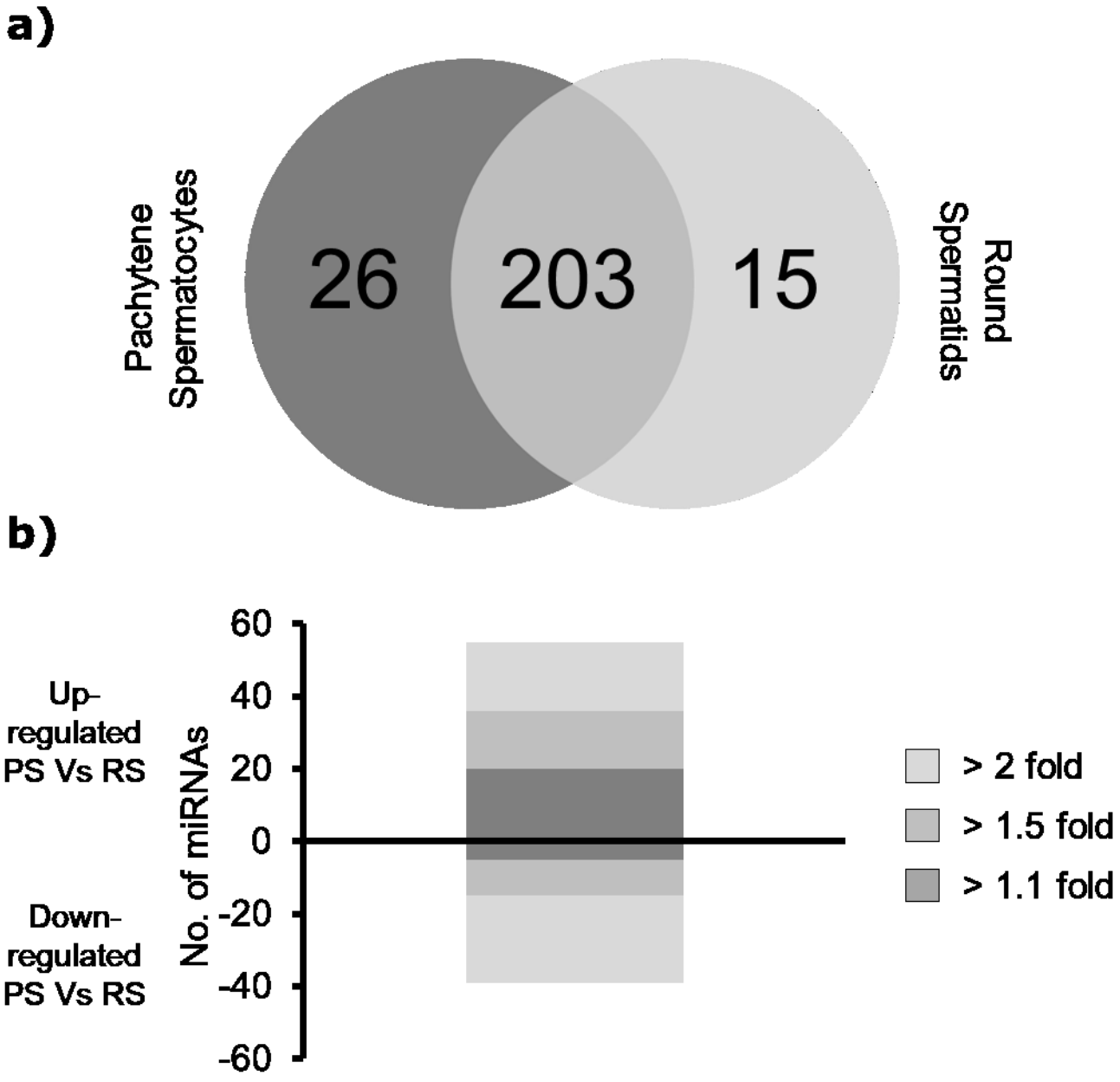

Figure 2-2. MiRNA expression in pachytene spermatocytes and round spermatids.

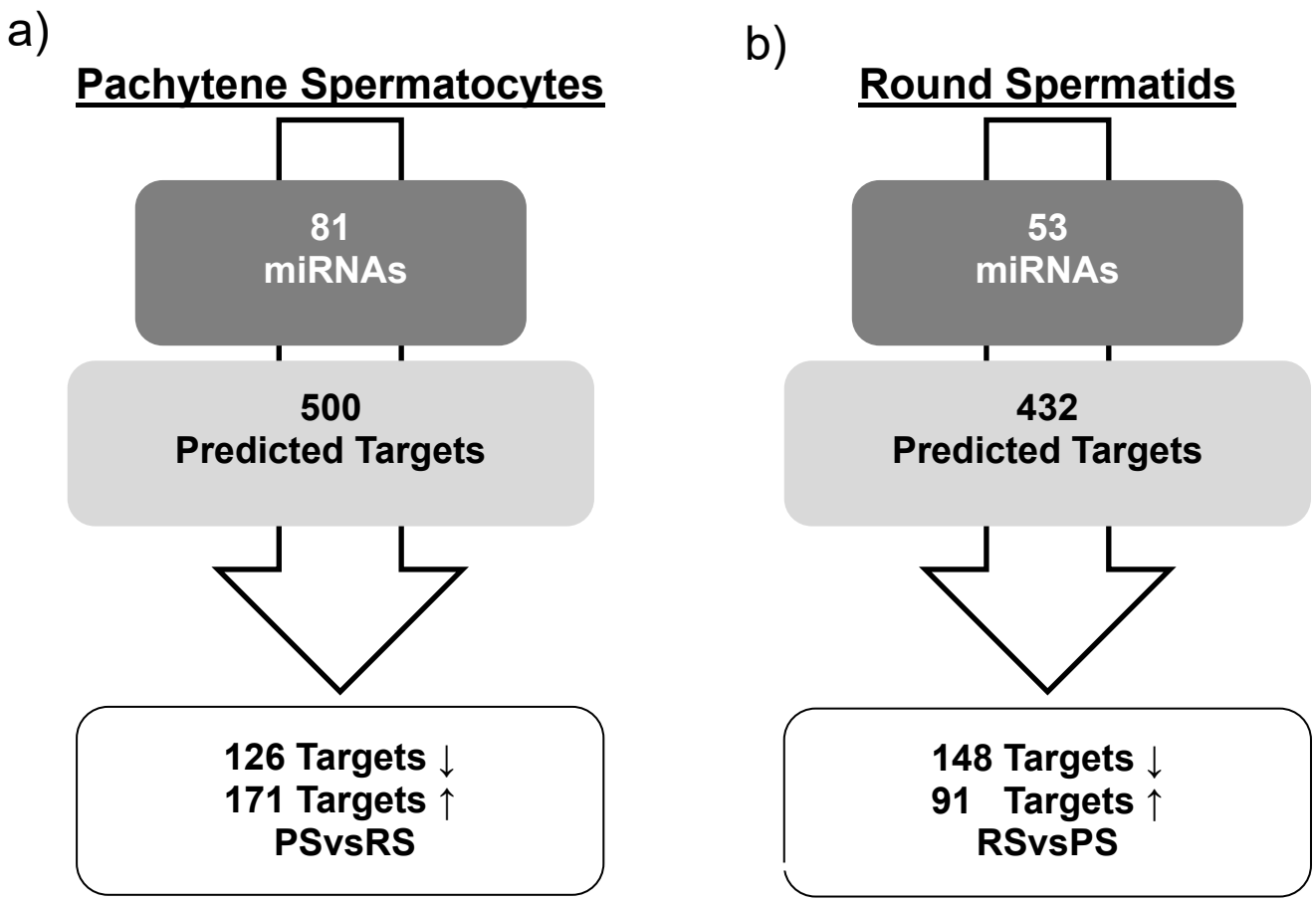

Figure 2-3. Target prediction of diffrentially expressed miRNAs and differentially expressed mRNA targets between pachytene spermatocytes and round spermatids.

a)

b)

pachytene spermatocytes and round spermatids.

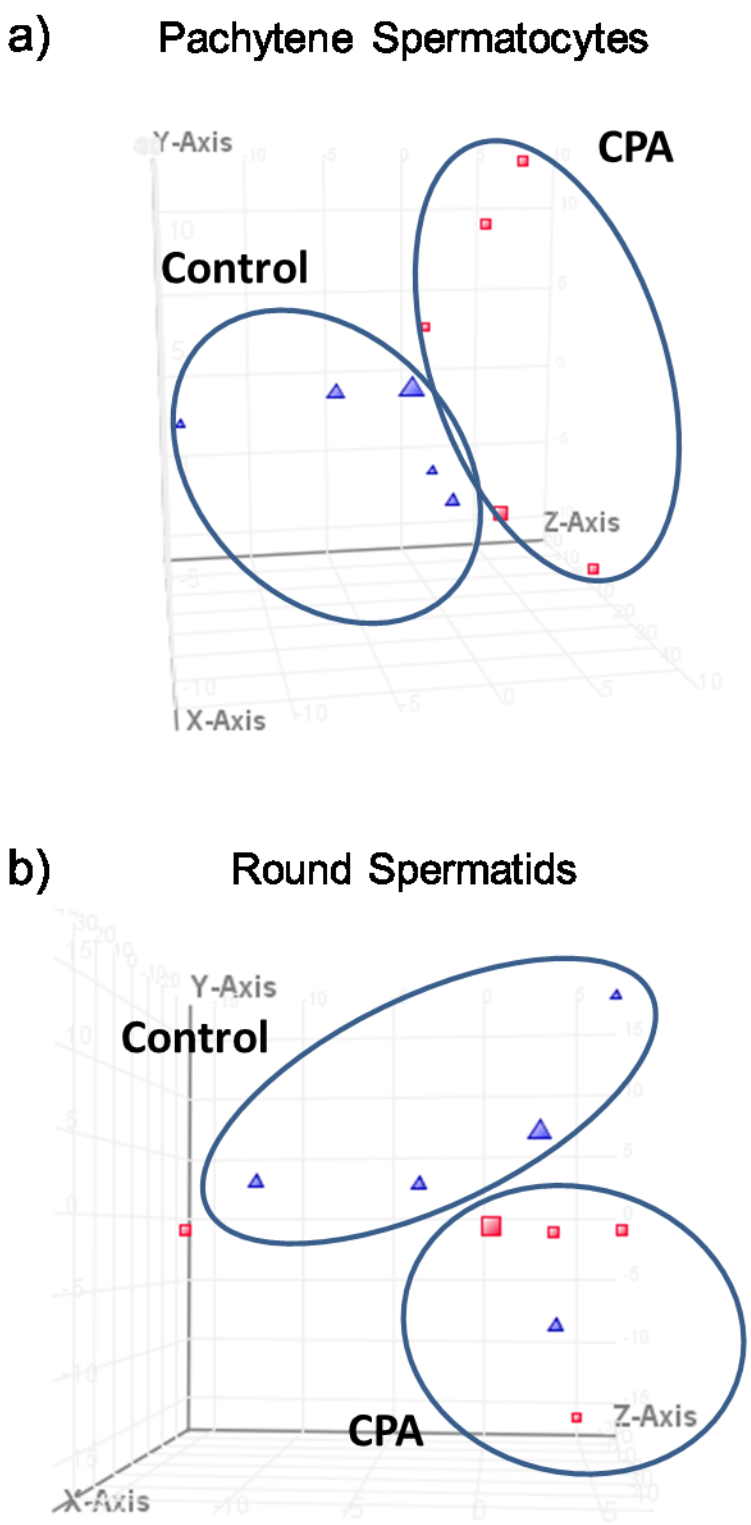

Figure 2-5. Principle component analysis (PCA) showing distribution of CPA-treated and control pachytene spermatocytes and round spermatid samples.

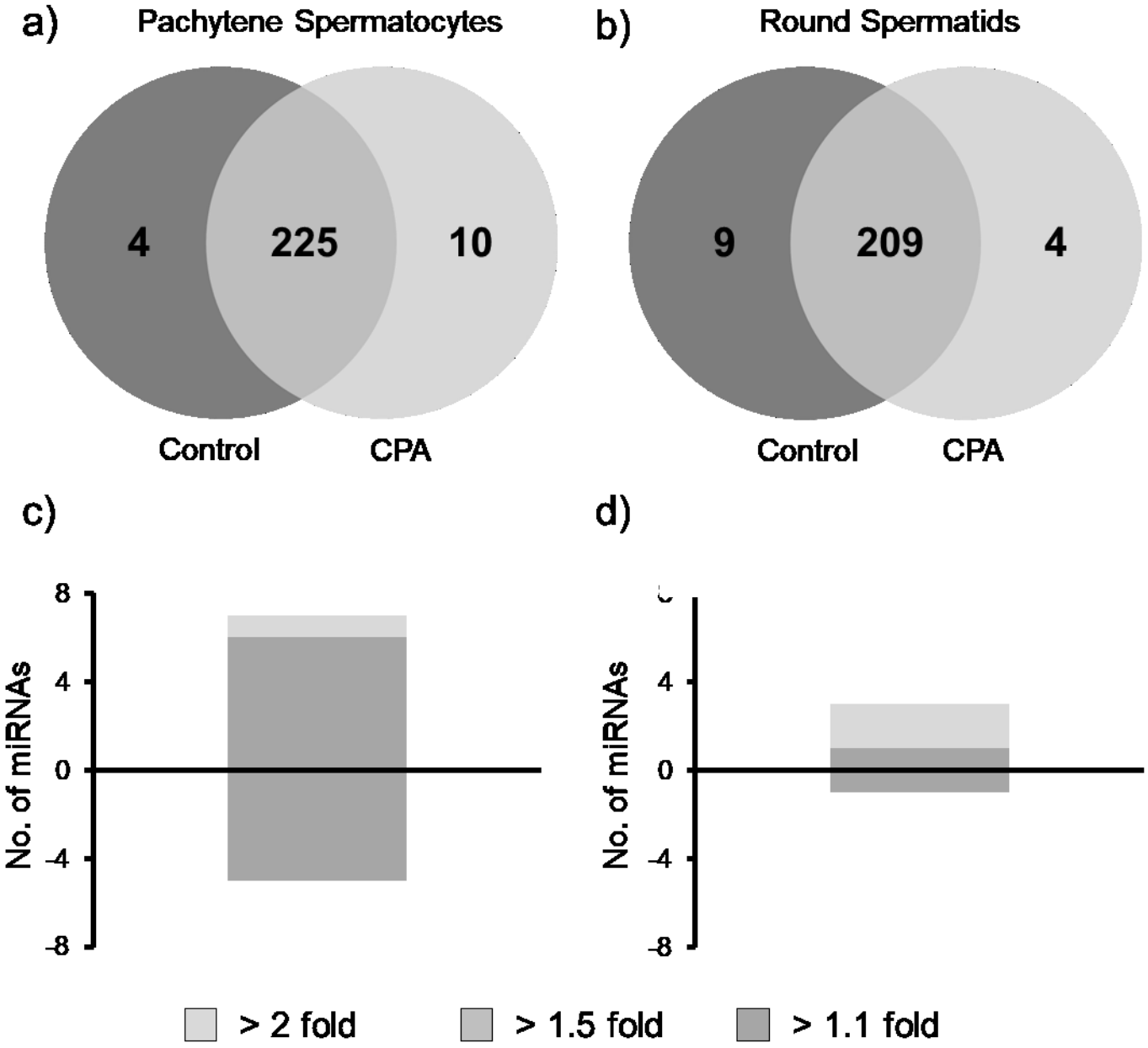

Figure 2-6. Changes in miRNA expression after CPA treatment.

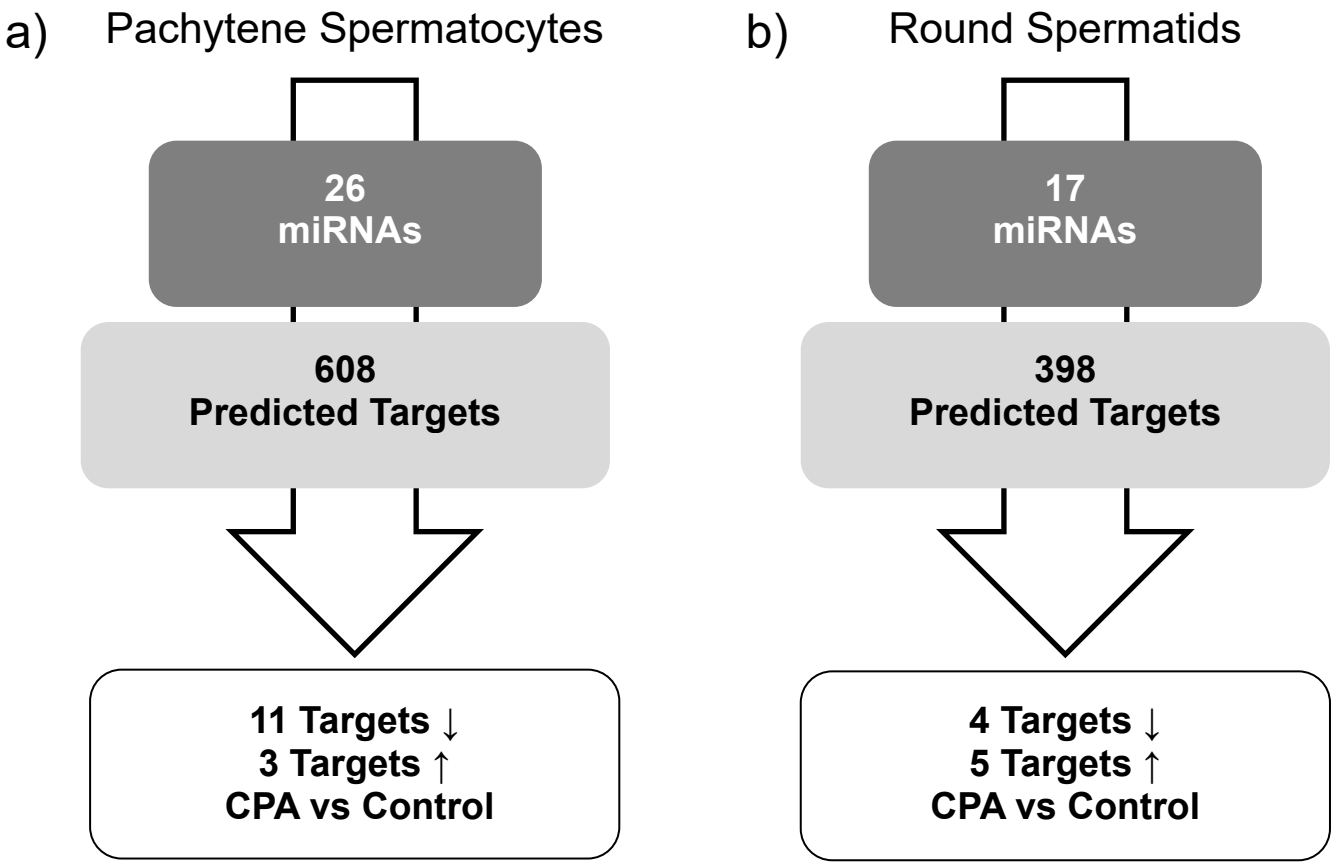

Figure 2-7. Target prediction of CPA altered miRNAs and mRNA targets that are altered after CPA treatment in pachytene spermatocytes and round spermatids.

a)


RegulationofCellAdhesion
SpermPart
ResponsetoToxicSubstance
ResponsetoDNADamage
ResponsetoDrug
DNABinding
ChromatinOrganization
ZinclonBinding
CellDivision
RegulationofTranscription
DNARepair
RegulationofCellDeath
SpermMobility
ZincBinding
CellJunction
ResponsetoStress
CellDifferentiation
Spermatogenesis
ResponsetoOxidativeStress
CellAdhesion
RegulationofCellCycle

b)


RegulationofCellDifferentiation
DNABinding
Reproduction
ResponsetoStress
DNARepair
MaleGameteGeneration
RegulationofTranscription
RegulationofCellDeath
ResponsetoDrug
Spermatogenesis
ResponsetoChemical
ResponsetoDNAdamage

CONNECTING TEXT

The studies in Chapter 2 of this manuscript examined the expression of miRNAs in isolated male germ cells, specifically pachytene spermatocytes and round spermatids and the effect of chronic CPA treatment on miRNA profiles in these cells. These studies suggest differences in miRNA profiles in both cell types which could influence germ cell response to toxic exposure. Importantly, these studies also demonstrate for the first time that CPA treatment can alter the expression of miRNAs that are involved in the response to oxidative stress, DNA damage and spermatogenesis. In Chapter 3, the germ cell response to CPA treatment and the effects on the transcriptome are further examined by whole genome gene expression analysis in isolated pachytene spermatocytes and round spermatids.

CHAPTER 3

ZINC TRANSPORT DIFFERS IN RAT SPERMATOGENIC CELL TYPES AND IS AFFECTED BY TREATMENT WITH CYCLOPHOSPHAMIDE

Anne Marie Downey[1], Barbara F. Hales[1], Bernard Robaire[1,2]

Departments of Pharmacology and Therapeutics[1] and Obstetrics and Gynecology[2],

McGill University, Montréal, Québec, Canada

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ABSTRACT

Adequate zinc levels are required for proper cellular functions and for male germ cell development. Zinc transport is accomplished by two families of zinc transporters, the ZIPs and the ZnTs, that increase and decrease cytosolic zinc levels, respectively. However, very little is known about zinc transport in the testis. Furthermore, whether cytotoxic agents such as cyclophosphamide (CPA), a known male germ cell toxicant, can affect zinc transport and homeostasis is unknown. We examined zinc transporter expression and zinc transport in pachytene spermatocytes (PS) and round spermatids (RS) in a normal state and after exposure to CPA. We observed differences in the expression of members of the ZnT and ZIP families in purified populations of PS and RS. We also observed that RS accumulate more zinc over time than PS. The expression of many zinc binding genes was altered after CPA treatment. Interestingly, we found that the expression levels of ZIP5 and ZIP14 were increased in PS from animals treated daily with 6 mg/kg CPA for 4 wk but not in RS. This up-regulation led to an increase in zinc uptake in PS but not in RS from treated animals compared to controls. These data suggest that CPA treatment may alter zinc homeostasis in male germ cells leading to an increased need for zinc. Altered zinc homeostasis may disrupt proper germ cell development and contribute to infertility and effects on progeny.

INTRODUCTION

Zinc is an essential trace element that is important for growth and development, metabolism, brain and immune system function, and reproduction [1]. It is present in varying amounts in different tissues and organs [2]. At the cell level, zinc is necessary for proper cellular function because it binds more than 10% of all proteins [3], is a co-factor for over 300 enzymes, and is required for more than 2000 transcription factors [4]. Zinc has an important function in modulating oxidative stress through its roles in antioxidant enzymes, the electron transport chain [5, 6], in preventing Fe/Cu Fenton reactions [7, 8], in endoplasmic reticulum ER protein misfolding [9, 10], and inhibition of NADPH oxidase activity [11, 12]. Moreover, apart from being important in DNA-protein binding through zinc finger proteins, DNA repair enzyme activity is influenced by zinc levels, indicating its importance in DNA damage repair [13-15].

Because zinc is an essential trace element, its levels need to be tightly controlled. This is accomplished by two families of zinc transporters: the ZIP family and the ZnT family of zinc transporters; these transporters work in an opposite fashion to regulate cytosolic zinc. The ZIP family is composeed of 14 members and increases cytosolic zinc by importing zinc from the extracellular spaces and releasing zinc from intracellular spaces [16]. The ZnT family is consists of 10 members and decreases cytosolic zinc by exporting zinc into the extracellular space and sequestering zinc into intracellular spaces [17].

The importance of zinc in reproduction is very apparent in the male reproductive system. Tissue zinc concentration is greatest in the prostate, and semen contains very

high concentrations of zinc [18]. Zinc is proposed to play an important role in sperm chromatin condensation by stabilizing chromatin structure [19]. Zinc is also abundant in the testis, where it is present in all stages of germ cells [20]. Zinc deficiency studies have been convincing in demonstrating the importance of zinc in male reproductive function, as zinc deficiency leads to increased oxidative stress, DNA damage, and apoptosis in the testis and an arrest in spermatogenesis [21-25]. On the other hand, zinc overload also has the same detrimental effects on spermatogenesis [26].

It is clear that proper control of zinc levels is essential in the testis; however, very little is known about zinc transport in male germ cells. Only two studies have described the presence of select members of both the ZIP and ZnT families in human and mouse testes [27, 28], while a third study measured the kinetics of zinc transport in spermatids [29]. No studies have investigated whether exposure to drugs or chemicals toxic to germ cells alters zinc transport.

Cyclophosphamide (CPA) is an alkylating agent commonly used in cancer and immunosuppression therapies and is a known male germ cell toxicant [30-38]. Men treated chronically with CPA have an increased incidence of azoospermia and oligozoospermia [30]. Studies in animal models have shown that CPA causes increased DNA damage [31] and oxidative stress [32] in male germ cells, as well as a decrease in sperm chromatin quality [33-36]. The detrimental effects on the male germ cells in turn lead to adverse progeny outcomes that are time-specific and dose-dependent [37]. Post-implantation loss is greatest at 4 wk after the initiation of treatment, whereas pre-implantation loss is greatest 5 to 6 wk after the initiation of treatment [37], respectively corresponding to targeting spermatids and spermatocytes [38]. The underlying

molecular mechanisms of CPA toxicity and how male germ cells respond to such an insult remain to be elucidated.

Given the important role of zinc in spermatogenesis and in mediating oxidative stress and DNA damage and the lack of knowledge of zinc transporters in germ cells, we examined the expression of ZIP and ZnT family members and zinc transport in purified populations of male germ cells in a normal state and after treatment with CPA.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (350–400 g; Charles River Canada, St-Constant, Qc, Canada) were maintained on a 12L:12D cycle and had access to food and water ad libitum. After 1 wk of acclimatization, rats were randomly assigned to 1 of 2 treatment groups and gavaged with saline (vehicle) or CPA (CAS 6055-19-2; Sigma Chemical Co., St. Louis, MO), 6 mg/kg body weight, 6 days per week for 4 wk. All animal care and handling were done in accordance with the guidelines outlined by the Canadian Council on Animal Care (McGill Animal Resources Centre protocol 2144).

Germ Cell Isolation and Separation

After 4 wk of treatment, rats were euthanized by CO2 asphyxiation. Spermatogenic germ cells were isolated and separated using a velocity sedimentation method (STA-PUT; Proscience, Don Mills, ON, Canada) as described by Bellve et al. [39] and modified by Aguilar-Mahecha et al. [40]. Briefly, testes were removed, decapsulated, and digested by incubation with collagenase (Sigma Chemical). After a

brief washing, seminiferous tubules were further digested by incubation with trypsin (type 1; T8003; Sigma Chemical) and DNase I (product DN-25; Sigma Chemical). Seminiferous tubules were subsequently dissociated in the presence of DNase I and filtered through 70-μm nylon mesh, followed by washing with RPMI 1640 medium (Life Technologies, Grand Island, NJ) containing 0.5% bovine serum albumin (BSA; Sigma Chemical) and filtering once more through 55-μm nylon mesh. A total of 5.6×10^8 cells suspended in 25 ml of RPMI medium containing 0.5% BSA was loaded into a velocity sedimentation cell separator apparatus and separated by unit gravity sedimentation with a 2%–4% BSA/RPMI gradient. Pachytene spermatocyte- and round spermatid-containing fractions were identified by phase-contrast microscopy. Fractions with more than 80% (pachytene spermatocyte) and 85% (round spermatid) purity were pooled and either pelleted and frozen at −80°C for further processing or cultured overnight (see below).

RNA Extraction and Microarray

Total RNA was extracted from frozen pachytene spermatocyte and round spermatid fractions using TRIzol reagent (Life Technologies) and cleaned using RNeasy mini kit columns (Qiagen, Mississauga, ON, Canada). Total RNA concentration was determined by spectrophotometry (Nanodrop 2000; Nanodrop Technologies, Wilmington, DE), and quality was determined by electrophoresis (BioAnalyzer 2100 Expert; Agilent Technologies, Santa Clara, CA). Only RNA samples with an RNA integrity number higher than 8 were used for microarray and quantitative reverse transcriptase-PCR (qRT-PCR) experiments. Total RNA was reverse transcribed, labeled, and hybridized to rat gene expression arrays (SurePrint G3 rat GE 8 × 60K

microarrays; Agilent Technologies) following the manufacturer's instructions (one-color microarray-based gene expression analysis protocol; Agilent). Raw data were quantile-normalized and further analyzed using GeneSpring version 12.0 software (Agilent Technologies). Student t-test was used to determine statistically significant differences in gene expression between treated and control samples. Genes that were significantly altered were further filtered using a 1.5-fold cutoff. Gene Ontology analysis (GeneSpring software) and Ingenuity Pathway Analysis software (Qiagen) were used to further characterize altered transcripts. All data were placed in Gene Expression Omnibus database (under accession number GSE79471; NCBI).

Real-time qRT-PCR

Real-time qRT-PCR validation of microarray results was done by two-step qRT-PCR. Reverse transcription was done with 50 ng of total RNA input using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA), following the manufacturer's instructions. Complementary DNA (cDNA) was diluted 1:2, and qRT-PCR was carried out using TaqMan gene expression assays and reagents (Applied Biosystems), following the manufacturer's instructions (OneStepPlus real-time PCR system; Applied Biosystems). The list of accession numbers for the primers is available in Table 1. The expression levels of all genes of interest were determined using the cycle threshold (ΔΔCt) method and normalized to the expression of 18S rRNA [41]. All samples were run in triplicate for each primer.

Protein Extraction and Western Blotting

Total protein was extracted from both the pachytene spermatocytes and round spermatids by using transmembrane protein extraction reagent (Fivephoton Biochemicals, San Diego, CA), following the manufacturer's instructions; total protein concentrations were determined by Bradford assay using a protein assay reagent (Bio-Rad, Saint-Laurent, Qc, Canada). Samples were resolved on 10% (w/v) polyacrylamide gels and then transferred onto polyvinylidene fluoride membranes. Membranes were blocked in 10% non-fat cow's milk in TBS 0.1%-Tween-20. Proteins were detected using antibodies specific for ZIP5 (1:1000 dilution; product ARP44143_P050; Aviva Systems Biology, San Diego, CA) and β-actin (1:5000 dilution; code sc-1616; Santa Cruz Biotechnology, Dallas, TX) diluted in 5% non-fat milk/TBS-0.1% Tween and incubated overnight at 4°C. Primary antibodies were followed by horseradish peroxidase-linked secondary antibodies (donkey anti-rabbit immunoglobulin G [product NA93V]; 1:25000 dilution; GE LifeSciences, Mississauga, ON, Canada; donkey anti-goat immunoglobulin G; 1:10000 dilution; product sc-2056; Santa Cruz Biotechnology) diluted in 5% non-fat milk/TBS-0.1% Tween-20, incubated for 2 h at room temperature. Protein bands were detected by electrochemiluminescence prime Western blotting detection reagent (GE LifeSciences).

Germ Cell Culture

After germ cell separation, pachytene spermatocytes and round spermatids were seeded (pachytene spermatocytes at 100×10^5 cells/well; round spermatids at 300×10^5 cells/well) into 96-well cell culture plates (Costar 3595; Corning Life Sciences, Tewksbury, MA) in phenol red-free Dulbecco modified Eagle medium/F12 medium; Life Technologies) with streptomycin and penicillin G added and supplemented with HEPES,

lactic acid, and fetal bovine serum, as adapted from the method of LaSalle et al. [42]. Cells were cultured overnight for approximately 11 hours at 32°C in 5% CO_2.

Zinc Uptake Assay

After overnight culture, cells were removed from culture plates and transferred to 1.5-ml microcentrifuge tubes. Cells were incubated with 1 μM Fluozin3-AM (Molecular Probes, Eugene, OR) in Live Cell Imaging solution (Molecular Probes) for 1 h at 32°C. Following incubation and washing, cells were resuspended in imaging solution containing Hoechst nuclear stain (2,5′-bi-1H-benzimidazole, 2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]; Invitrogen, Burlington, ON, Canada) and incubated for 5, 15, 30, or 45 min at 32°C with 100 μM ZnSO4. After 45 min, TPEN (500 μM N,N,N′,N′-tetrakis[2-pyridylmethyl] ethane-1,2-diamine; Sigma Chemical) was added for 30 min to chelate zinc. Propidium iodide (PI; Invitrogen) was used to evaluate cell viability. After a second wash in imaging solution, cells were transferred to a 96-well cell carrier (PerkinElmer, Woodbridge, ON, Canada) plate with an optically clear bottom. The plate was spun down at 300g at 4°C for 5 min and immediately scanned (Operetta HTS imaging system; PerkinElmer) at 20× magnification, with 15 fields of view per well. Image analysis software (Columbus version 2.2; PerkinElmer) was used to quantify the mean fluorescent signals from individual cells in each well.

Statistical Analyses

Results are expressed as mean ± standard error of the mean (SEM) and were analyzed using Student t-test in Prism version 6 software (GraphPad Software, Inc., LaJolla, CA).

RESULTS

Pachytene Spermatocytes and Round Spermatids Show Differences in Gene Expression Profiles

We assessed the differences in gene expression in purified pachytene spermatocytes and round spermatids, two germ cell populations at different stages of differentiation. Principle component analysis (PCA) was used to reduce the dimensionality of the gene expression data sets and allow for easier discernment of the general relationships of groups (Fig.3-1). As expected, PCA indicated clear differences, accounting for approximately 78% of the variance in gene expression profiles between pachytene spermatocytes and round spermatids.

Expression of the ZIP Family Zinc Transporters in Pachytene Spermatocytes and Round Spermatids

The expression of members of the ZIP family of zinc transporters, responsible for increasing cytosolic zinc, in pachytene spermatocytes was evaluated and compared with that in round spermatids. All members of this family of zinc transporters were expressed to various degrees in both of the germ cell types (Fig. 3-2a). The most abundantly expressed member in both of the cell types was ZIP3, followed by ZIP4; both members were present at levels up to 2 orders of magnitude higher than the lowest expressed member, ZIP2. These differences in expression of ZIP transporters are even more evident when visualized on the linear scale (Supplemental Fig. S3-1a; all Supplemental Data are available online at www.biolreprod.org). Additionally, the expression levels of the different ZIP family members vary between the two cell types

(Fig. 3-2b). The largest difference in expression was seen with ZIP12, which was greater than 5 times more abundant in round spermatids than in pachytene spermatocytes, followed by ZIP11, which was almost 4 times more abundant in round spermatids. In contrast, ZIP7 was almost 3.5 times more abundant in pachytene spermatocytes than in round spermatids. ZIP4, the second most abundant ZIP member in the two cell types, was present in pachytene spermatocytes at levels more than 2 times those found in round spermatids.

Expression of the ZnT Family Zinc Transporters in Pachytene Spermatocytes and Round Spermatids

The expression of members of the ZnT family of zinc transporters, responsible for decreasing cytosolic zinc, in pachytene spermatocytes was evaluated and compared with that in round spermatids (Fig. 3-2c). ZnT family members were expressed to various degrees in both of these cell types. The most abundantly expressed ZnT family members in both cell types were ZnT3 and ZnT1, which were expressed at levels more than 2 orders of magnitude higher than the lowest expressed members, ZnT2, ZnT4, and ZnT5. These differences in the expression of ZnT transporters were even more evident when visualized on a linear scale (Supplemental Fig. S3-1b). The expression of ZnT members differed significantly between the two cell types, with the exception of ZnT3, ZnT9, and ZnT10 (Fig. 3-2d). The largest difference was seen with ZnT6, which was greater than 5 times more abundant in pachytene spermatocytes than in round spermatids. In addition, ZnT1, the second most abundant ZnT member in both cell types, was expressed at a level almost 5 times higher in round spermatids than in pachytene spermatocytes.

Zinc Transport in Pachytene Spermatocytes Compared to Round Spermatids

Considering the differences in expression of zinc transporters, we next evaluated zinc transport over time in both pachytene spermatocytes and round spermatids by live cell imaging using a fluorescent zinc probe. Within 5 min after the addition of zinc, the mean fluorescent signal in both cell types increased by 10% above baseline levels and continued to increase steadily (Fig. 3-3, a, b, and c). There were statistically significant differences in zinc uptake between the two cell types at 45 min after the addition of zinc; at this time the mean fluorescent signal in round spermatids was increased above that of pachytene spermatocytes by 18% above baseline (Fig. 3-3c). The addition of TPEN, an intracellular zinc chelator, decreased the fluorescence signal to levels well below baseline, indicating that the observed fluorescence signal was indeed from zinc (Fig. 3-3d). If alterations in membrane integrity were to account for the differences seen in zinc accumulation, round spermatids would be expected to have decreased membrane integrity. However, membrane integrity, as assessed by PI staining, showed a decreased number of cells with PI signal in round spermatids compared to that in pachytene spermatocytes (5% vs. 16% of cells, respectively) (Supplemental Fig. S3-2a), supporting the results obtained in the zinc uptake assay.

Chronic Low Dose CPA Treatment Alters Gene Expression in Pachytene Spermatocytes and Round Spermatids

We assessed the impact of chronic low-dose CPA treatment on gene expression in pachytene spermatocytes and round spermatids by using whole-rat genome microarrays. Principal component analysis was used to examine relationships between

samples (Fig. 3-4, a and b). Samples from CPA-treated animals and control animals separated along the y and z axes. The analysis also revealed differences in gene expression profiles after CPA treatment in both of the cell types.

Of the 30507 probe sets present on the arrays, 20449 (67%), corresponding to 13524 known genes, were considered expressed in both CPA-treated and control pachytene spermatocyte samples (Fig. 3-5a). A number of these known genes were significantly altered and had a 1.5 or greater fold change with respect to treatment (Fig. 3-5c). Nearly twice as many genes were down-regulated than were up-regulated after treatment. Additionally, more than 10 times more genes were reduced to undetectable levels after CPA treatment than were induced (Fig. 3-5a). These results suggest that CPA treatment may have a repressive effect on gene expression in pachytene spermatocytes.

In round spermatids, 20972 probe sets (68.8%), corresponding to 13939 known genes, were present in both treated and control samples (Fig. 3-5b). The number of known genes significantly altered over 1.5-fold after treatment in round spermatids and was similar to the number in pachytene spermatocytes (Fig. 3-5d). However the numbers of known genes that were down- and up-regulated after CPA treatment were approximately equal. Similarly, although the number of genes that was induced after treatment in round spermatids was greater than the number of genes repressed, the differences were not as striking as in the pachytene spermatocytes (Fig. 3-5d). The comparable numbers of transcripts or known genes that were induced or up-regulated and repressed or down-regulated suggest that CPA treatment has a greater effect on gene expression in round spermatids.

Expression of Members of the Slc39a (ZIP) Family of Zinc Transporters Is Increased in Pachytene Spermatocytes After CPA Treatment

Bioinformatic analysis using ingenuity pathway analysis was used to further characterize transcripts that were significantly altered by CPA treatment. As expected, genes involved in the response to stress, response to DNA damage, DNA repair, regulation of cell death, and spermatogenesis were altered after CPA treatment in pachytene spermatocytes and round spermatids (Fig. 3-6, a and b). Unexpectedly, many genes involved in ion transport and zinc binding were altered in both of the cell types after CPA treatment. Supplemental Tables S3-1 and S3-2 list the genes involved in zinc binding and transport. In both of the cell types, treatment altered the expression of many genes involved in transcription, including multiple zinc finger proteins (Fig. 3-6, c and d). Both estrogen-related receptor alpha (Essra) and PR domain containing 13 (Prdm13) were altered by CPA treatment in both cell type, although in opposite directions. Of particular interest were four members of the ZIP family of zinc transporters, Zip5, Zip6, Zip13, and Zip14, which were all significantly up-regulated in pachytene spermatocytes after CPA treatment (Figs. 3-6a and and 3-7, a–d). The expression of these zinc transporters, or any others, was not altered in round spermatids (Supplemental Fig. S3-3).

Real-time qRT-PCR assay was used to validate the microarray results in pachytene spermatocytes and confirmed the significantly increased expression of Zip5 and Zip14 (Fig. 3-7, e and h). However, Zip6 and Zip13 were not significantly up-regulated (Fig. 3-7, f and g). The results from the qRT-PCR experiment were

remarkably similar to those from the microarray results, reflecting the sensitivity of the microarray experiment.

Western blots were analyzed to determine whether the changes in expression of Zip5 and Zip14 transcripts translated to proteins. The results revealed that ZIP5 levels were 2.26-fold up-regulated in pachytene spermatocytes (Fig. 3-8a). Protein expression levels were also verified in the round spermatids to determine whether altered transcript levels in pachytene spermatocytes could carry over into the more differentiated germ cells; ZIP5 levels in round spermatids were not significantly affected by CPA treatment (Fig. 3-8b). The protein levels of ZIP14 were not assessed due to the lack of a specific antibody.

Chronic Low-Dose CPA Treatment Results in an Increase in Zinc Uptake in Pachytene Spermatocytes

Whether the increased expression of ZIP5 and ZIP14 resulted in an increase in zinc accumulation was assessed by live cell imaging using a fluorescent zinc probe and monitoring the fluorescence signal over time after the addition of zinc (Fig. 3-9, a and c). The mean fluorescence signal in pachytene spermatocytes from CPA-treated animals was significantly increased above that of control cells by 24% above baseline levels at 45 min after addition of zinc. Addition of the intracellular zinc chelator TPEN decreased the fluorescence signal to levels well below baseline, indicating the specificity of the fluorescent zinc probe (Fig. 3-9e). The number of cells with a positive PI signal was not significantly different between the two treatment groups (Supplemental Fig. S3-2b), indicating that the differences in zinc accumulation between cells from CPA treated

animals and control cannot be explained by a detrimental effect of CPA on membrane integrity.

Chronic Low Dose CPA Treatment Does Not Increase Zinc Uptake in Round Spermatids

The effect of CPA treatment on zinc uptake was also determined in round spermatids which did not have altered expression of zinc transporters (Fig. 3-9, b and d). After the addition of zinc the fluorescent zinc signal increased over time in round spermatids from both treated and control animals; however, there was no significant difference in fluorescent signal between the two groups at any time point. Again, consistent with the pachytene spermatocytes, there were no significant differences in the number of cells with PI signal between round spermatids from CPA-treated and control animals (Supplemental Fig. S3-2c). These results indicate that CPA treatment does not affect zinc transport in round spermatids.

DISCUSSION

Despite clear evidence of the importance of zinc in male germ cell development and fertility, there is little understanding of zinc transport in the testis. Here we examined the expression of ZIP and ZnT family members and zinc transport in purified populations of male germ cells in a normal state and after treatment with CPA. We found that both pachytene spermatocytes and round spermatids expressed many members of both families of zinc transporters and that the level of expression of some transporters differed between the two cell types. Additionally, we found that round spermatids accumulated more zinc over time than pachytene spermatocytes. Treatment

with the alkylating agent CPA led to an increase in the expression of ZIP5 and ZIP14 in pachytene spermatocytes but not in round spermatids. Consistent with our expression data, CPA treatment resulted in an increase in zinc uptake in pachytene spermatocytes but not in round spermatids.

Many studies have investigated differences in gene expression between different germ cell types. However, no studies have investigated zinc transporters in particular. Furthermore, only a few studies have addressed the expression of zinc transporters in the testis. To our knowledge, our study is the first to evaluate the expression of all members of the ZnT and ZIP families in purified populations of pachytene spermatocytes and round spermatids. Other studies have investigated the expression of select transporters in whole-testis homogenates or immunohistochemically [27, 28, 43-47].

In previous studies, ZnT1 was detected immunohistochemically in Sertoli cells but not in germ cells in mouse and human testes [27, 43], whereas ZnT7 showed strong immunoreactivity in mouse spermatocytes and spermatids [44]. At the mRNA level, ZnT1, ZnT2, and ZnT3 have been found in mouse testis homogenates and purified Sertoli cells [45, 46]. We found that both ZnT1 and ZnT3 are highly expressed in both pachytene spermatocytes and round spermatids. All other ZnT family members were also expressed in both cell types with the exception of ZnT2 in pachytene spermatocytes and ZnT8 and ZnT10 in round spermatids.

ZIP family members 5, 6, and 8 have been detected in spermatogonia, spermatocytes, spermatids and spermatozoa in human testis biopsies [27]. ZIP1 was

detected only in spermatids and spermatozoa and ZIP14 was not detected in any cell of the human testis [27]. In mice, ZIP14 has been localized to spermatogonia; ZIP5, ZIP8 and ZIP10 have been localized to spermatocytes; ZIP6, ZIP8 and ZIP10 have been localized in round spermatids; ZIP1 and ZIP6 have been localized in elongating spermatids; and ZIP5 has been localized in Sertoli cells [28]. At the transcript level, the testis has shown the highest level of expression of ZIP3 [47]. Similarly, we found ZIP3 was the most abundant ZIP transporter in both germ cell types, pachytene spermatocytes and round spermatids. In our study we also found the expression of ZIP5, ZIP6, ZIP8 and ZIP10 in pachytene spermatocytes and ZIP6, ZIP8 and ZIP10 in round spermatids at the mRNA level. Transcripts for all other members of the ZIP family were also found.

The expression of most of the different ZnT and ZIP family members in germ cells may serve a compensatory role as ZnT or ZIP knock out models have not shown any obvious reproductive phenotype [16, 17]. In fact, testis zinc levels are not altered in either ZnT3 or ZIP3 knockout mice [48, 49]. However, fertility was not specifically evaluated in these models.

The expression of many of the zinc transporters differed between pachytene spermatocytes and round spermatids. Considering this, we next examined whether there were differences in zinc accumulation in these cells. We found that round spermatids took up more zinc over time than pachytene spermatocytes. To our knowledge, this is the first study to compare zinc transport in these germ cell types. A previous study investigated the kinetics of zinc transport in spermatids (round and elongating) using ^{65}Zn labeling, and found a temperature-independent fast kinetic

component which corresponded to extracellular zinc binding, followed by a temperature-dependent slower kinetic component which corresponded to an active transport of zinc into the cell [29]. Inside the cell, zinc was primarily bound to organelles or large cytoskeletal elements. Zinc efflux was slower than influx, presumably due to a slow release of zinc from intracellular organelles and binding sites. Our study was not designed to assess the kinetics of zinc influx and efflux in the germ cells. However, from our results we can postulate that the zinc kinetics of pachytene spermatocytes are different from those of round spermatids.

The observed differences between zinc transporter expression and zinc uptake in pachytene spermatocytes and those in round spermatids likely reflect cell- and stage-specific zinc requirements. Because zinc plays an important role in sperm chromatin condensation, it is possible that round spermatids may accumulate more zinc for use in later stages of spermiogenesis than pachytene spermatocytes do. The significance of the differences in zinc transporter expression and zinc transport, as well as the specific roles played by zinc in these two germ cell types, warrant further investigation.

We also evaluated the effect of CPA on global gene expression in pachytene spermatocytes and round spermatids. We found that CPA treatment repressed and down regulated many genes in pachytene spermatocytes, suggesting a global repression of transcription. The dysregulation of transcripts in round spermatids was more evenly distributed. As expected CPA treatment altered the expression of genes involved in the response to stress. The results from this study are consistent with those of a previous study from our laboratory where we showed that chronic CPA treatment resulted in a decrease in the expression of transcripts involved in the stress response in

pachytene spermatocytes and round spermatids [50]. Interestingly, acute CPA treatment resulted in an increase in gene expression, particularly in round spermatids [51]. Other cancer treatment regimens, such as the combination of bleomycin, etoposide, and cis-platinum, have been shown to alter gene expression in male germ cells [52]. Unexpectedly, we found that many transcripts involved with zinc were altered after CPA treatment, including members of the ZIP family of zinc transporters.

Changes in zinc transporter expression have been associated with neurodegenerative diseases, immunological impairment, and cancer progression and metastasis [53]. The potential for a xenobiotic to modulate the expression of zinc transporters, to our knowledge, has not been tested. Here we show that chronic treatment with CPA up-regulated the expression of two zinc transporters, ZIP5 and ZIP14, in pachytene spermatocytes. ZIP5 was highly up-regulated after CPA treatment. ZIP5 has been localized to the basolateral membranes of intestinal enterocytes and pancreatic acinar cells [54, 55]. In periods of dietary zinc deficiency, ZIP5 is internalized and degraded [55]. These data and results from ZIP5 knockout mice suggest that ZIP5 plays a role in intestinal zinc excretion and zinc accumulation/retention in the pancreas [55, 56].

ZIP14 expression was also increased after CPA treatment. ZIP14 is most abundantly expressed in the liver where it has been localized to the plasma membrane of hepatocytes and is upregulated in response to inflammation through interleukin-6 (IL-6), IL-1β and nitric oxide, leading to increased hepatic zinc uptake and contributing to hypozincemia [57, 58]. ZIP14 has high expression in the duodenum, at the apical membrane of polarized cells, where it is thought to play a role in zinc absorption [59].

Additionally, ZIP14 may have a function in chondrocyte and adipocyte differentiation via signalling pathways involving zinc [60-62].

What roles these zinc transporters play in male germ cells are unknown. Neither the expression of ZIP5 nor of ZIP14 in the testis was affected by moderate zinc deficiency [28]. However, the expression of zinc transporters was assessed only in whole testis. Whether zinc deficiency affects the expression of different zinc transporters in germ cells at different stages of development is unknown.

We next asked whether the increased expression of ZIP5 and ZIP14 would lead to greater zinc uptake. Using a fluorescent zinc probe, we measured an accumulation of zinc that was greater in pachytene spermatocytes from CPA-treated animals than in controls. Consistent with the expression data, no increase in zinc uptake was observed over time in round spermatids. The fact that ZIP transporters and zinc transport were increased in pachytene spermatocytes but not round spermatids may be due to inherent differences in the two cell types. Perhaps pachytene spermatocytes are better suited to adapt and respond to CPA treatment. Indeed, although proliferating premeiotic and meiotic germ cells respond to alkylating agents, postmeiotic germ cells and spermatozoa are more susceptible [63, 64].

Previously we and others have shown that CPA treatment causes oxidative stress and DNA damage in male germ cells [31]. We propose that this stress leads to an increased zinc requirement in these cells. Pachytene spermatocytes may respond to a CPA-induced zinc deficiency by increasing the expression of ZIP5 and ZIP14, which leads to greater zinc uptake. A previous study showed that, in the testis, ZIP6 and

ZIP10 expression levels were decreased in response to moderate zinc deficiency [28]. However, it is possible that germ cells, in particular pachytene spermatocytes, respond differently to different stressors, in this case dietary zinc deficiency versus cellular stress caused by CPA, by altering the expression of different zinc transporters. ZIP5 and ZIP14 are not the most abundant zinc transporters expressed in the pachytene spermatocytes; however they may be the ZIP transporters most involved in responding to cellular insult by a toxic agent. Whether chronic low dose-CPA treatment causes zinc deficiency in male rats was not assessed in this study. However, considering the known importance of zinc in transcription, along with our observations of an apparent global repression in gene expression, and the altered expression of many transcription factors that we observed after CPA treatment may also indicate a state of zinc deficiency in pachytene spermatocytes. Additionally, high doses of CPA administered to male rats have been shown to result in reduced serum and testis zinc levels [65].

Sperm chromatin from CPA- treated rats has decreased protamination and sulfhydryl groups [34]. Because zinc plays an important role in sperm chromatin structure [19], we propose that a decrease in zinc levels in the germ cells may be responsible for the reduced sperm chromatin quality observed after CPA treatment.

Treatment with an acute high dose but not a chronic low dose of CPA impairs meiotic progression in pachytene spermatocytes [66]. It is possible that altered zinc homeostasis in pachytene spermatocytes is involved in this impairment. Zinc is important for meiotic progression in oocytes [67-69], but it is not known whether it plays an important role in meiosis in male germ cells. The lack of impairment after chronic CPA treatment may in fact be due to the cells adapting by increasing zinc uptake.

In conclusion, the expression of ZIP and ZnT family members as well as zinc transport differ in purified populations of pachytene spermatocytes and round spermatids. Importantly, we have shown that an alkylating agent such as CPA is capable of modulating the expression of zinc transporters in male germ cells, leading to changes in zinc uptake. Considering the importance of zinc in proper cellular function and spermatogenesis, any changes in the regulation of zinc levels have the potential to alter normal spermatogenesis. How these changes affect germ cell survival, development, and quality will need to be examined further. These novel data have important implications for understanding damage to male germ cells from toxic agents as they suggest that zinc homeostasis is altered after CPA treatment. Zinc supplementation could potentially be an interesting strategy to protect male germ cells from damage induced by toxic agents such as CPA.

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TABLES

Table 3-1. Primers used in qRT-PCR

Gene Name	Accession no.	TaqMan Assay no.
Slc39a5	NM_001108728.1	Rn01527167_m1
Slc39a6	NM_001024745.1	Rn01405813_m1
Slc39a13	NM_001039196.1	Rn01485759_m1
Slc39a14	NM_001107275.1	Rn01468336_m1
18s	NM_213557.1	Rn01428913_gH

Supplemental Table 3-1. Genes involved with zinc that are significantly altered by CPA treatment in pachytene spermatocytes. $p<0.05$

Down-Regulated After CPA Treatment		Up-Regulated After CPA Treatment	
Fold Change	Gene Symbol	Fold Change	Gene Symbol
-1.63	Mmp11	2.25	Slc39a5
-1.52	Ace	1.62	Slc39a14
-1.52	Zfp169	1.51	RGD1564243
-1.5	Adam4	1.33	Limd1
-1.48	Triml1	1.32	Erap1
-1.43	Clip1	1.22	Champ1
-1.43	Prdm13	1.21	Trim32
-1.42	Adam24	1.2	Sirt5
-1.39	Lhx2	1.2	Gpatch8
-1.38	March7	1.2	Gtf2b
-1.38	Sp2	1.17	Ubr7
-1.36	LOC100360593	1.17	Nr2c2
-1.33	Sun1	1.16	Rnf180
-1.31	Zfp385d	1.14	Slc39a6
-1.3	Chd5	1.14	Akap8
-1.28	Prkcg	1.13	Zfp622
-1.28	Dpf3	1.13	Zfp191
-1.27	Sec23a	1.13	Kdm2a
-1.26	Zfp36l1	1.13	Srek1ip1
-1.24	Ring1	1.13	Slc39a13
-1.23	Zdhhc1	1.11	Esrra
-1.23	Zhx3	1.08	Brpf1
-1.23	Usp44		
-1.21	Man2b2		
-1.2	Glis1		
-1.2	Foxp4		
-1.2	Rc3h1		
-1.19	Zfp385a		
-1.18	Nrd1		
-1.11	Sharpin		

Supplemental Table 3- 2. Genes involved with zinc that are significantly altered by CPA treatment in round spermatids. $p<0.05$

Down-Regulated After CPA Treatment		Up-Regulated After CPA Treatment	
Fold Change	Gene Symbol	Fold Change	Gene Symbol
-1.87	Zc3h12b	2.69	Fbxo40
-1.54	Zfp40	2.24	Trim58
-1.38	Rarg	1.54	Zbtb33
-1.34	Egr4	1.51	Vdr
-1.33	Zfp532	1.5	Nrap
-1.26	Zfp142	1.43	Pparg
-1.23	Mtr	1.41	Rapsn
-1.22	Zfp655	1.37	Zfp385c
-1.18	Deaf1	1.36	Zfp219
-1.16	Car7	1.36	Mmp8
-1.16	Zfr	1.34	Adamts9
-1.16	Zfp318	1.31	Zbtb41
-1.13	Rc3h2	1.31	Prdm13
-1.11	Lasp1	1.3	Mss51
-1.11	Esrra	1.3	Lonrf2
-1.11	Rnf216	1.3	LOC680200
-1.1	Hinfp	1.26	Zfp280d
		1.26	Scrt1
		1.24	Nupl2
		1.23	Mmel1
		1.23	Zfp513
		1.22	Zzef1
		1.21	Zfp263
		1.21	Rnf44
		1.19	Zfp827
		1.18	Rnf5
		1.17	RGD1565844
		1.15	Zfp652
		1.14	Zfp414

FIGURE LEGENDS

Figure 3-1. Principal Component Analysis (PCA) showing distribution of pachytene spermatocytes and round spermatids in a 3D plot. Pachytene spermatocyte (n=5) and round spermatids (n=6) occupy distinct spaces.

Figure 3-2. Expression of zinc transporters in male germ cells. Log scale mRNA expression levels of (a) ZIP family and (c) ZnT family of zinc transporters in pachytene spermatocytes and round spermatids. Fold change of expression levels of (b) ZIP members and (d) ZnT members in pachytene spermatocytes versus round spermatids. N=5 pachytene spermatocytes, N=6 round spermatids. *P<0.05

Figure 3-3. Zinc transport in pachytene spermatocytes and round spermatids. High throughput imaging of fluorescent zinc probe Fluozin-3am bright field in (a) pachytene spermatocytes (n=8) and (b) round spermatids (n=5) incubated with 100 nm $ZnSO_4$ for 0, 5, 15, 30 or 45 minutes and after the addition of the zinc chelator TPEN. (c) Quantification of fluorescence intensity over time and (d) after addition of TPEN, as a percentage of baseline values. *P<0.05. Scale Bar 50μM.

Figure 3-4. Principle component analysis showing distribution of CPA treated and control (a) pachytene spermatocyte (n=5) and (b) round spermatid (n=6) samples.

Figure 3-5. Changes in mRNA expression of known genes after CPA treatment. Venn diagram of known genes that are expressed in both control and CPA treated samples and those that are exclusively expressed in one treatment group in (a) pachytene spermatocytes (n=5) and (b) round spermatids (n=6). Numbers of known genes that are

significantly altered over 1.5 fold after CPA treatment in (c) pachytene spermatocytes and (d) round spermatids. P>0.05

Figure 3-6. Functional characterization of genes significantly altered by CPA treatment. Pie charts show the prevalence of different GO terms associated with genes altered by CPA treatment in (a) pachytene spermatocytes and (b) round spermatids. Functional analysis of altered genes involved in zinc ion binding in (c) pachytene spermatocytes and (d) round spermatids.

Figure 3-7. CPA increases the mRNA expression of members of the ZIP family of zinc transporters. The expression of ZIP family members is significantly increased after CPA treatment in pachytene spermatocytes as shown by microarray results (a-d) and qRT-PCR validation (e-h). N=5, *P<0.05.

Figure 3-8. CPA treatment increases ZIP5 expression at the protein level. The protein expression of ZIP5 is increased in (a) pachytene spermatocytes but not in (b) round spermatids after CPA treatment. N=3, *P<0.05

Figure 3-9. Zinc Transport in pachytene spermatocytes and round spermatids after CPA treatment. High throughput imaging of fluorescent zinc probe Fluozin-3am and bright field of (a) pachytene spermatocytes and round spermatids (b) from treated and control animals, incubated with 100 nm $ZnSO_4$ for 0, 5, 15, 30 or 45 minutes and after the addition of the zinc chelator TPEN. Quantification of fluorescence intensity over time in (c) pachytene spermatocytes and (d) round spermatids as a percentage of baseline values. Quantification of fluorescence after addition of TPEN, as a percentage of baseline values in (e) pachytene spermatocytes and (f) round spermatids. Pachytene

spermatocytes N=9 CPA, N=8 control; round spermatids N=5. *P<0.05. Scale Bar 50 μM.

Figure S3-1. Linear mRNA expression of (a) ZIP and (b) ZnT family members in pachytene spermatocytes and round spermatids. Pachytene spermatocytes N=5, round spermatids N=6 *P<0.05

Figure S3-2. PI staining in male germ cells. Analysis of membrane integrity by propidium iodide staining in (a) control pachytene spermatocytes and round spermatids and CPA treated (b) pachytene spermatocytes and (d) round spermatids. *p<0.05

Figure S3-3. mRNA expression of zinc transporters was not altered after CPA treatment in round spermatids. Microarray results for (a) ZIP5 (b) ZIP6 (c) ZIP13 (d) ZIP14.

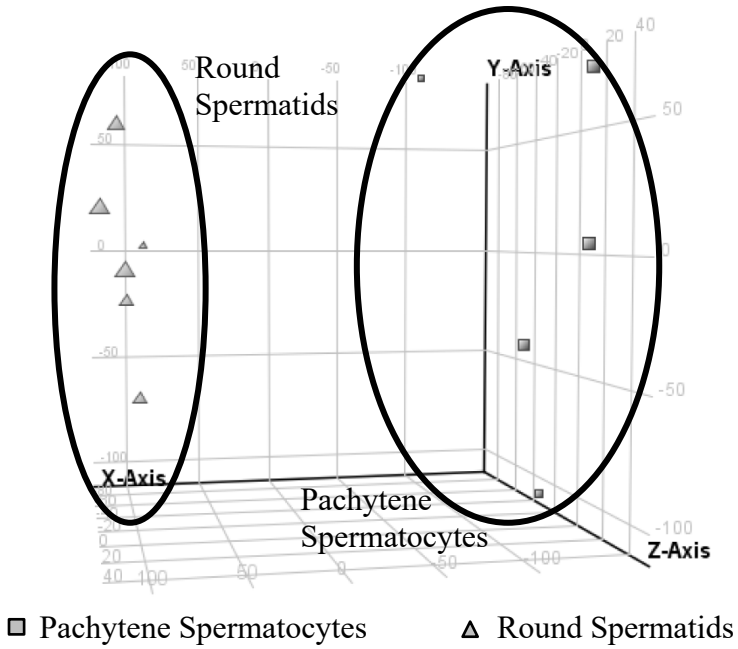

Figure 3-1. Principal Component Analysis (PCA) showing distribution of pachytene spermatocytes and round spermatids in a 3D plot.

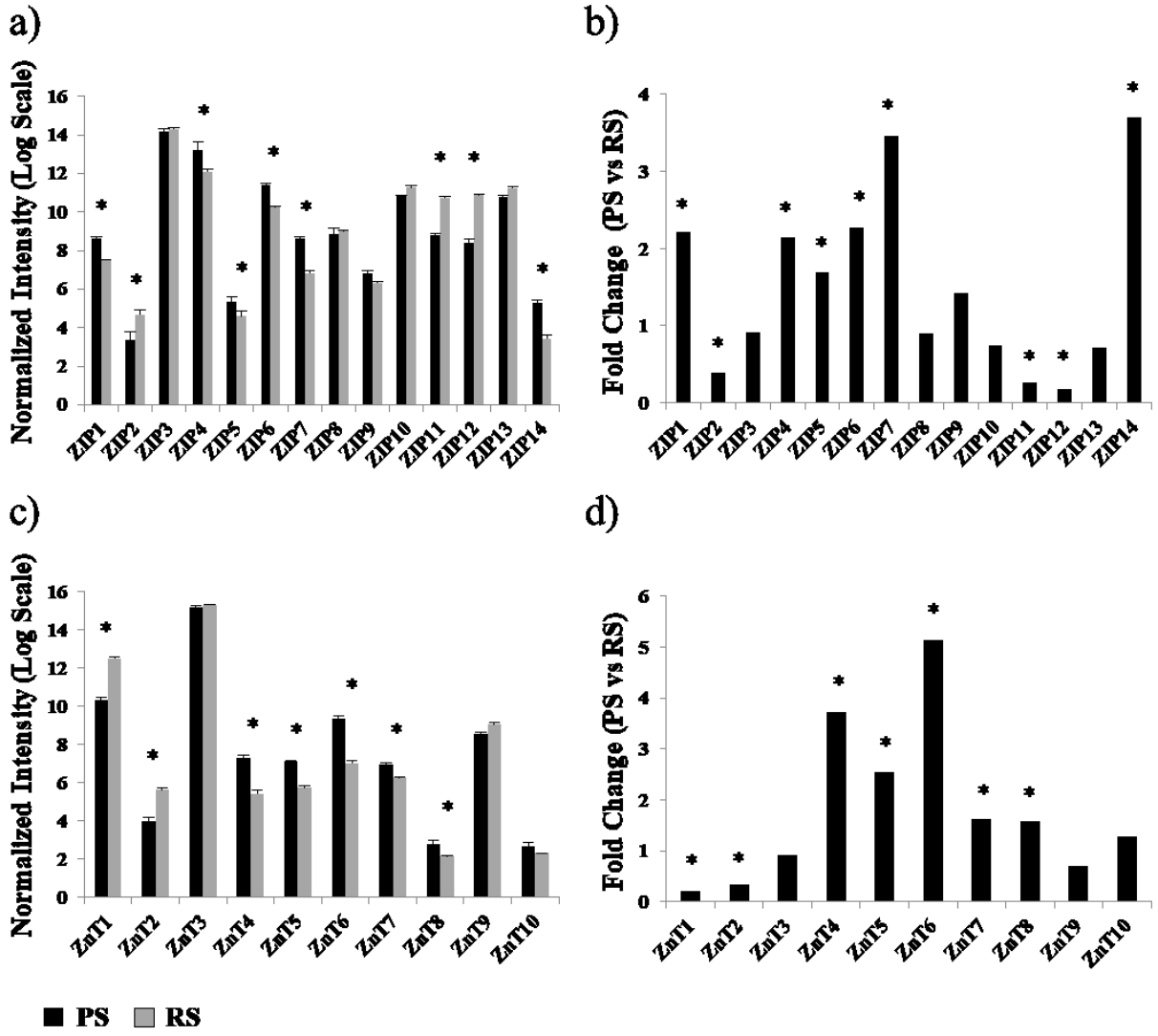

Figure 3-2. mRNA Expression of zinc transporters in male germ cells.

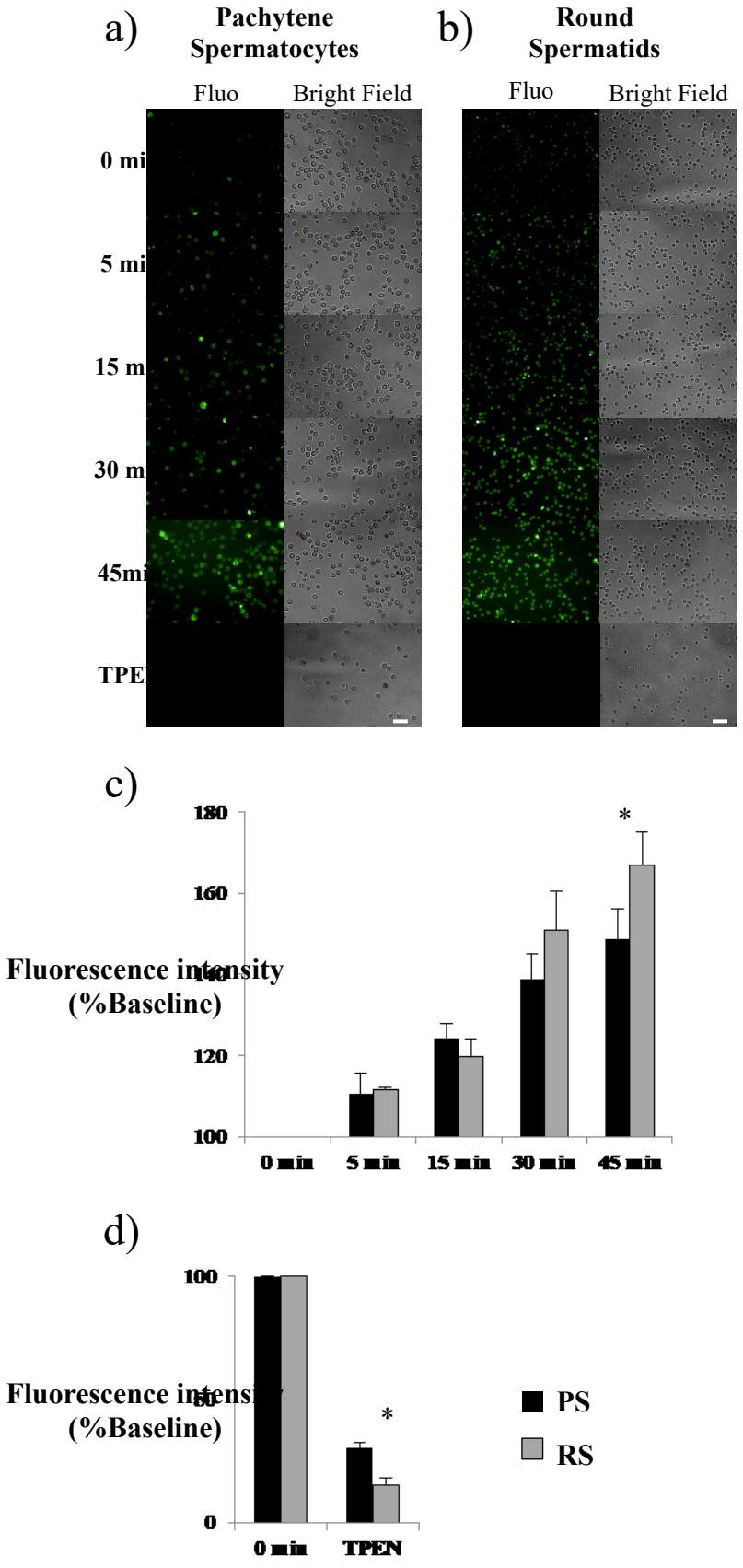

Figure 3-3. Zinc transport in pachytene spermatocytes and round spermatids.

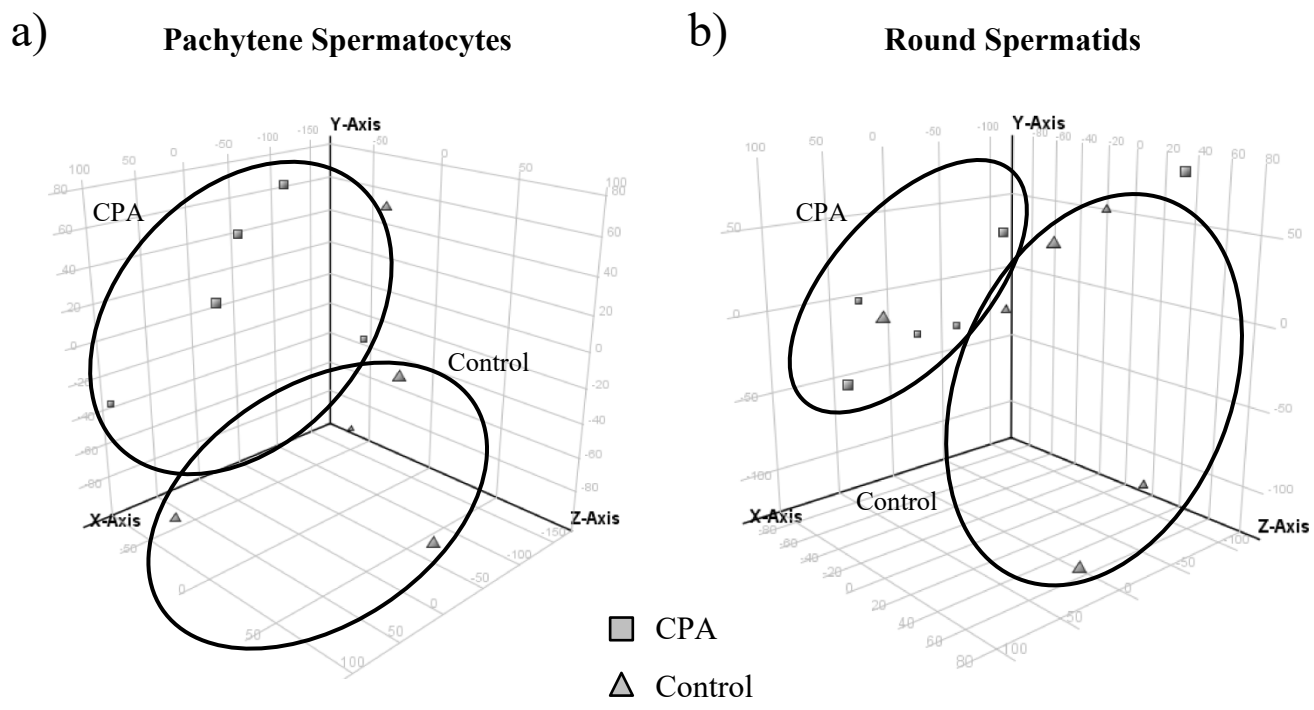

Figure 3-4. Principle component analysis showing distribution of CPA treated and control pachytene spermatocyte

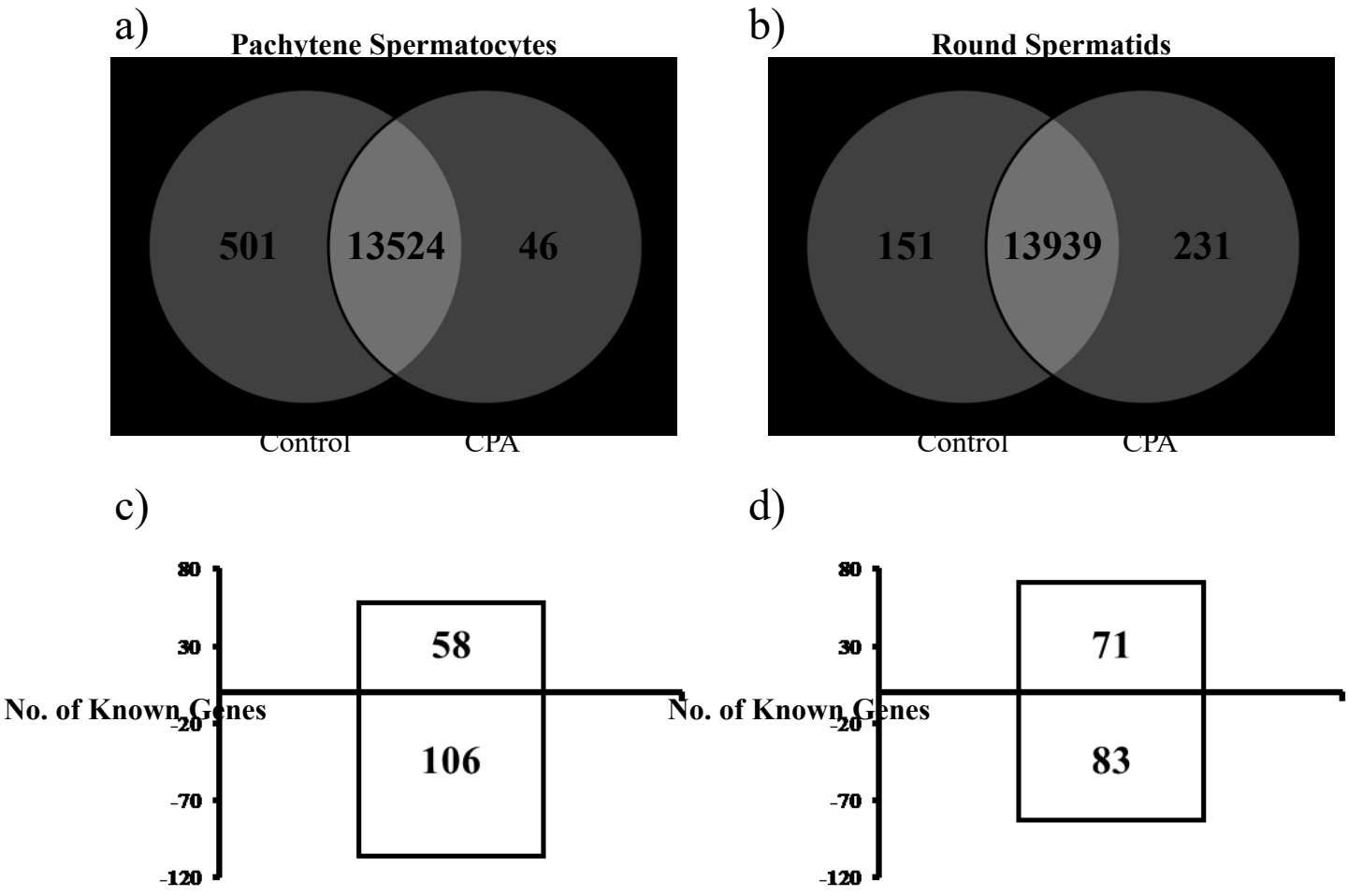

Figure 3-5. Changes in mRNA expression of known genes after CPA treatment.

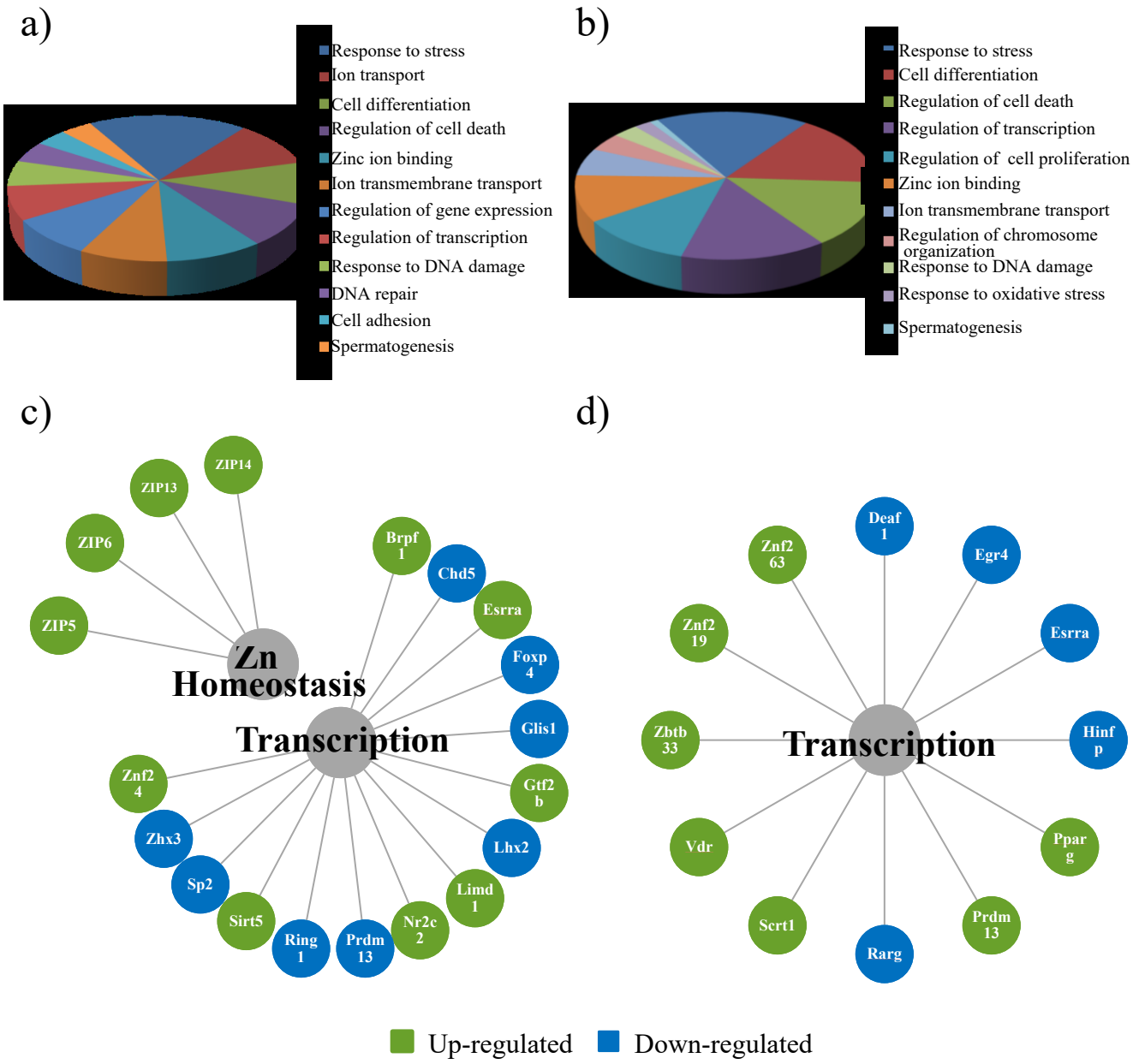

Figure 3-6. Functional characterization of genes significantly altered by CPA treatment.

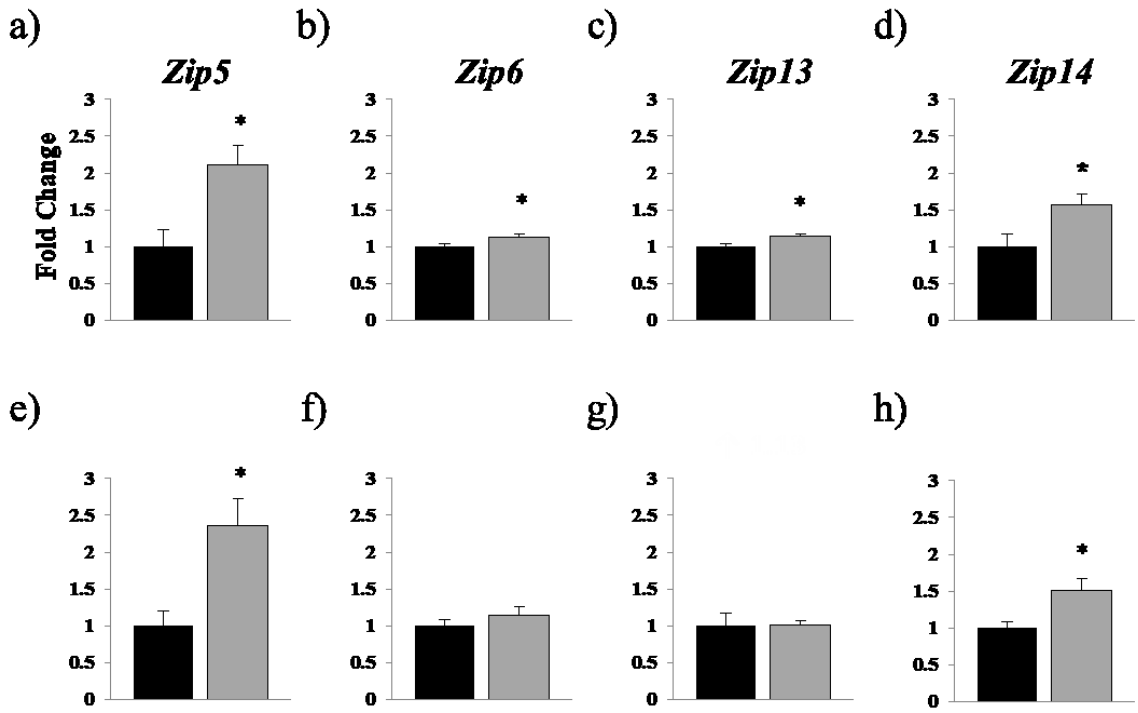

Figure 3-7. CPA increases the mRNA expression of members of the ZIP family of zinc transporters.

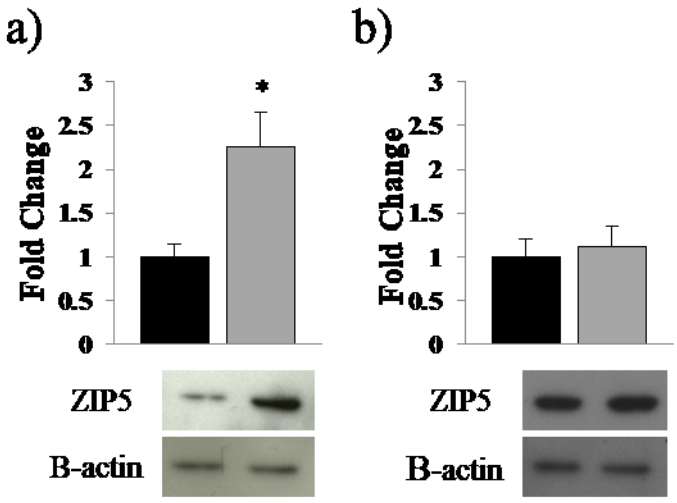

Figure 3-8. CPA treatment increases ZIP5 expression at the protein level.

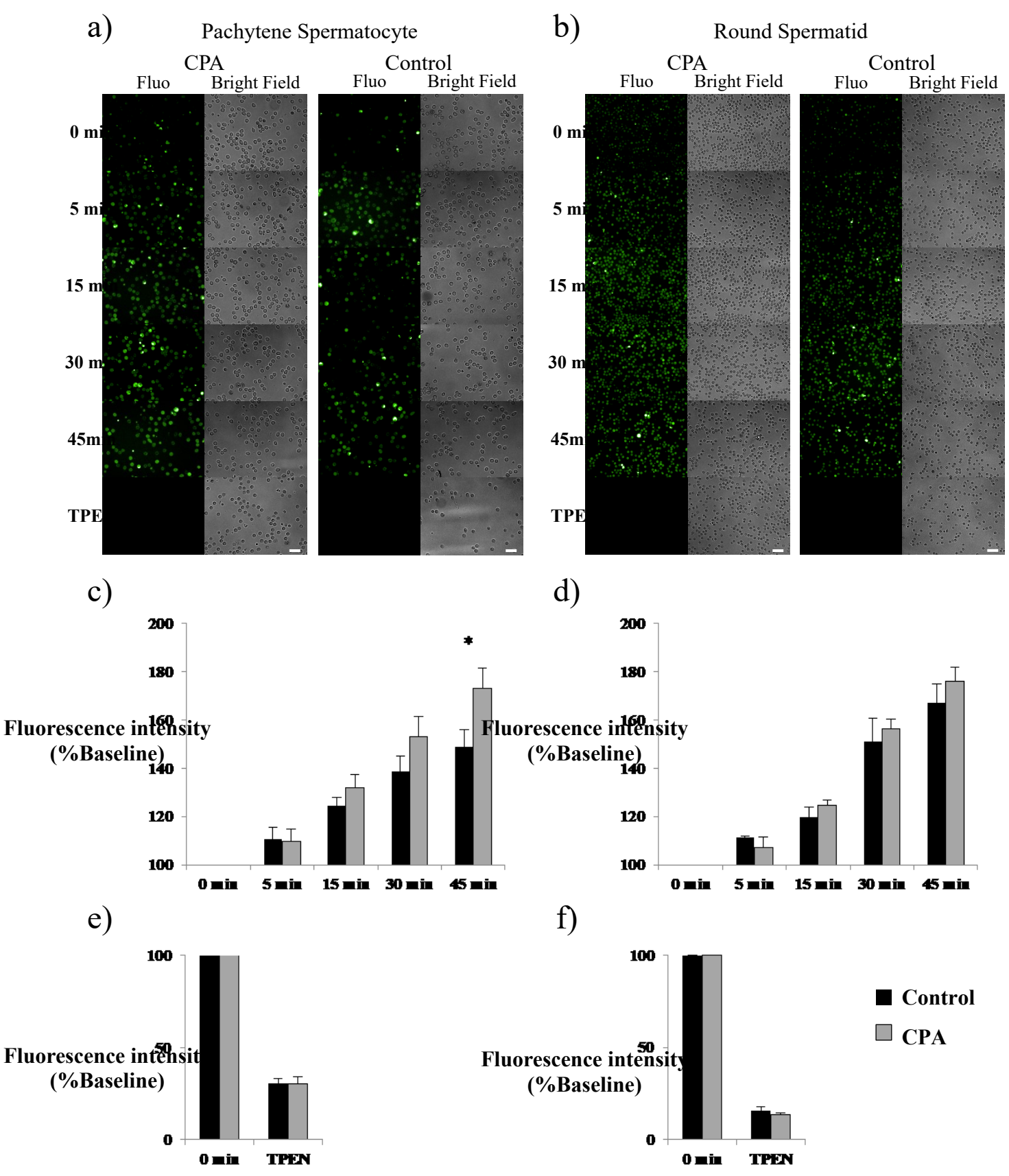

Figure 3-9. Zinc Transport in pachytene spermatocytes and round spermatids after CPA treatment.

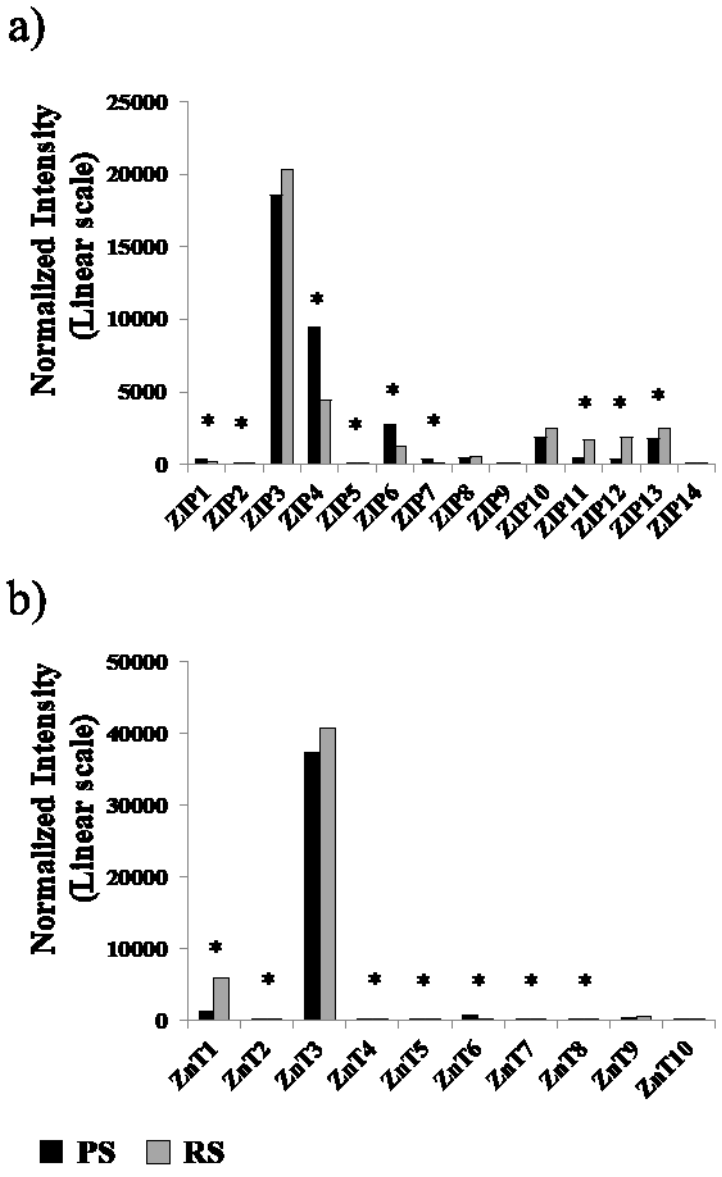

Figure S3-1. Linear expression of ZIP and ZnT family members in pachytene spermatocytes and round spermatids.

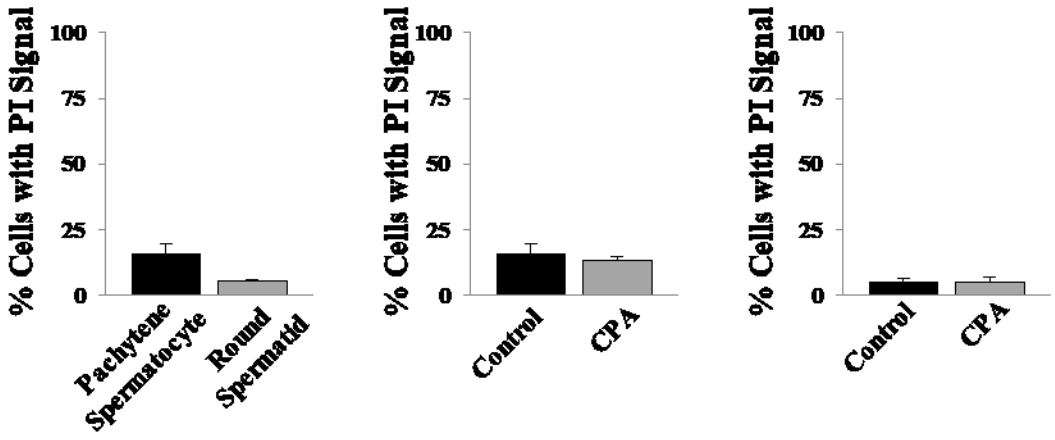

Figure S3-2. PI staining in male germ cells.

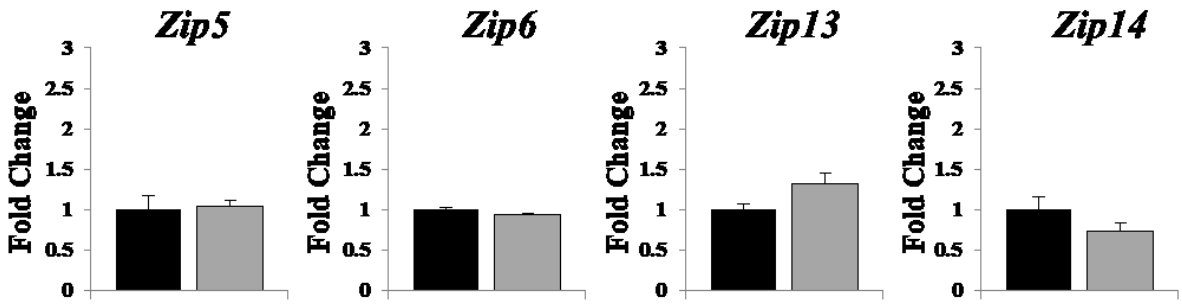

Figure S3-3. Expression of zinc transporters was not altered after CPA treatment in round spermatids.

CONNECTING TEXT

The results presented in Chapter 3 indicate that chronic low dose CPA treatment alters the expression of numerous transcripts involved in zinc binding including members of the ZIP family of zinc transporters. Additionally, zinc uptake was increased in germ cells isolated from CPA treated males. These results, along with altered expression of miRNAs predicted to target zinc binding proteins (Chapter 2), suggest that zinc may play a role in the germ cell response to CPA treatment. We proposed that the stress incurred by male germ cells from chronic CPA treatment leads to an increased requirement for zinc. We hypothesized that zinc supplementation may be able to alleviate some of the toxic effects of CPA treatment.

In Chapter 4 of this manuscript we examined the ability of zinc to protect male germ cells from CPA toxicity. This was accomplished by supplementing male rats with zinc along with chronic CPA treatment. At the end of the treatment period, the ability of zinc to protect male germ cells against CPA induced oxidative stress and DNA damage was assessed by live cell imaging and confocal microscopy.

CHAPTER 4

ZINC SUPPLEMENTATION PROTECTS MALE GERM CELLS AGAINST CYCLOPHOSPHAMIDE INDUCED OXIDATIVE STRESS AND DNA DAMAGE

Anne Marie Downey[1], Barbara F. Hales[1], Bernard Robaire[1,2]

Departments of Pharmacology and Therapeutics[1] and Obstetrics and Gynecology[2],

McGill University, Montréal, Québec, Canada, H3G 1Y6

ABSTRACT

There is a need to develop strategies to protect male germ cells against toxic agents used in chemotherapy. Cyclophosphamide (CPA), a commonly used chemotherapeutic and immunosuppressant drug and a germ cell toxicant, leads to an increase in zinc uptake in male germ cells, suggesting an increased requirement for zinc. Zinc is an essential trace element that plays important roles in the defence against oxidative stress and DNA damage. In the present study we tested the hypothesis that zinc supplementation would protect male germ cells from CPA induced oxidative stress and DNA damage. We found that zinc supplementation reduced oxidative stress in isolated pachytene spermatocytes and round spermatids when compared to CPA treatment. The γH2Ax signal and foci size, as an indication of DNA damage, and 53BP1 signal, as an indication of DNA repair, were also decreased in animals that received zinc supplementation when compared to CPA alone. The results from this study suggest a potential role for zinc supplementation in protecting male germ cells against CPA insult.

INTRODUCTION

The incidence of cancers that affect men of reproductive age has risen worldwide [1-3]. Although advancements in treatment regimen have drastically improved cancer survival rates [3], the therapies used are gonadotoxic and leave many men infertile [4]. This is an important issue for the quality of life for survivors, as these men often have a strong desire to have children. Currently, the only available fertility preservation strategy is sperm cryopreservation prior to the start of treatment and later use with assisted reproductive technologies [5]. However, fertility preservation is often overlooked during the development of a treatment plan [6, 7]. Therefore, strategies to protect male germ cells from the gonadotoxic agents used in chemotherapy are needed.

One such agent is cyclophosphamide (CPA), an alkylating drug and known germ cell toxicant. Men who are treated with this drug often become oligozoospermic or azoospermic [8-10]. Animal models have shown that CPA treatment leads to increased oxidative stress [11] and DNA damage [12, 13] in the developing male germ cells that cause a decrease in sperm chromatin quality [14-16]. This in turn results in adverse effects on embryo development and progeny outcome [17-22]. Thus, there is a need to better understand how male germ cells respond to such toxic insult.

We have previously shown that male germ cells respond to CPA insult by increasing the expression of members of the ZIP family of zinc transporters and increasing zinc uptake [23]. Zinc is an essential trace element that plays important roles in the defence against oxidative stress and DNA damage. Zinc is involved in mediating oxidative stress by acting as a co-factor for SOD1 [24], providing structural stability to

enzymes that influence oxidative stress levels (such as subunits of the cytochrome c oxidase in the electron transport chain)[25], protecting sulfhydryl groups against oxidation [26] and by competing with the redox active copper and iron ions to prevent Fenton reaction mediated lipid peroxidation [27, 28]. Zinc is also an important structural component for DNA binding proteins including many involved in DNA repair and zinc levels have been shown to influence their activity [29-32]. Additionally, zinc is essential for spermatogenesis as zinc deficiency leads to increased germ cell apoptosis, oxidative stress and DNA damage and can also lead to an arrest in spermatogenesis [33-40]. Therefore, we hypothesized that zinc supplementation would protect male germ cells against CPA induced oxidative stress and DNA damage.

Materials and Methods

Animals

Adult male Spraque-Dawley rats (300-350g) were purchased from Charles River Canada (St-Constant, Québec). Animals were maintained on a 12 hours light/12 hours dark cycle and had access to food and water *ad-libitum*. After one week of acclimatization animals were randomly assigned to one of four treatment groups (Fig. 4-1) and received the following by gavage: 0.9% saline 6 days/week for 5 weeks(SAL/SAL), $ZnCl_2$ (20mg/kg, Sigma Chemical Co., St. Louis, MO) 6 days/week for 5 weeks (ZN/SAL), saline 6 days for 1 week followed by CPA (6 mg/kg, CAS 6055-19-2, Sigma Chemical Co., St. Louis, MO) 6 days/week for 4 weeks (SAL/CPA), or $ZnCl_2$ (20mg/kg) 6 days for 1 week followed by $ZnCl_2$ (20 mg/kg) in combination with

CPA (6mg/kg) for 4 weeks (ZN/CPA). The CPA dose of 6 mg/kg is a clinically relevant dose and this treatment regimen has been used in previous animal studies examining the effect of CPA on the male germ cells [14, 16, 41-43] and leads to altered expression of genes involved in zinc binding and increased zinc transport in male germ cells [23]. The dose and treatment paradigm of zinc supplementation is based on a previous study that observed that daily gavage with 20 mg/kg of $ZnCl_2$ reduced the toxic effects of cigarette smoke on the reproductive system of male rats [44, 45]. Animals were either euthanized for germ cell collection (n=10) or anaesthetized for blood and tissue collections and perfusions (n=6). All animal care and handling were done in accordance with the guidelines outlined by the Canadian Council on Animal Care (McGill Animal Resources Centre protocol #2144).

Tissue and blood collection

At the end of treatment animals were anaesthetized with Isofluorane (induction: 5%, O_2 1.0L/min; maintenance: 2.5%, 0.8L/min). Whole blood was collected from the saphenous vein, left to clot at room temperature and spun down at 1000 x g. Serum was collected and frozen at -80°C until further use. Right testes and epididymides were removed and weighed. Testes were flash frozen in liquid nitrogen and kept at -80°C until further use for testicular sperm count and zinc levels.

Testis Fixation

Left testes were fixed by whole animal perfusion of anaesthetized animals using modified Davidson's Fluid (mDF; 30% of 40% formaldehyde, 15% ethanol, 5% glacial

acetic acid and 50% distilled H_2O) [46] as described previously [47]. Briefly, a round ended gavage syringe was inserted through the left heart ventricle and clamped in the aorta of anaesthetized animals. Blood was cleared through an incision in the right atrium with saline before perfusing with mDF. Perfused testes were collected and fixed further in mDF for 24hrs followed by dehydration and paraffin embedding.

Testicular Sperm counts

Testicular sperm counts were done as previously described [48]. Briefly, a piece of frozen testis was weighed and homogenized (Polytron PT 10-35 GT, 15000 RPM; Brinkman Instruments) for two 15 second periods with a 30 second interval in 2ml of buffer containing 0.9% NaCl, 0.1% thimerosal (Sigma), and 0.5% Triton X-100 (Sigma). Sperm heads were counted using a hemocytometer.

Testosterone Levels

Serum testosterone levels were measure using an enzyme-linked immunosorbent assay (ELISA) kit (IBL America) according to the manufacturer's instructions.

Testicular Zinc Measurement

Fragments of flash frozen testes were carefully weighed, crushed, lyophilized overnight and then desiccated under vacuum another 24 hours. Tissue was digested to release zinc by the addition of nitric acid (67%) and incubated overnight. Lipids were dissolved by the addition of hydrogen peroxide (30%) dropwise over the course of 1 hour followed by incubation at 92°C for 1 hour. Digested samples were diluted to 50ml

and zinc levels were measured by inductively coupled plasma optical emission spectrometry (ICP-OES) (Agilent Technologies). Lobster hepatopancreas – TORT-3 (National Research Council Canada) was used as a certified reference material.

Germ cell separation and isolation

At the end of treatment, rats were euthanized by asphyxiation with CO_2 and then decapitated. Male germ cells were isolated using the STA-PUT velocity sedimentation method as previously described by Bellve et al. [49] and modified by Aguilar-Mahecha et al. [50]. Briefly, testes were removed, decapsulated and the seminiferous tubules were digested with collagenase (Sigma Chemical) and further digested with trypsin (type 1; T8003; Sigma Chemical). The seminiferous-tubules were then physically dissociated in the presence of DNAse (product DN-25; Sigma Chemical) and then filtered through 70μm nylon mesh. The mix germ cell suspension was washed three times in 0.5% bovine serum albumin (BSA) and filtered through a 55μm nylon mesh to obtain a single cell suspension. A total of 5.6×10^8 cells were loaded into the STAPUT velocity sedimentation chamber and separated on a 2%-4% BSA gradient. Fractions containing spermatocytes and round spermatids were identified by phase contrast microscopy. Fractions with over 80% purity for pachytene spermatocytes and over 85% purity for round spermatids were pooled. A total of six germ cell separations met the purity cut off and were used for further experiments.

Germ cell culture

After germ cell separation, pachytene spermatocytes and round spermatids were seeded (pachytene spermatocytes - 100×10^5 cells/well; round spermatids – 300×10^5

cells/well) into 96 well cell culture plates (Costar 3595; Corning Life Sciences, Tewksbury, MA) in phenol-red-free media (DMEM/F12 medium; Life Technologies, Grand Island, NJ) with Streptomycin and Penicillin G added and supplemented with HEPES, lactic acid and FBS, adapted from La Salle et al [51]. Cells were cultured overnight for approximately 11 hours at 32°C and 5% CO_2.

Germ Cell ROS Measurement

After overnight culture, germ cells were removed from culture plates and transferred to 1.5ml microcentrifuge tubes. Cells were incubated for 30min at 32°C in Live Cell Imaging Solution (MolecularProbes) with 50 μM CellRox DeepRed Reagent (Invitrogen, Burlington, ON) and Hoechst (2,5′-bi-1H-benzimidazole, 2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]; Invitrogen, Burlington, ON). After washing, cells were transferred to a 96-well Cell Carrier (PerkinElmer, Woodbridge, ON) plate with an optically clear bottom. Plate was spun down at 300 g at 4°C for 5 minutes and immediately scanned by the Operetta HTS imaging system (PerkinElmer, Woodbridge, ON) at 20X magnification with 15 fields of view per well. Columbus 2.2 image analysis software (PerkinElmer, Woodbridge, ON) was used to quantify the mean fluorescent signals from individual cells in each well.

Immunofluorescent Staining

Blocks of paraffin embedded testes were sliced into 5μM sections and mounted on charged slides. Tissue was deparaffinized (Histoclear, Diamed Inc) and rehydrated through a graded ethanol series. Antigen retrieval and permeabilization were achieved by boiling slides for 10 minutes in sodium citrate buffer (0.01 M sodium citrate, 0.05%

Tween-20, pH 6.0). Non-specific binding was blocked with 10% normal goat serum for 1hr at room temperature, primary antibodies (γH2Ax 1:500,Millipore; 53BP1 1:500, Novus Biologicals) for 1 hour at 37°C, secondary antibodies (goat anti-mouse Alexa 488, goat anti-rabbit Alexa 546 1:1000, Thermofisher) for 45 minutes at room temperature, 4'-6'-diamidino-2-phenylindole (DAPI, 1:1000) combined with lectin PNA from Arachis hypogaea (peanut) Alexa Fluor 647 conjugate (1:400, Thermofisher) for 10 minutes at room temperature. Slides were mounted with Permafluor antifade mountant (Thermofisher), left at room temperature to dry for 24 hours before being stored at 4°C until imaging.

Image Analysis

Images were captured using a multiphoton Leica TCS SP8 MP microscope with 20x and 63x objectives. Images from Z-stacks were reconstituted into three-dimensional images and analyzed using Imaris Software version 9.1.2 (Bitplane, Switzerland). Quantification of the number of positive cells, mean fluorescence per cell, and co-localization for both γH2AX and 53BP1 was done with images at lower magnification. A minimum of 100 tubules per animal were selected manually and analyzed independently. Individual cells were identified by DAPI nuclear staining using the Imaris surfaces function and proper identification was verified visually. The fluorescent signal from the channels of both γH2AX and 53BP1 was obtained for each cell in each tubule. Cells were considered positive if the signal was above the set threshold. Data from controls with no primary antibody were used to set threshold. Fluorescence signal was analyzed across all tubules and also by seminiferous cycle stage. Tubules were visually

staged by the presence and appearance of PNA staining of the acrosome and grouped into 4 stage categories: stages I-IV, V-VIII, IX-XI and XII-XIV (Supp. Fig 1.).

Analysis of γH2AX foci volume was done with reconstituted three-dimensional images at higher magnification. Individual foci were identified and the volumes of a minimum of 300 foci were obtained per animal using the Imaris surfaces function. The average foci volume was calculated. The distribution of foci volume was determined by the percentage of the total number of foci in different volume categories: <0.1, 0.11-0.49, 0.5-0.99, 1-2.49, 2.5-4.99, 5.00-9.99, 10.00-14.99, 15.00-19.99, 20-24.99, 25-29.99 μm^3 and >30 μm^3.

Statistical Analysis

Data were analysed using one-way ANOVA with Tukey's multiple comparison correction with Graphpad Prism version 5. Results are expressed as means with standard error of the means.

RESULTS

Effect of cyclophosphamide treatment and zinc supplementation on physiological parameters

The effects of chronic CPA treatment and zinc supplementation on body and tissue weights are shown in Figure 4-2. Body weight was assessed weekly to determine the effect of chronic CPA treatment and zinc supplementation on the overall health of the animals. Neither CPA treatment nor zinc supplementation had a significant effect on

body weight at the end of treatment (Fig. 4-2a). However, the amount of weight gained, shown as the percentage of initial body weight, was lower in both the CPA treated and zinc supplemented groups (Fig 4-2b). On average rats in these groups gained 26% less weight compared to control rats and rats that receive zinc only.

The weights of the testes were not altered by CPA treatment or zinc supplementation (Fig. 4-2c). Epididymal weights were slightly increased by 16% in rats that received CPA with zinc supplementation (Fig. 4-2d). However, this weight increase was not accompanied by an increase in sperm production (Fig. 4-2e). Testicular sperm counts were not altered by either CPA treatment or zinc supplementation.

Serum testosterone levels were directly assessed by ELISA at the end of treatment. There was no significant difference in testosterone levels between any of the groups indicating that neither CPA treatment nor zinc supplementation affects steroidogenesis (Fig. 4-3a).

Elemental zinc levels were measured spectroscopically in whole testis. As expected, the zinc supplemented rats tended to have more elevated zinc levels than non-zinc supplemented rats (Fig.4-3b). However, this trend did not reach statistical significance.

Zinc supplementation reduces CPA induced oxidative stress

The ability of zinc supplementation to protect male germ cells against CPA induced oxidative stress was examined by live cell imaging with a fluorescent probe for reactive oxygen species. Representative fluorescent images of cells from each treatment group are shown in Figure 4-4 (a-d). Pachytene spermatocytes from control

and zinc only animals displayed similar fluorescent signal that were not statistically different, indicating that zinc supplementation alone does not cause additional oxidative stress in these cells (Fig. 4-4e). The mean fluorescent signal from pachytene spermatocytes from CPA-treated animals was significantly increased above the signal from saline and zinc control cells by 43% and 51% respectively indicating that CPA treatment leads to elevated levels of reactive oxygen species in these cells. The mean fluorescent signal from cells from animals treated with CPA and supplemented with zinc was 34% lower than the fluorescent signal from CPA only cells. Furthermore, the fluorescent signal from the zinc supplemented cells was not significantly different from controls. These data indicate that zinc supplementation reduces CPA induced reactive oxygen species to levels that are similar to those in control, thus protecting pachytene spermatocytes from oxidative stress.

The effect of zinc supplementation on oxidative stress was also assessed in round spermatids by live cell imaging. Representative images of the cells from each treatment group are shown in figure 4-5 (a-d). The fluorescent signal from cells from the zinc control group was similar to that of the saline control cells, indicating that zinc does not cause oxidative stress in these cells. Similar to pachytene spermatocytes, CPA treatment caused a 23% and 25% increase in fluorescent signal above saline and zinc control levels (Fig. 4-5e). The fluorescent signal in round spermatids from zinc supplemented animals was reduced to 68% of the signal observed in cells from animals treated with CPA alone and was similar to levels found in controls. Although this trend is similar to what was observed for the pachytene spermatocytes, these results did not reach statistical significance.

Zinc supplementation protects male germ cells against CPA induced DNA damage

Cyclophosphamide alkylates DNA causing DNA double strand breaks and crosslinks[52]. This damage leads to the phosphorylation of histone variant H2AX (γH2AX) initiating a cascade of downstream events, including recruitment of proteins such as 53BP1, that culminates in the repair of the damaged DNA[53]. We examined the effect of CPA treatment and zinc supplementation on DNA damage and repair by immunofluorescent staining of seminiferous tubules for γH2AX and 53BP1.

Representative images of tubules stained for γH2AX from each treatment group are shown in figure 4-6 (a-d). The intensity of γH2AX staining differed between the 4 treatment groups (Fig. 4-6e). Fluorescent intensity was highest in tubules from animals treated with CPA and was nearly double that of the saline control and triple the zinc control. The number cells per tubule that were positive for γH2AX was also increased by over 1.5 fold in the CPA group compared to controls, indicating an increase in DNA damage after CPA treatment (Fig. 4-6g). Interestingly, γH2AX signal and the number of positive cells in the zinc control was only 60% that of the saline control, suggesting that zinc supplementation can reduce the endogenous levels of DNA damage. Most importantly, fluorescent signal for γH2AX in tubules from the zinc supplemented group was reduced to approximately 30% of the levels observed in CPA only group and was similar to the signal observed in the zinc control. The number of cells positive for γH2AX signal was also reduced 2-fold compared to CPA only.

To further analyze these findings, we staged the tubules based on the appearance PNA staining of the acrosome and grouped them into 4 categories: stages I-IV, V-VIII, IX-XI and XII-XIV (Supplemental Fig. S4-1). Representative images of γH2AX staining in the different stages in all 4 treatment groups are shown in supplemental figures S4-2 to S4-5. We observed a similar trend when the data were broken down into stages. The γH2AX intensity and number of cells positive for γH2AX signal were consistently highest in animals treated with CPA only across all 4 stage groupings (Fig. 4-6f and h). The zinc control also consistently had lower signal intensity and less positive cells compared to the saline control. Finally, across all 4 stage groupings, the γH2AX signal intensity and number of positive cells was reduced in the zinc supplemented group compared to CPA only, and similar to the saline and zinc controls. The highest intensity value for all four treatment groups was found in tubules that were stages XII-XIV. This corresponds to stages that contain spermatogonia, meiotic germ cells (zygotene, pachytene and secondary spermatocytes) and late step spermatids (12-14) [54]. The strongest staining was seen in cells along the basement membrane, suggesting damage to spermatogonia and early spermatocytes.

While these data display strong trends consistently indicating that CPA causes increase DNA damage that is decreased with zinc supplementation, the limited number of samples analyzed did not permit these trends to reach statistical significance.

The sizes of γH2AX foci are indicative of the level of damage : small foci indicate regular cellular functions whereas larger foci indicate elevated DNA damage from a toxic substance [55]. Thus, γH2AX foci volume was analyzed in sections from all treatment groups. Representative images are shown in Figure 4-7 (a-d). In both control

samples, the distribution of foci volume was similar, with a majority of the foci (82% for saline control; 77% for zinc control) falling between 0.1-10μm^3 (Fig. 4-7e and f). The average volume of γH2AX foci in the zinc control was approximately 6μm^3, slightly larger than the saline control which was around 4μm^3 (Fig. 4-7i). However, this difference is negligible compared to the differences observed in samples from CPA treated animals. The distribution of foci volume from CPA treated animals appears broader when compared to controls, with 59% of γH2AX foci falling in between 0.1 and 10 μm^3.(Fig. 4-7g). Most strikingly, 15% of γH2AX foci were over 30μm^3, while less than 1% in the saline control and only approximately 3% in zinc control were larger than 30μm^3. The average foci volume in CPA samples was 15μm^3 and significantly different from both saline and zinc controls. This indicates that CPA causes an increase in DNA damage in male germ cells. Zinc supplementation led to a γH2AX foci size distribution resembling those of controls (Fig. 4-7h). Similar to controls, the majority of foci (82%) fell between a volume of 0.1-10μm^3 and just over 1% of foci had a volume larger than 30μm^3. Additionally, zinc supplementation significantly decreased the average volume of foci over 3-fold compared to CPA treatment only (Fig. 4-7i). These results indicate that CPA treatment causes an increase in DNA damage in male germ cells that can be reduced with zinc supplementation.

To determine if there was any treatment effect on DNA repair we also assessed the expression of 53BP1 by immunofluorescence in seminiferous tubules. Similar to results for γH2AX signal, fluorescent staining for 53BP1 was highest in animals treated with CPA and increased over 1.5 fold compared to saline control and over 3 fold compared to zinc control (Supplemental Fig. S4-6e). The number of cells positive for the

marker was increased in CPA treated animals compared to controls (Supplemental Fig.S4-6g). In line with the previous results, the signal and number of cells positive for 53BP1 were reduced in the zinc control compared to the saline control. Zinc supplementation decreased 53BP1 signal to levels similar to control and to 40% of the signal observed from CPA treatment alone. The number of cells positive for the marker was also decreased by 23% in the zinc supplemented group compared to CPA alone. These results indicate that DNA repair activity is increased in response to CPA treatment and decreased with zinc supplementation.

Representative images of 53BP1 staining in the different stages in all 4 treatment groups are shown in supplemental figures S4-2 to S4-5. When the results are broken down by stage, the same trends remain across all stage categories, with 53BP1signal and positive cells being highest in the CPA treated (Supplemental Fig. S4-6f and h) . However, in contrast to γH2AX results, there does not appear to be any differences in 53BP1 expression between the groups of stages.

Co-localization of the γH2AX foci with 53PB1 signal was analyzed in order to relate the increase in DNA damage with the DNA repair response (Fig. 4-6l). In all treatment groups the majority of γH2AX signal co-localized with 53BP1, indicating DNA repair at the sites of damage. However the number of cells positive for γH2AX and 53BP1 was slightly lower in the two controls than in the CPA treated and zinc supplemented groups. Similar trends were observed when the data were broken down into different spermatogenesis stages (Fig. 4-6j, supplemental Fig. S4-2 to S4-5). Additionally, as with the 53BP1 staining, there were no differences between the stages in the number of cells that were positive for γH2AX and 53BP1. These results suggest

that DNA repair pathways are activated in response to DNA damage and not altered by CPA or zinc treatment.

Similar to the analysis of γH2AX fluorescence intensity, across all tubules and the different tubule stages, consistent trends were observed with 53BP1 fluorescence intensity and the colocalization of the two markers. These trends suggest DNA repair activity in response to CPA induced DNA damage. However these results did not reach statistical significance due to the limited sample size.

DISCUSSION

Zinc is an essential trace element that is important for normal cellular and physiological functions, including spermatogenesis. In the present study, male rats were treated with CPA and supplemented with 20 mg/kg of $ZnCl_2$. This dose has previously been shown to protect male germ cells against cigarette induced oxidative stress and improve sperm quality [44, 45]. Here we show that zinc supplementation protects male germ cells against oxidative stress and DNA damage caused by chronic low dose CPA treatment.

We have previously shown that chronic low dose CPA treatment has adverse effects on male germ cells and progeny outcome without any overt effects on the male reproductive system [17, 56]. Here we confirm these findings, showing that CPA treatment does not alter serum testosterone or testis and epididymis weights. In addition, daily treatment with zinc had no effect on reproductive parameters. Although CPA treatment with zinc supplementation led to a slight increase in epididymal weights,

this was not accompanied by an increase in sperm production. Our previous studies showed a decrease in testicular sperm counts after 9 weeks of chronic CPA treatment [17] which was not observed after this 4 week treatment period for any of the treatment groups. It is possible that, similar to the above mentioned studies with cigarette smoke, that zinc supplementation could improve sperm counts after 9 weeks of CPA treatment.

CPA treatment has been shown in many studies to increase levels of lipid peroxidation and decreased the expression of SOD, catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPX) in whole testis [11]. However the oxidative stress response in specific testicular cell types is less well known. We have previously shown that the expression of genes involved in the defence against oxidative stress is decreased in isolated pachytene spermatocytes and round spermatids after chronic low CPA treatment, indicating an impaired antioxidant status [57]. In the present study we show that CPA treatment causes increased levels of ROS in isolated pachytene spermatocytes and round spermatids. Furthermore, we show that zinc supplementation reduces levels of ROS to control levels, thus protecting male germ cells from oxidative damage. Other non-enzymatic antioxidants, including alpha-tocopherol-succinate (pro-vitamin-E)[58], lipoic acid [59, 60], ascorbic acid (vitamin C) [61], melatonin [62] and lycopene [63], and natural plant derived products, such ginseng [64] and green tea [65], have also been shown to protect male germ cells against CPA induced oxidative stress. The antioxidant activity of these compounds is different from zinc, and they act mainly by scavenging ROS and inducing the expression and activity of antioxidant enzymes.

The main mechanism of action of CPA is DNA damage via alkylation creating inter- and intra-strand crosslinks [66]. The phosphorylation of histone variant H2Ax is an

early marker of DNA damage and also plays an additional role in the formation of the sex body, as evidenced by the strong signal observed in sex chromosomes in spermatocytes [67]. Additionally, the sex body fails to form in H2Ax deficient mice leading to defects in meiotic pairing [68]. As expected, in our study we observed an increase in γH2AX signal, an early marker of DNA double strand breaks [53], in tubules from CPA treated animals compared to controls. This increased signal was the result from DNA damage caused CPA treatment as γH2AX signal in the sex body in spermatocytes was observed across all treatment groups. Furthermore, we analyzed the volume of the γH2AX foci: small foci indicate regular cellular function while larger foci indicate elevated damage from toxic substances [55]. We observed an increase in the volume of γH2AX foci in tubules from CPA treated animals indicating increased DNA damage from CPA treatment. An increase in large γH2AX foci has also been observed in the decondensing sperm chromatin in the paternal pronucleus of zygotes sired by CPA treated male rats [69, 70]. In addition to its role in the defence against oxidative stress, zinc is also essential for many DNA binding proteins including proteins involved in the DNA damage response such as XPA, RPA, PARP, OGG1, BRCA1, APE and P53 [29-32]. In the present study, we found that zinc supplementation in CPA-treated rats decreased γH2AX signal and foci sizes to levels similar to controls, indicating that zinc can protect male germ cells against CPA induced DNA damage.

DNA repair activity was examined by 53BP1 immunoreactivity. We that found DNA repair was active in all treatment groups but highest after CPA treatment. The number of cells that were positive for γH2AX and 53BP1 was highest in the CPA treated compared to the zinc supplemented and the control groups, suggesting that there is a

proportion of sites of H2AX phosphorylation in the zinc supplemented and control samples that do not recruit downstream DNA repair proteins. We propose that this difference is because the observed foci in these group are mostly due to normal cellular processes rather than detrimental DNA damage. In support of this, it has been shown that the smaller sized, normally occurring, γH2AX foci do not recruit DNA repair proteins, including 53BP1[55].

The protective effect of zinc against chemotherapy toxicity has also been shown in other studies. Zinc L-carnosine is a mucosal protective agent and was shown to decrease oxidative stress and inflammation in the bladder of mice treated with a single high dose of cyclophosphamide [71]. Zinc has also been shown to protect against cisplatin induced nephrotoxicity [72]. These studies indicate that zinc may not only play a protective role in preventing off target damage to the testis but also other tissues. Zinc, in the form of zinc acetate, also improves Sertoli cells function after cisplatin treatment [73]. Whether the CPA dose used in our study specifically affects Sertoli cells has not been investigated. However, acrolein, an active CPA metabolite, has been shown to alter Sertoli cell function [74]. The combination of antioxidants, including zinc, selenium and vitamins C and E, has been shown to improve sperm motility and morphology and decrease germ cell loss in rats treated with the chemotherapy cocktail bleomycin, etoposide and cisplatin [75]. Thus the use of antioxidants such as zinc may be useful in mediating some of the toxic side effects in patients undergoing chemotherapy. In one small study, zinc supplementation in conjunction with chemo- and radiation therapy in patients with advanced nasopharyngeal carcinoma was shown to decrease tumour reoccurrence and also improve patient survival [76]. Although more

mechanistic studies would be required, this suggests that zinc supplementation could help mediate some of the toxic side effects in patients without decreasing the efficacy of the drug treatment.

In conclusion, we have shown that zinc supplementation can reduce CPA induced oxidative stress and DNA damage. Whether zinc supplementation can also reverse the previously observed effects of paternal CPA treatment on sperm quality and progeny outcomes remains to be seen. However this study suggests that zinc may be useful as a supplement to include in treatment plans for men with cancer to help protect male germ cells.

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FIGURE LEGENDS

Figure 4-1. CPA treatment and zinc supplementation paradigm. Rats were divided into 4 treatment groups: Saline control (SAL/SAL): 0.9% saline x 5 weeks; zinc control (ZN/SAL): 20 mg/kg $ZnCl_2$ x 5 weeks; CPA treated (SAL/CPA): 0.9% saline x 1 week, then 6 mg/kg x 4 weeks; zinc supplemented (ZN/CPA): 20 mg/kg $ZnCl_2$ only x 1 week followed by 20 mg/kg $ZnCl_2$ and 6 mg/kg CPA x 4 weeks. Rats were treated 6 days per week by oral gavage.

Figure 4-2. Effects of CPA treatment and zinc supplementation on weights and sperm counts. The mean a) body weight; b) weight gained (percentage gain from starting weight); c) epididymis weight; d) testis weight; and g) testicular sperm count, are displayed (n=6) $p<0.05$

Figure 4-3. Effect of CPA treatment and zinc supplementation on testosterone and zinc level. The mean a) serum testosterone and b) testicular zinc levels are displayed. (n=6).

Figure 4-4. Zinc supplementation protects pachytene spermatocytes from CPA induced oxidative stress. Representative images from of live cells incubated with a fluorescent probe for ROS from a) saline control, b) CPA treated, c) zinc control and d) zinc supplemented. Quantification of fluorescent signal from images is shown in e). (n=6); Bar = 10μm; $p<0.05$.

Figure 4-5. Zinc supplementation protects round spermatids from CPA induced oxidative stress. Representatiive images from of live cells incubated with a fluorescent probe for ROS from a) saline control, b) CPA treated, c) zinc control and d) zinc

supplemented. Quantification of fluorescent signal from images is shown in e). Bar = 10μm; (n= 6)

Figure 4-6. Zinc supplementation decreases CPA induced DNA damage in male germ cells. Representative images for a) saline control, b) CPA treatment, c) zinc control and d) zinc supplemented. Quantification of γH2AX Intensity (e,f), number of γH2AX positive cells per tubule (g,h) and number of cells positive for γH2AX and 53BP1 (i,j) in all tubules (left) and per tubule stage (right). (n=3); Bar = 100μM

Figure 4-7. γH2AX foci volume are reduced with zinc supplementation. Representative images of foci in a) saline control, b) zinc control, c) CPA treated, and d) zinc supplemented and distribution of foci volume for each group (e-h). Average volume of foci is displayed in i). White arrow = small foci; yellow arrow = sex body; red arrown = large foci, blue arrow = foci in elongating spermatids. (n=3) Bar = 10μm; $p<0.05$

Figure S4-1. Staging of seminiferous tubules. Stages were determined by PNA staining of the acrosome and grouped into 4 categories. Adapted from Dym and Clermont (1970).

Figure S4-2. Immunofluorescent staining for γH2AX and 53BP1 in stages I-IV (Category 1). Bar = 40μm

Figure S4-3. Immunofluorescent staining for γH2AX and 53BP1 in stages V-VIII (Category 2). Bar = 40μm

Figure S4-4. Immunofluorescent staining for γH2AX and 53BP1 in stages IX-XI (Category 3). Bar = 40μm

Figure S4-5. Immunofluorescent staining for γH2AX and 53BP1 in stages XII-XIV (Category 4). Bar = 40μm

Figure S4-6. Zinc supplementation decreases the level of DNA repair in male germ cells. Representative images for a) saline control, b) CPA treatment, c) zinc control and d) zinc supplemented. Quantification of 53BP1 Intensity (d,f), number of 53BP1 positive cells per tubule (g,h) and in all tubules (left) and per tubule stage (right). (n=3); Bar = 100μM

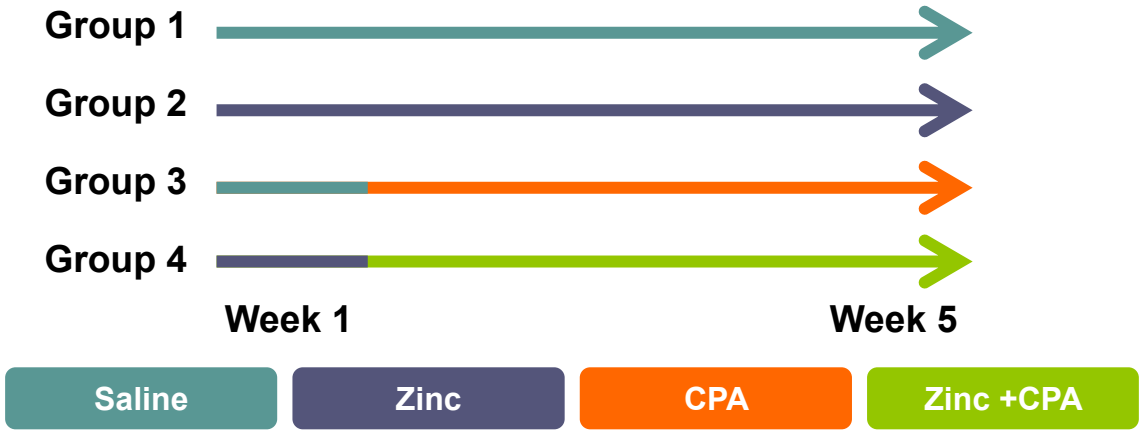


Figure 4-1. CPA treatment and zinc supplementation paradigm.

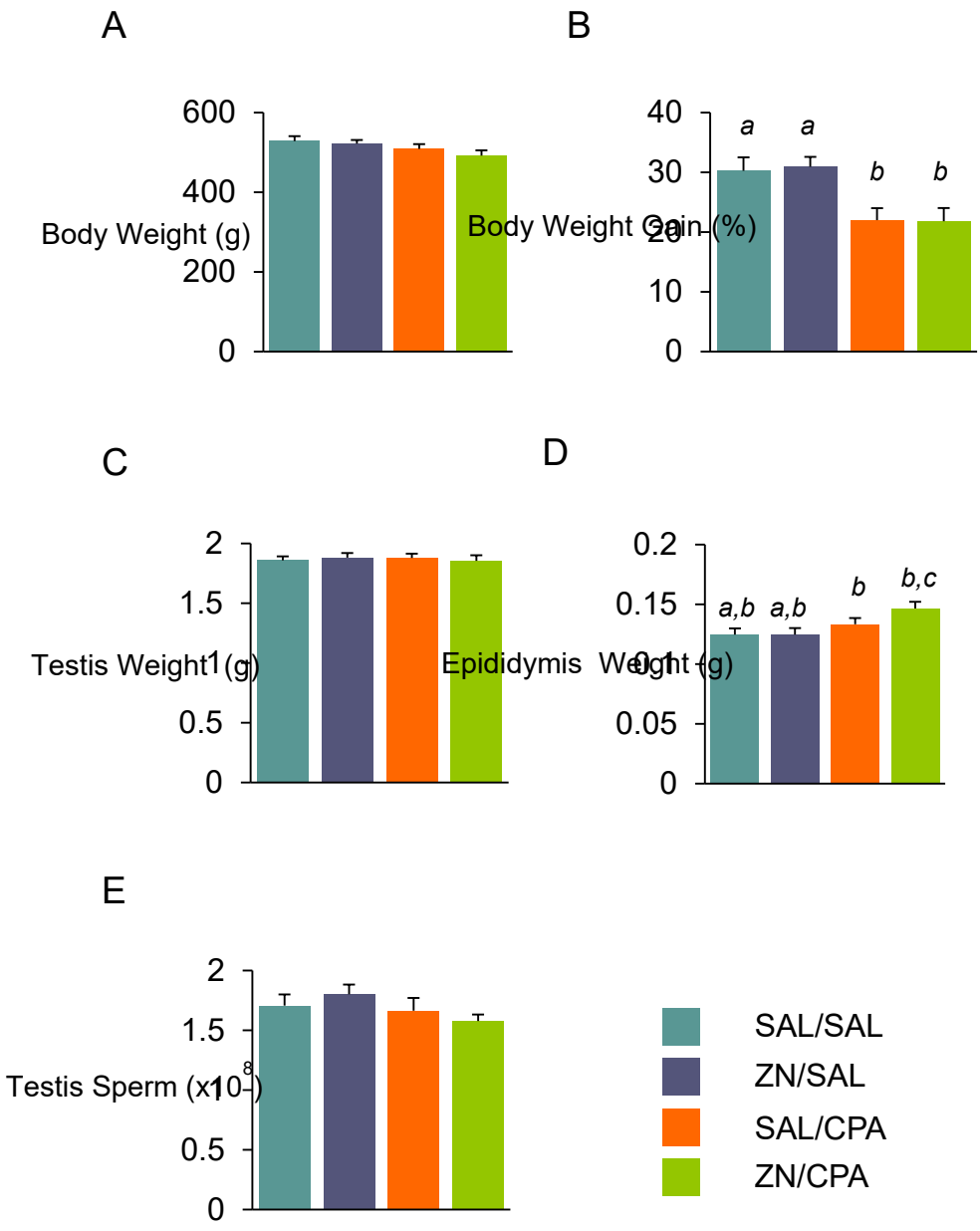


Figure 4-2. Effects of CPA treatment and zinc supplementation on weights and sperm counts.

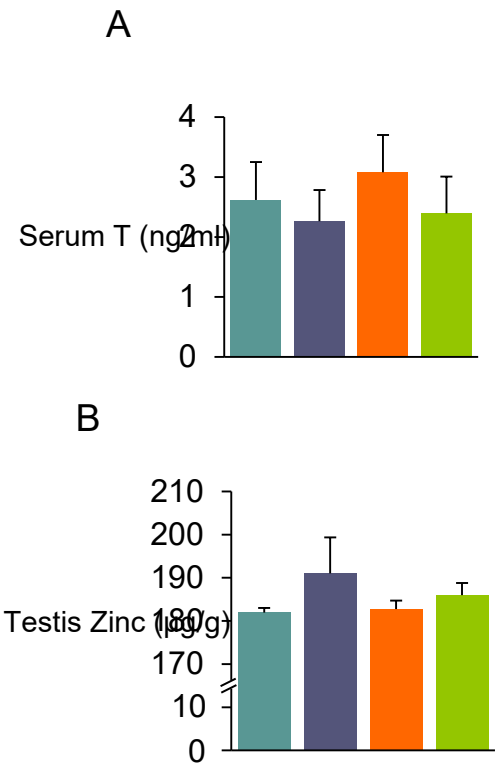


Figure 4-3. Effect of CPA treatment and zinc supplementation on testosterone and zinc level.

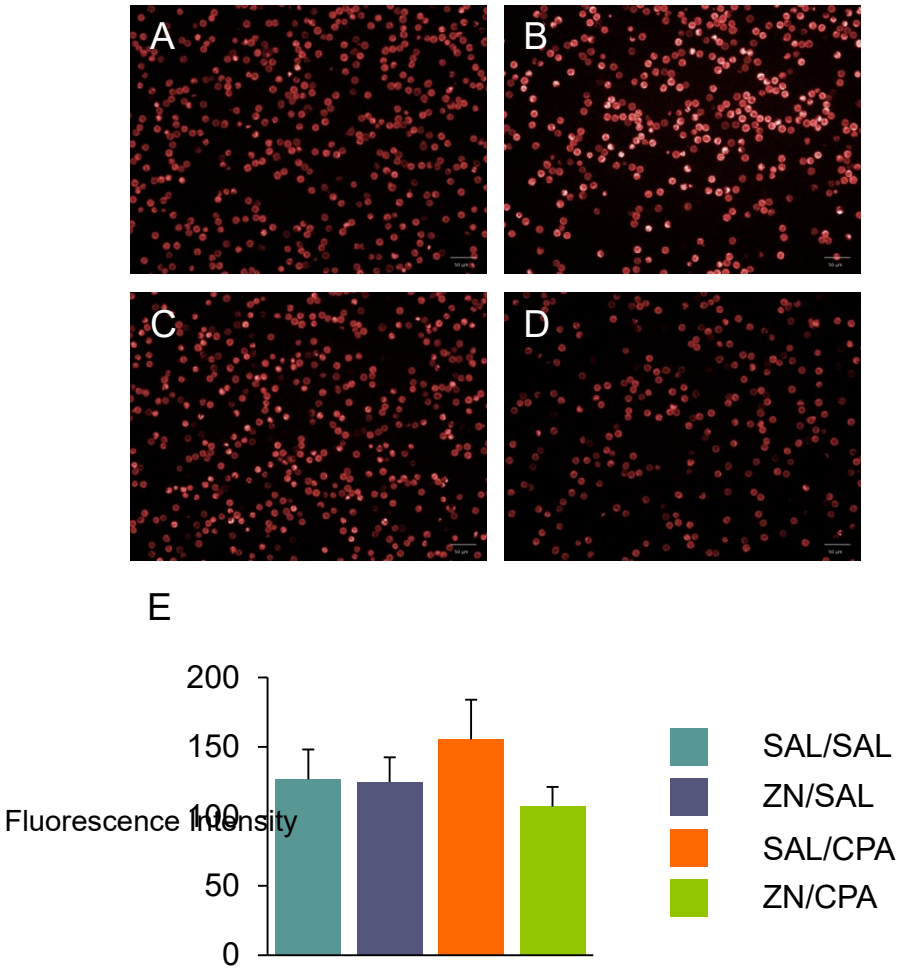


Figure 4-4. Zinc supplementation protects pachytene spermatocytes from CPA induced oxidative stress.

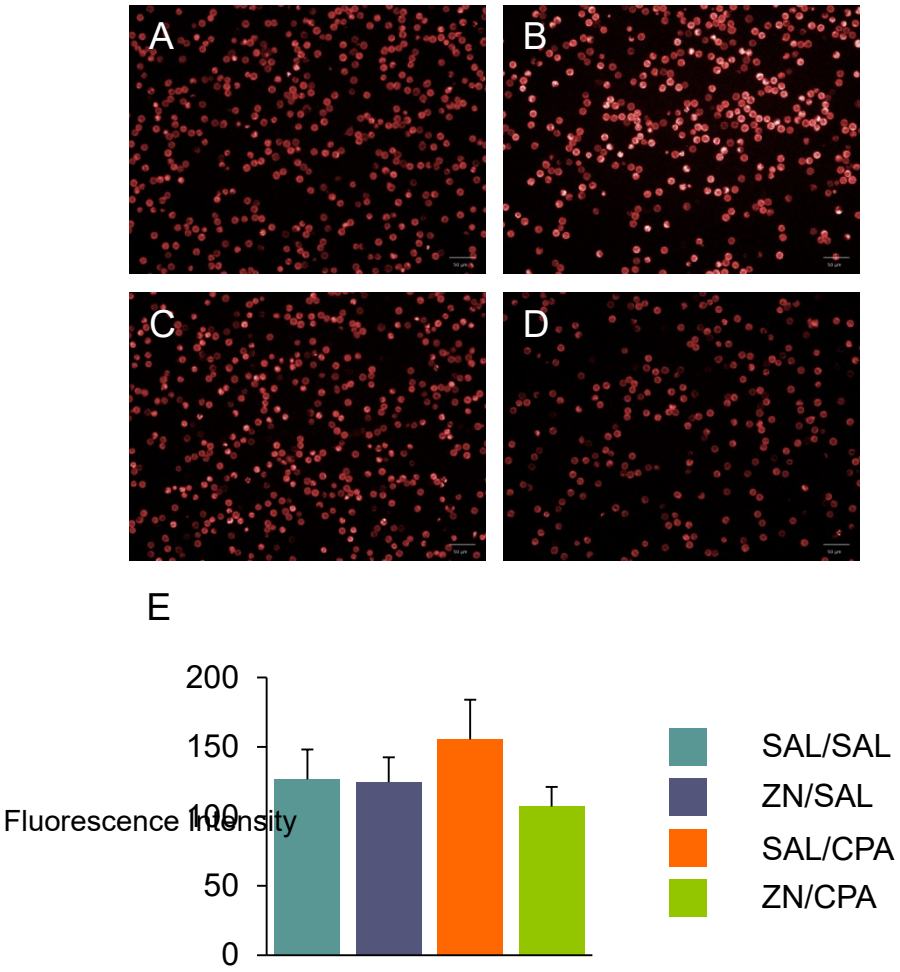


Figure 4-5. Zinc supplementation protects round spermatids from CPA induced oxidative stress.

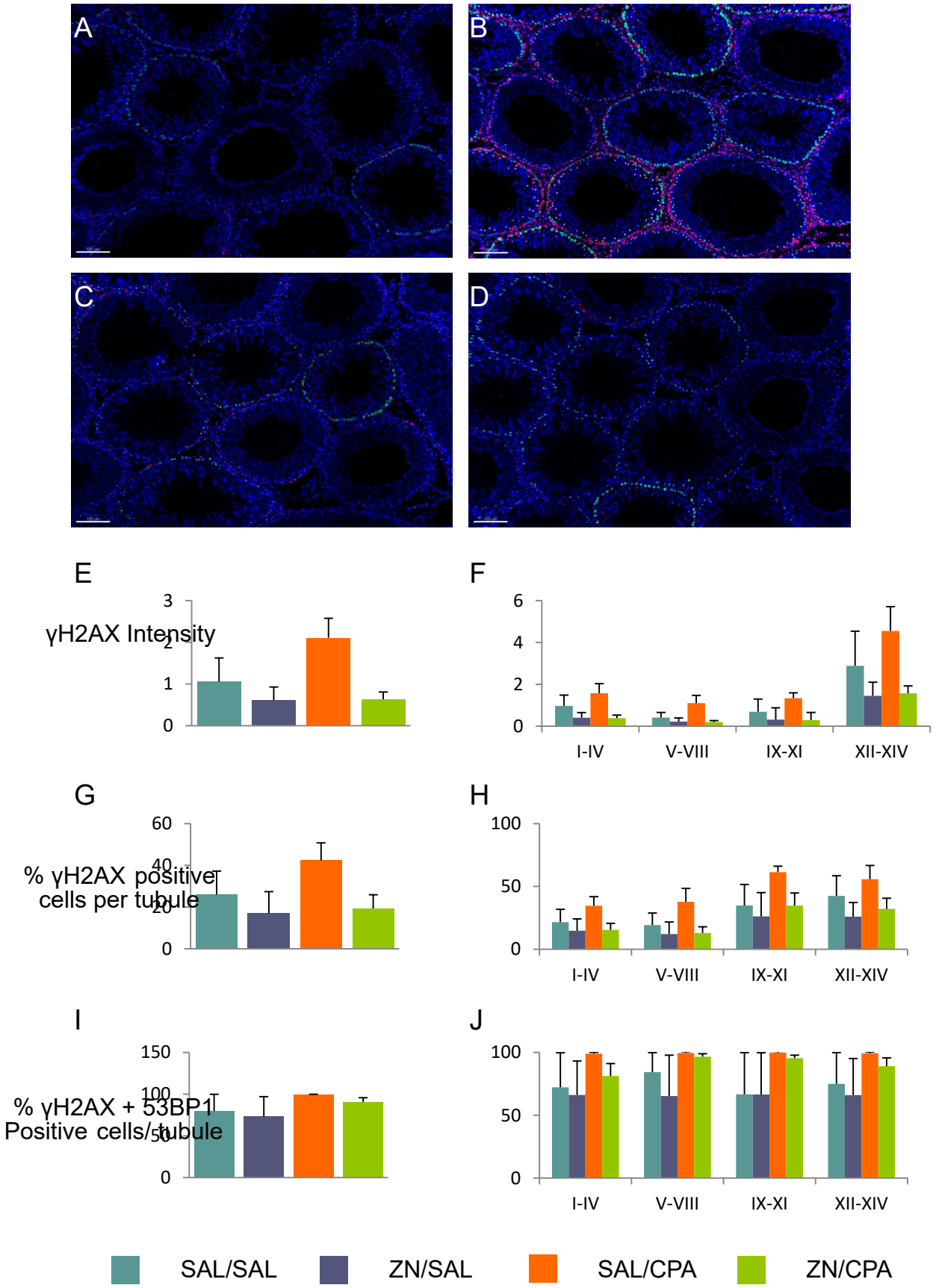


Figure 4-6. Zinc supplementation decreases CPA induced DNA damage in male germ cells.

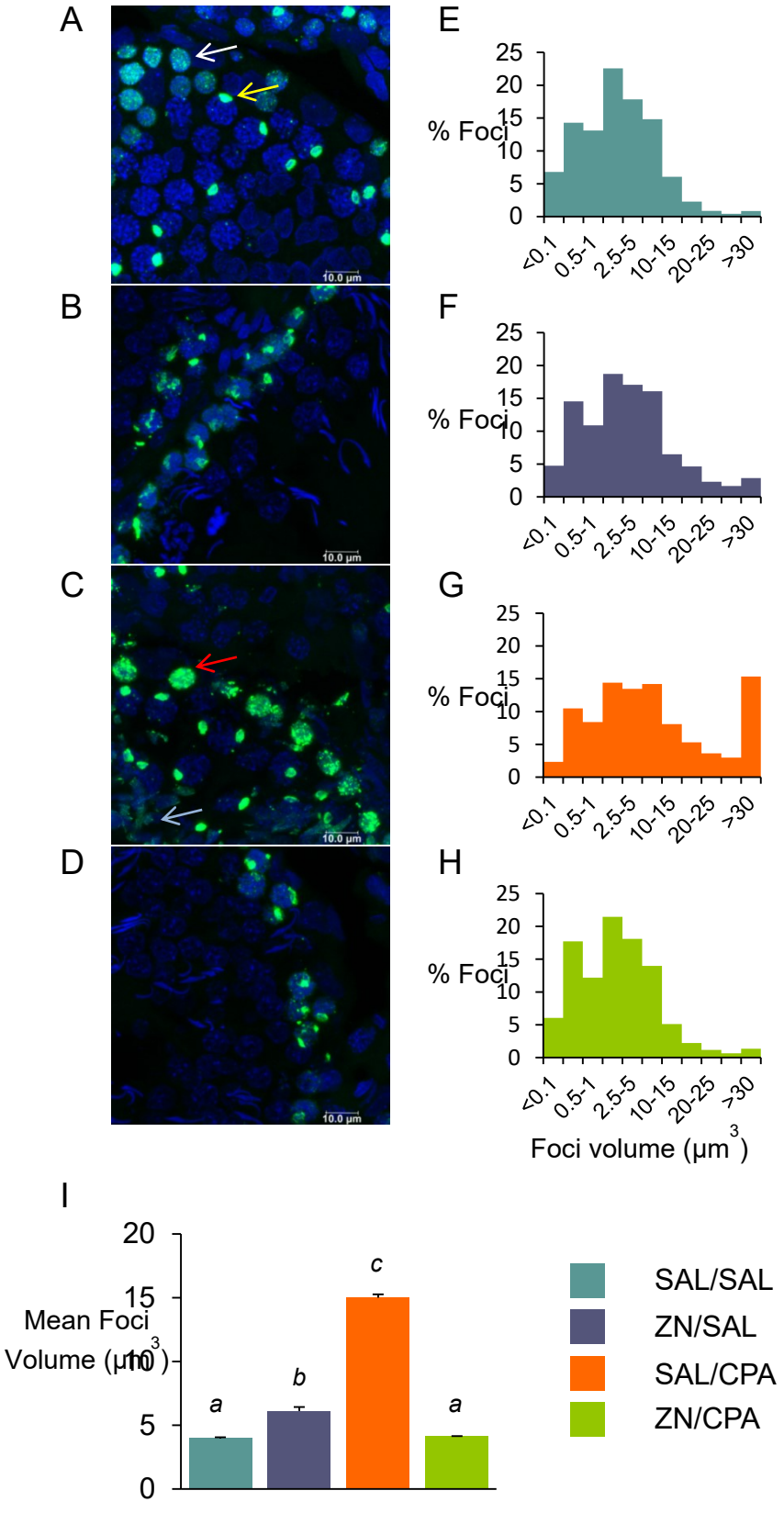
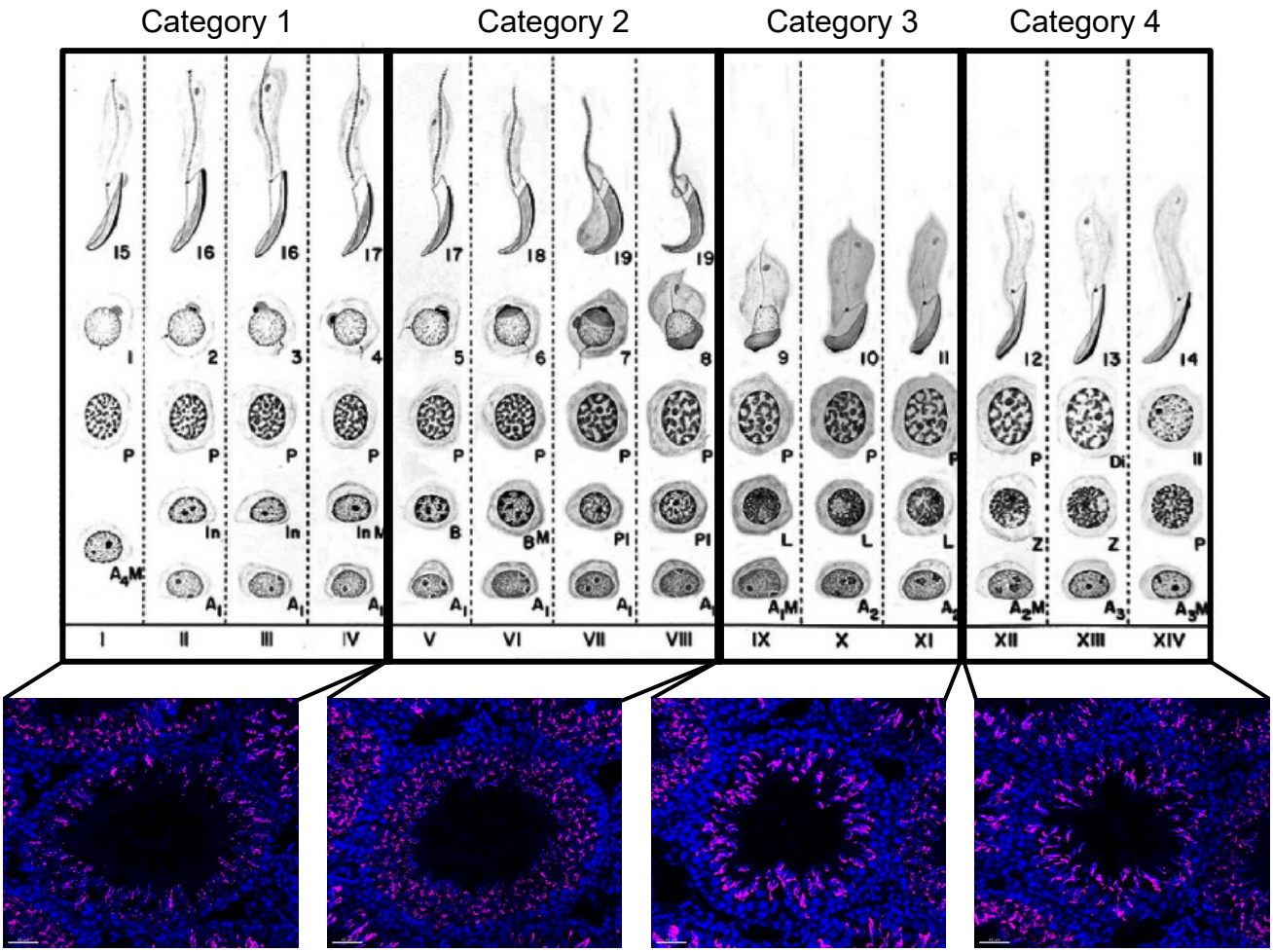


Figure 4-7. γH2AX foci volume are reduced with zinc supplementation.



Adapted from Dym and Clermont, 1970

Figure S4-1. Staging of seminiferous tubules.

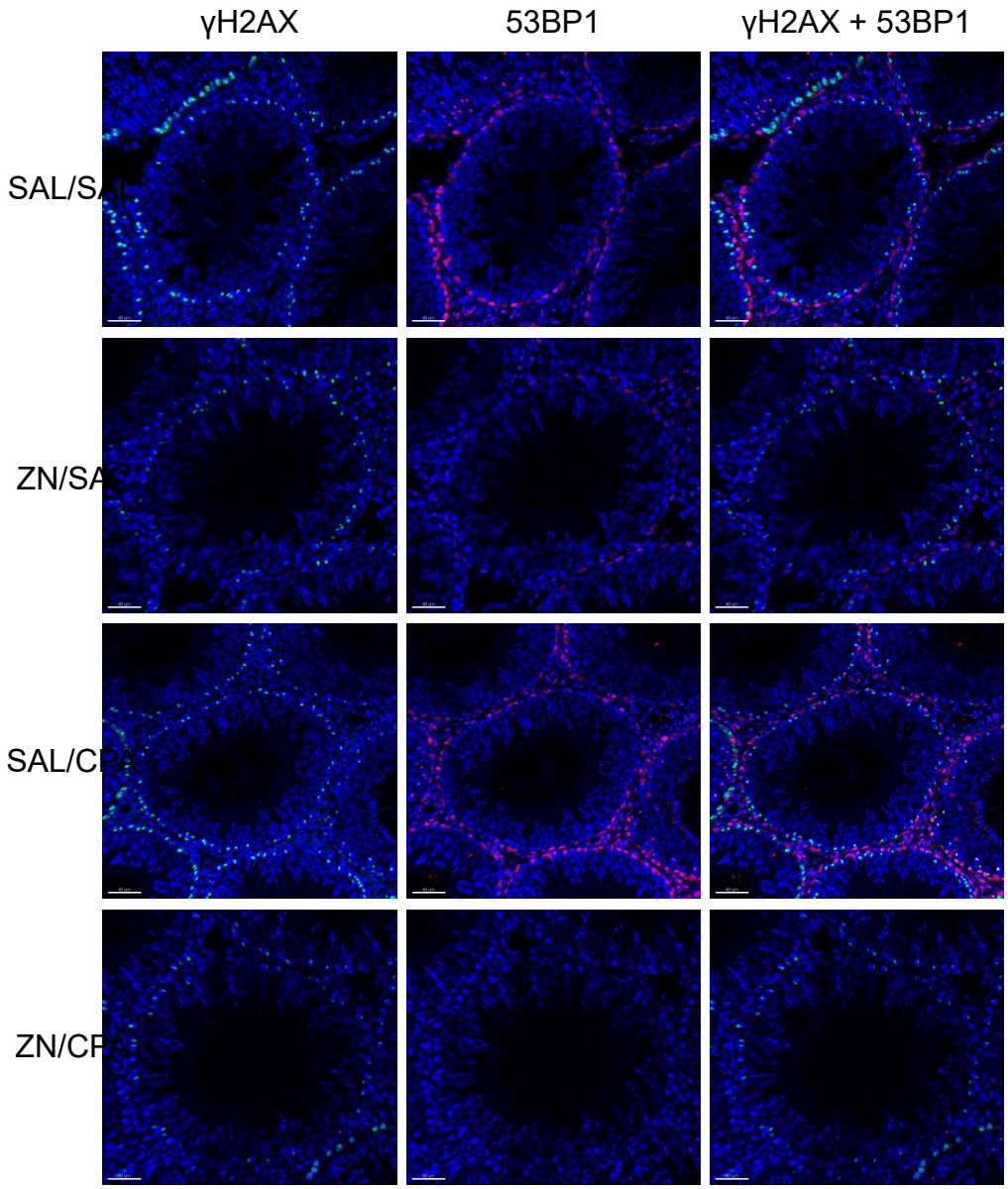


Figure S4-2. Immunofluorescent staining for γH2AX and 53BP1 in stages I-IV (Category 1).

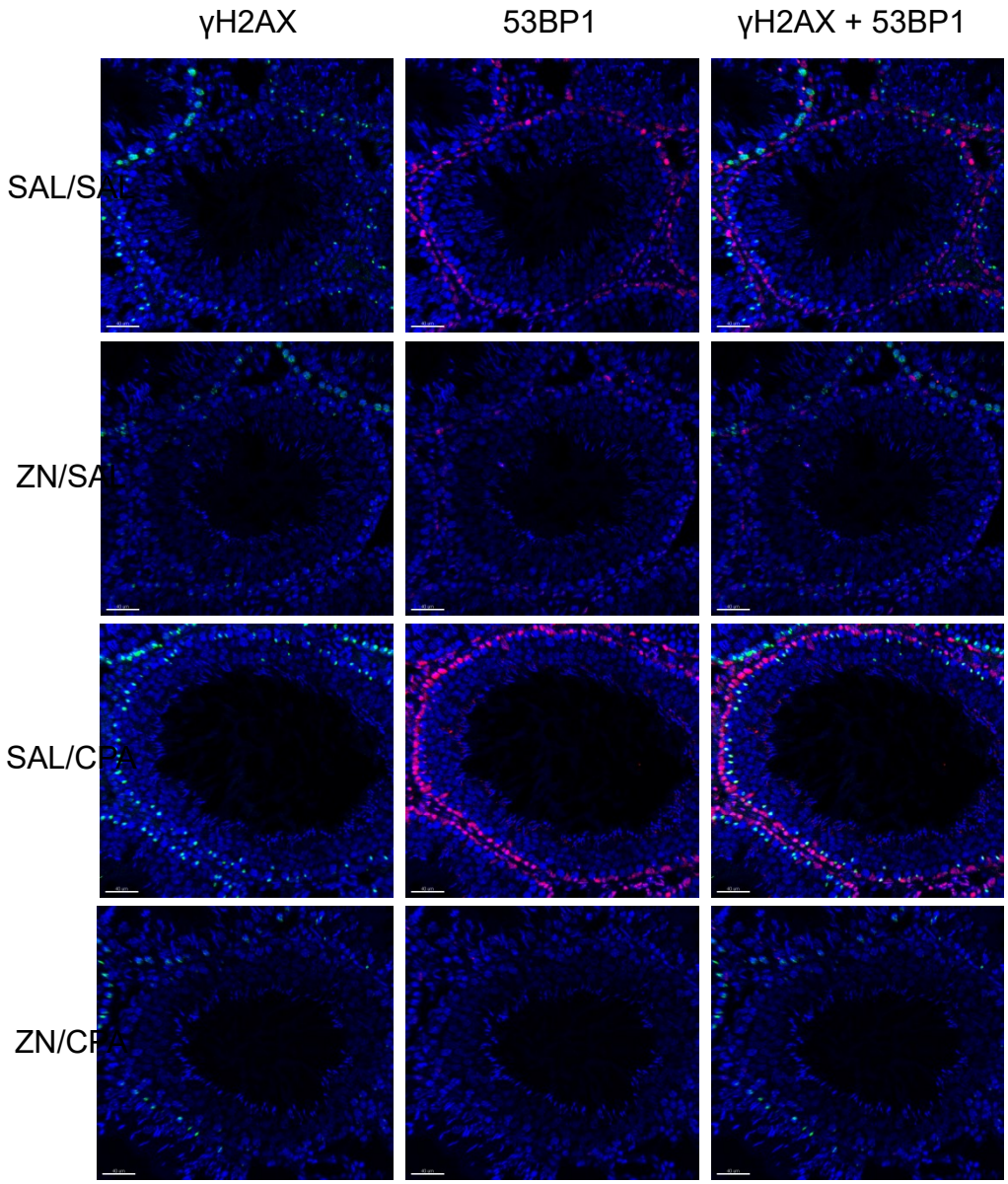


Figure S4-3. Immunofluorescent staining for γH2AX and 53BP1 in stages V-VIII (Category 2).

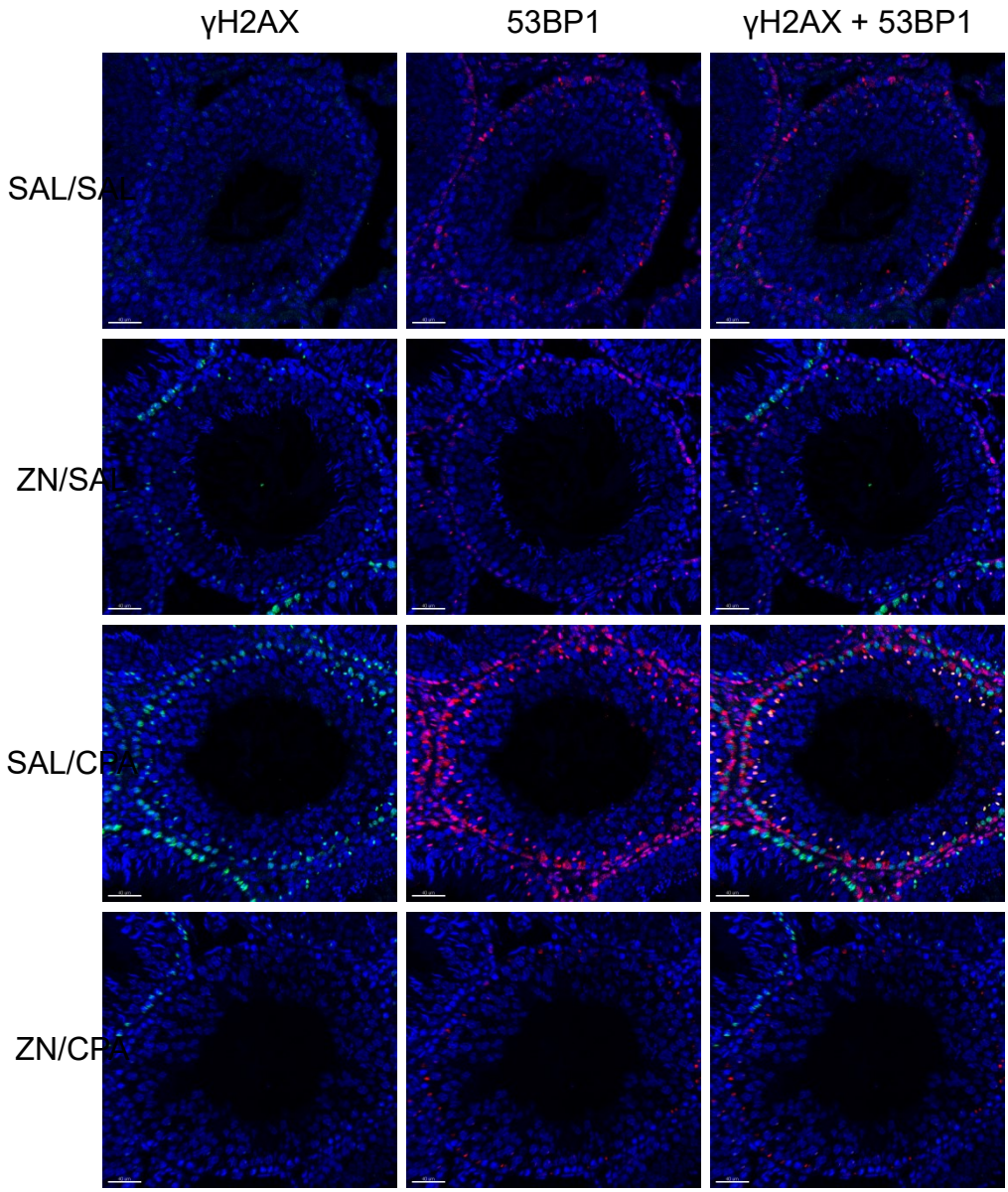


Figure S4-4. Immunofluorescent staining for γH2AX and 53BP1 in stages IX-XI (Category 3).

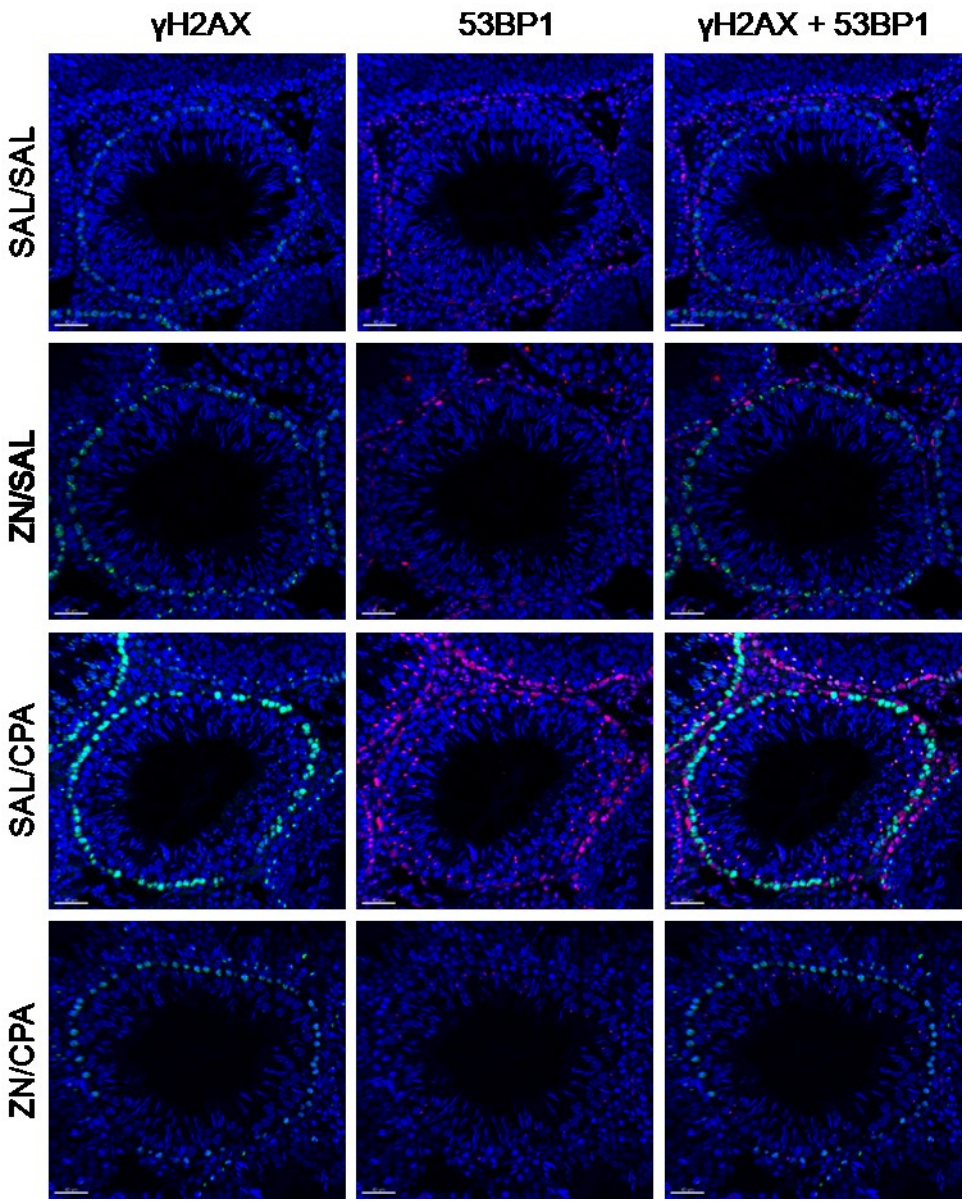


Figure S4-5. Immunofluorescent staining for γH2AX and 53BP1 in stages XII-XIV (Category 4).

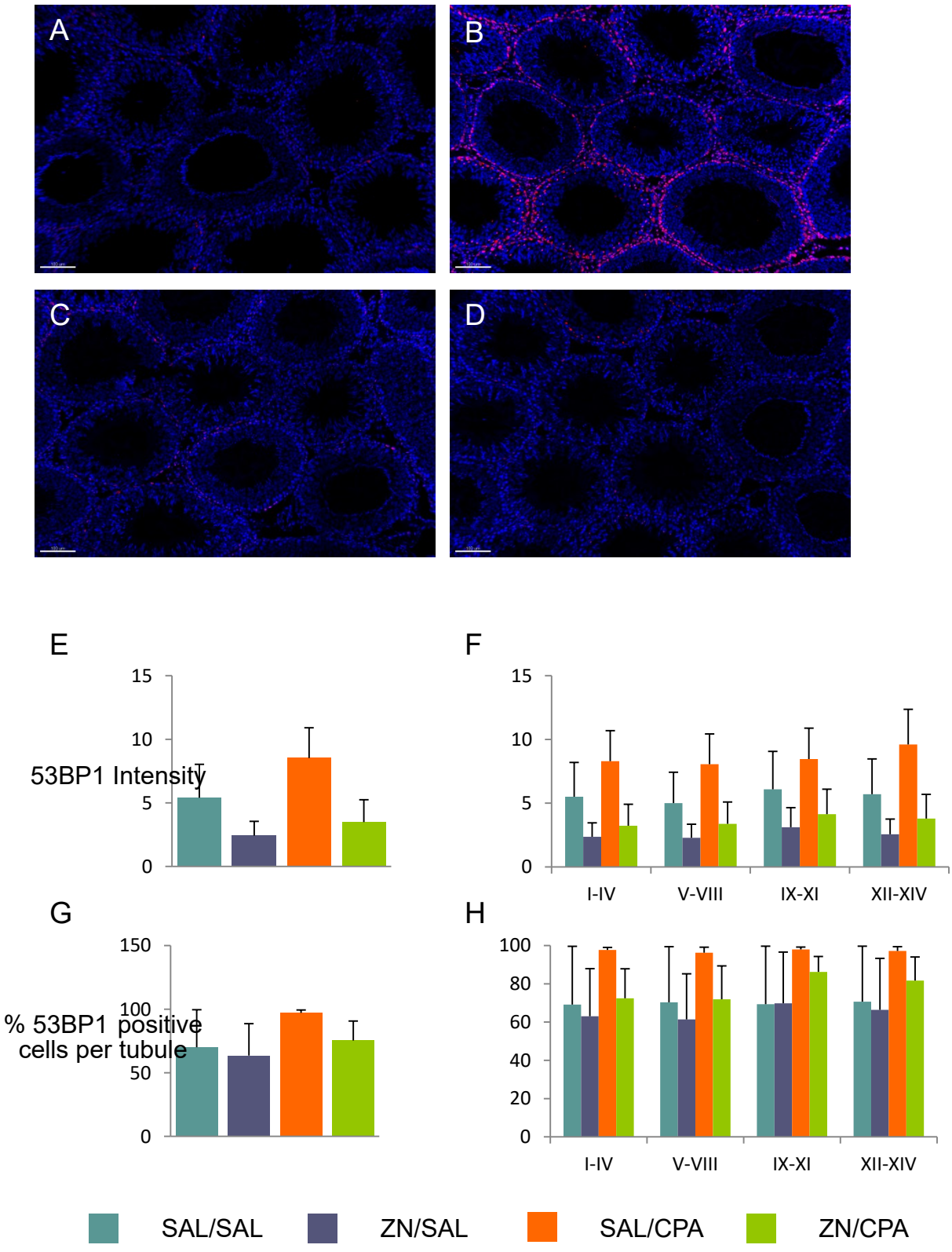


Figure S4-6. Zinc supplementation decreases the level of DNA repair in male germ cells

CHAPTER 5

DISCUSSION

5.1 Summary

The main objective of this thesis was to provide a greater understanding of the male germ cell response to the toxic effects of CPA. The first chapter introduced background information on several topics that are fundamental to this thesis, such as spermatogenesis, germ cell transcriptome, germ cell defense mechanisms and male mediated reproductive toxicology. Furthermore, this introductory chapter reviewed the current knowledge of the effects of paternal CPA treatment on male germ cells, embryo development and progeny outcome.

The work presented in this thesis began by profiling the transcriptome of male germ cells after chronic exposure to low dose cyclophosphamide treatment. In the second chapter of this thesis we describe the miRNA profiles in isolated pachytene spermatocytes and round spermatids, highlighting key differences in expression patterns between the meiotic and post meiotic germ cells. Importantly we also show that miRNA profiles are altered by CPA treatment. To our knowledge, this is the first study to 1) profile miRNAs in isolated rat pachytene spermatocytes and round spermatids and 2) examine the effect of a therapeutic drug on miRNA profiles in developing male germ cells. The altered miRNAs in both cell types were predicted to target transcripts involved in basic cellular function and proper germ cell development as well as processes important for the response to toxic agents. Interestingly, many miRNA targets in both cell types were also involved in zinc ion binding.

In the third chapter, whole genome gene expression profiles were examined in isolated pachytene spermatocytes and round spermatids. Previous studies have only

profiled a subset of stress response genes in isolated germ cells. We show that CPA treatment not only alters the expression of genes involved in stress response but also that of other unexpected genes that are involved in zinc binding and transport. Using live cell imaging and a fluorescent zinc probe, we also showed that zinc transport was increased in pachytene spermatocytes from CPA treated animals.

The common finding of miRNA targets and transcripts involved in zinc binding and transport from chapters two and three led us to the hypothesize that zinc may be playing an important role in the germ cell response to CPA exposure. Specifically, we propose that CPA treatment alters zinc requirements due to elevated levels of oxidative stress and DNA damage. In order to compensate, the expression of zinc transporters and zinc transport is increased in male germ cells. The observed changes in expression of miRNAs and transcripts related to zinc may reflect changes in zinc homeostasis. In the fourth chapter of this thesis we investigated whether zinc could alleviate the gonadotoxic effects of CPA treatment. We found that zinc supplementation protects male germ cells against CPA induced oxidative stress and DNA damage.

This final chapter will address some of the key findings presented in this thesis and discuss their significance as well as the clinical implications. Potential future research directions that further build upon the current findings will also be outlined. Additionally, concerns and limitations pertaining to the experiments and design will be considered.

5.2 Integrating miRNA expression into toxicogenomic studies

It is well established that miRNAs play a significant role in male germ cell development. Before exploring the effect of CPA on miRNA expression, we profiled miRNA expression in isolated pachytene spermatocytes and round spermatids from untreated Sprague-Dawley rats. The importance of this work is highlighted by the fact that previous studies have typically examined miRNA profiles in whole testes [1, 2], while a handful have looked at the expression of miRNAs in isolated germ cells [3-5]. The advantage of examining miRNAs in isolated germ cells is that unlike in whole testis experiments, we can relate miRNA expression to the unique processes that the different germ cells undergo during development. While the majority of previous studies were done in mice, we looked at miRNAs in the Sprague Dawley rat, the standard model for toxicology testing. Thus the data we generated provides baseline miRNA profiles that can be used to further understand the role miRNAs play in these two germ cell types during normal germ cell development and how they may be perturbed by exposures to toxic agents.

The finding that miRNA profiles differed between the pachytene spermatocytes and round spermatids is not surprising and reflects the different developmental stages of these cells. Our data indicate that pachytene spermatocytes and round spermatids utilise different miRNAs to regulate similar processes. It also suggests that the observed difference in miRNA expression might be involved in the differential response of the two cell types to CPA treatment. MicroRNA expression was altered in both pachytene spermatocytes and round spermatids after CPA treatment. Whether these altered miRNAs are the result of a specific adaptive response or general dysregulation due to a toxic insult is unclear.

Other studies have identified changes in miRNA expression in other tissues after exposure to toxic substances, including benzo(a)pyrene [6], alcohol [7, 8] , tobacco [9, 10], cocaine [11], cisplatin [12], valproic acid [13] and acetaminophen [14]. Some of the miRNAs associated with these toxic exposures were also altered in male germ cells in our dataset after CPA treatment. However, similarly to our work, specific roles for many of the miRNAs altered by these toxic exposures have yet to be identified. Various miRNAs have been identified as biomarkers for neurotoxicity, liver toxicity, kidney toxicity and cardiotoxicity (reviewed in [15]). Additionally, miRNA profiles have been examined to predict the outcome of chemotherapy for various cancers. For example, several miRNAs have been associated with large B cell lymphoma resistance to the combined chemotherapy R-CHOP (rituximab, CPA, doxorubicin, vincristine and prednisone) and are predictive of poor survival outcomes [16-18]. The dysregulated miRNAs that we identified in male germ cells after CPA treatment could also be used as biomarkers for male germ cell toxicity and used by the pharmaceutical industry and regulatory agencies to assess the gonadotoxic potential of drugs and chemicals.

Target prediction algorithms are useful tools to determine what transcripts a miRNA could regulate [19, 20] and what processes it could influence; in-vitro luciferase reporter assays can further validate the target prediction [21]. However, this simplistic prediction is complicated by a number of variables. First, miRNAs can target multiple transcripts and a given transcript can be targeted by multiple miRNAs [22]. Second, the predicted target mRNA must also be expressed in the same cell as the miRNA. Finally, miRNA targeting causes not only transcript translation repression and degradation but can also act in other ways such as regulating N^6-methyladenosine (m^6A) modifications of RNAs

[23]. This modification on mRNA can influence not only the transcript stability but also its localization within the cell [24]. Thus the predicted target transcripts of a miRNA may be modulated at the level of the transcript, protein or cellular localization. Surprisingly there was very little overlap between the predicted targets of CPA altered miRNAs and the differentially expressed mRNAs after CPA treatment. We propose that this could be the result of miRNAs acting at the translational level of gene expression. Therefore, proteomic studies should be undertaken to further understand how miRNAs that are altered by CPA treatment might influence gene expression.

To better understand the roles or consequences of CPA altered miRNAs, over-expression and knock-down experiments could be done and the effects on cell survival, DNA damage, meiotic progression and chromatin reorganization assessed. In cell lines or primary cultures of many cells, *in-vitro* over expression or knockdown can easily be accomplished using miRNA mimics and antagonists. However these types of studies are very challenging in male germ cells. Although efforts and advancements have been made, *in-vitro* spermatogenesis is still not possible [25]. Additionally, male germ cells do not adhere to the culture dish, making it difficult to properly wash the cells. Furthermore, the above techniques typically work in mitotically active cells. Although spermatocytes can be pushed to progress through meiosis [26], round spermatids do not divide. Finally, germ cells do not survive in culture for extended periods of time and survival is reduced under stressful conditions such as advanced age and oxidative stress [27]. These difficulties could be overcome by artificially over-expressing or knocking down miRNAs in-vivo by intra-testicular injection of miRNA mimics or antagonists. This method has successfully been used to assess the role of miR-124 in the biogenesis of

the acrosome in male germ cells [28] and miR-150 in steroidogenesis in Leydig cells [29]. Thus this method is suitable for examining miRNA function in both testicular somatic and germ cells. Future experiments to assess the roles of altered miRNAs on germ cells could include the use of intra-testicular injection of miRNA mimics and antagonists.

In our experiments, commercial whole genome microarrays were used to profile miRNAs. The advantage of using microarrays is the ease and affordability with which one can assess a large number of RNA transcripts. However, one disadvantage is that the expression profiling is limited to the probes that are present on the arrays. At the time of our miRNA profiling experiments, the microarrays used were based on miRBase release 16.0 and contained probes for the 677 miRNAs identified in the rat at the time. New miRNAs are being identified regularly and the miRBase miRNA database is updated to reflect these. We are currently at miRBase release 22.0 and 764 mature miRNA sequences have been identified to date in the rat. In contrast, 1978 miRNAs have been identified in mouse and 2654 miRNAs have been identified in humans [30-34]. Thus it is conceivable that the newly identified miRNAs that have not yet been identified in rat and that were not on our microarrays could also be altered by CPA treatment.

An alternative method to examine miRNA expression is RNAseq. With continuous technological advancement, RNAseq is becoming more affordable and the large data sets that these experiments generate more manageable. The advantage of RNAseq is that data obtained is not limited to currently identified transcripts [35]. New miRNAs as

well as other non-coding RNA species can be identified based on the sequences and the structures these transcripts are predicted to form.

These other non-coding RNAs (piRNAs, lncRNAs, circRNAs, endo-siRNAs and tsRNAs) have all been identified in the testis and could potentially be affected by CPA treatment. The ability of a drug to modulate the expression of these RNA species in male germ cells has not been examined. In contrast, paternal diet has been shown to alter tsRNA expression in mature spermatozoa in mice [36, 37]. Similarly, sperm miRNAs can be altered by paternal diet and stress [38-42]. These altered sperm born tsRNAs and miRNAs can transmit the dietary and stress phenotypes. Extraordinarily, the simple injection of tsRNAs or miRNAs isolated from the sperm of male mice fed high fat/low protein diets or chronically stressed into control zygotes was capable of recapitulating the dietary and stress phenotypes [36, 38, 40, 41]. Future studies to understand how chronic paternal CPA treatment leads to the negative embryo development and progeny outcomes we have previously observed should include the investigation of altered sperm born miRNAs and other RNA species such as tsRNAs.

5.3 Gene expression in male germ cells: profiling the response to toxic exposures

Male germ cells display distinct gene expression profiles that reflect their stage of development [43-47]. These differences in gene expression indicate that different germ cells may display different susceptibilities to toxic exposures. For example, previous studies have shown that spermatids are most susceptible to the damaging effects of

CPA treatment [48-50] while spermatogonia are most susceptible to the effects of radiation [51]. Indeed, the expression of stress response genes has previously been shown to differ between pachytene spermatocytes, round spermatids and elongating spermatids [52]. The expression of stress response genes differed between these three cell types following acute and chronic CPA treatment [53, 54]. Similarly, gene expression profiles differ between different cancers and even between patients with the same type of cancer, and have been used to predict the susceptibility and resistance of different cancers to chemotherapy, including CPA [55-59]. Additionally studies have shown that the development of chemo-resistance is accompanied by changes in gene expression profiles. For example, the development of CPA resistance in prostate tumours is accompanied by changes in expression of genes involved in coagulation [60]. Large databases have been created to curate all the data that have been generated from these types of studies in order to facilitate the correlation of differences in gene expression signatures with diagnosis, prognosis and prediction of therapy sensitivity and resistance [61-64].

In chapter 3, we profiled whole genome gene expression in pachytene spermatocytes and round spermatids. We found that both cell types displayed distinct gene expression profiles following CPA treatment. Many of these differentially expressed genes were involved in processes important for the response to CPA treatment such as the response to stress, response to DNA damage, DNA repair, regulation of cell death, and spermatogenesis. It would be interesting to compare the gene expression results from our study with the available data in the databases mentioned above. The information obtained from such a bioinformatic analysis could

provide additional information on the mechanisms of action of CPA, and the differential response of male germ cells to the toxic effects of CPA treatment. Furthermore, this information could also help predict the effect of other chemotherapeutic agents on male germ cells.

Strikingly, we found that CPA treatment has an apparent repressive effect on gene expression in pachytene spermatocytes. In a previous study, the expression of stress response genes was also decreased in male germ cells after chronic CPA treatment [53]. The reason behind this is unclear. Indeed DNA crosslinks and damage would be expected to interfere with transcription. GO analysis of the altered genes and targets of the altered miRNAs revealed that CPA affects genes involved in transcriptional regulation. The changes in zinc binding proteins and potential effect of CPA treatment on zinc homeostasis (discussed in chapters 3 and 4 and below) may also affect transcription.

Interestingly, the DNA lesions induced by the bifunctional alkylating agents nitrogen mustard, melphalan, chlorambucil and cisplatin have been shown to result in *in-vitro* transcription termination [65, 66]. Conversely the cyclophosphamide derivative 4-S-(propionic acid)-sulfidocyclophosphamide did not have the same effect [65]. However, RNA synthesis was decreased by CPA in an in-vitro tumour system, and differed between CPA sensitive and resistant tumours [67]. CPA functions by not only alkylating DNA but also by alkylating proteins creating DNA-protein crosslinks [68, 69]. Proteomics analysis in human fibrosarcoma cells exposed to the active CPA metabolite phosphoramide mustard has revealed that a large number of the proteins that form the DNA-protein crosslinks are involved in transcriptional regulation, mRNA/RNA

processing and chromatin organization [70]. Thus alkylation of these proteins may contribute to the decrease in transcription that we observed. Interestingly, other chemotherapeutic agents have been shown to affect components of the transcriptional machinery. The DNA intercalating drug doxorubicin down regulates the expression and inhibits the activity of RNA polymerase II [71, 72]. In addition, bleomycin reduces RNA synthesis [73] and mediates the cleavage of a variety of RNAs including mRNAs [74]. How CPA treatment or other chemotherapeutic agents affect transcription in male germ cells is unknown.

Our results indicate that transcription may be more affected in pachytene spermatocytes than in round spermatids. The reason for this is unclear. Future experiments to assess transcription in male germ cells need to be done. It will be interesting to assess if zinc supplementation reverses the effect of CPA on gene expression through zinc binding proteins either by microarray or RNAseq. The ability of CPA to affect RNA synthesis could be assessed and compared in isolated pachytene spermatocytes and round spermatids using commercially available Click-It RNA labelling kits to monitor the incorporation of 5-ethynyl uridine (EU). Additionally, modification of these kits allows the capture of nascent RNAs for further analysis of gene expression. Proteomic analysis of CPA induced DNA-protein crosslinks in isolated germ cells to assess whether CPA is directly affecting transcriptional machinery by alkylation could further shed light on the effect of CPA on gene expression. Additionally, these experiments could also indicated whether DNA-protein crosslinks are altering chromatin remodelling events that occur in male germ cells during meiosis and spermiogenesis.

Finally, as mentioned in the above section about miRNA expression, the effect of CPA treatment on mRNA profiles in mature sperm should be assessed. Other toxic substance such as carbendazim (a fungicide) and 2,5-hexanedione (a component of gasoline) have been shown to alter the mRNAs contained in sperm from rats [75]. Additionally, studies have shown that mRNA (and miRNA) profiles are different between fertile and infertile and smoking and non-smoking men [76-81](and reviewed in [82]). While roles for sperm born RNAs in embryo development are widely unknown, altered RNA signatures could be used as biomarkers for testicular toxicity. These signatures could be used in drug development and in clinical practice to predict pregnancy outcomes.

5.4 Zinc: An essential trace metal for the male germ cell response to CPA?

The advantage of using transcriptomics approaches is the possibility of uncovering novel mechanisms in the cellular response to toxic agents such as CPA. It is not surprising that paternal CPA treatment altered the expression of genes involved in stress response mechanisms such as DNA repair, oxidative stress and heat shock proteins in male germ cells [53]. Unexpectedly, using a whole genome profiling, we found CPA treatment altered the expression of many transcripts that code for proteins involved in zinc binding and transport.

Zinc is an essential trace metal that is found ubiquitously in the body [83]. Importantly, male reproductive tissues display high concentrations of zinc [84]. Physiologically, zinc is necessary for growth and development, lipid metabolism and

normal brain, immune and reproductive function [85]. The importance of zinc at the molecular level is evident as over 10% of proteins are zinc binding [86]: these include more than 2000 transcription factors and other DNA binding proteins as well as 300 enzymes that require zinc to function properly [87].

Cellular zinc levels need to be tightly controlled, and this is accomplished by two families of zinc transporters: The ZIP (Slc39a) and ZnT (Slc30a) families. ZIP members function in zinc influx into the cytoplasm from extracellular spaces [88] and intracellular organelles while ZnT members function in zinc efflux from cell and sequester cytosolic zinc into intracellular organelles [89].

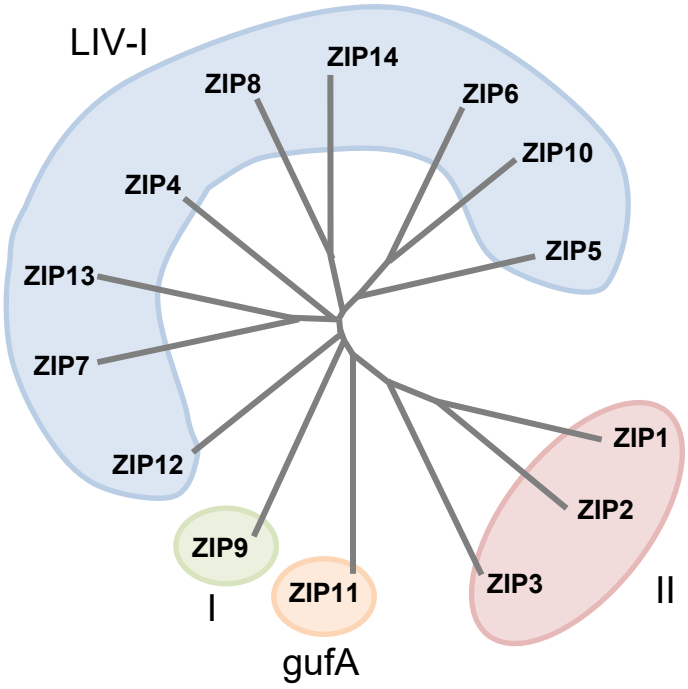
Relative to other tissues, especially the brain, knowledge about these transporters in the testis is sparse [90-93]. Considering the importance of adequate zinc levels for proper spermatogenesis [94-100], this is surprising. The work presented in this thesis is significant because it is the first to examine the expression of all members of the ZIP and ZnT zinc transporters the testis let alone isolated pachytene spermatocytes and round spermatids. We found that members from both these families are expressed in both cell types to different degrees. The significance of the levels of expression of individual transporters and of the observed differences in expression between the cell types is unclear.

At the structural level, ZIP transporters contain eight transmembrane domains with the N- and C- termini facing the extracellular space. Histidine-rich loop domains are present between transmembrane domains 3 and 4. The particularly amphipathic transmembrane domains 4 and 5 are thought to form the cavity through which zinc is

transported. The conserved residues of these regions are believed to be crucial for zinc transport function [101]. The 14 ZIP transporters are subdivided into 4 classes based on their sequences (Figure 5-1) [88, 102]. ZIP 9 is the sole member of subclass I. Subclass II contains ZIPs 1, 2 and 3. ZIP2 is only found in mammalian cells suggesting that it is the most recently evolved ZIP member [103]. The sole member of GufA subclass, ZIP11 family appears to be most ancestral ZIP and contains less histidine residues than other ZIP members [103].The majority of the mammalian ZIP transporters (ZIP 4,5,6,7,8,10,12,13 and 14) are members of the LIV-1 subclass which contain additional histidine residues and a highly conserved putative metalloprotease motif.

The ZnT family of zinc transporters are composed of six transmembrane domains, with N- and C- terminals facing the intracellular space [88]. The conserved histidine/serine rich loop between transmembrane domains 4 and 5 is thought to be important for zinc binding [104]. The 10 ZnT members can be classified into 4 subfamilies based on their sequence (Figure 5-1) [89]: subclass I contains ZnT 5 and 7, subclass II contains ZnT2,3,4 and 8, subclass III contains ZnT1 and 10 and subclass IV contains ZnT6 and 9 [102].

Mammalian ZIP Transporters



Mammalian ZnT Transporters

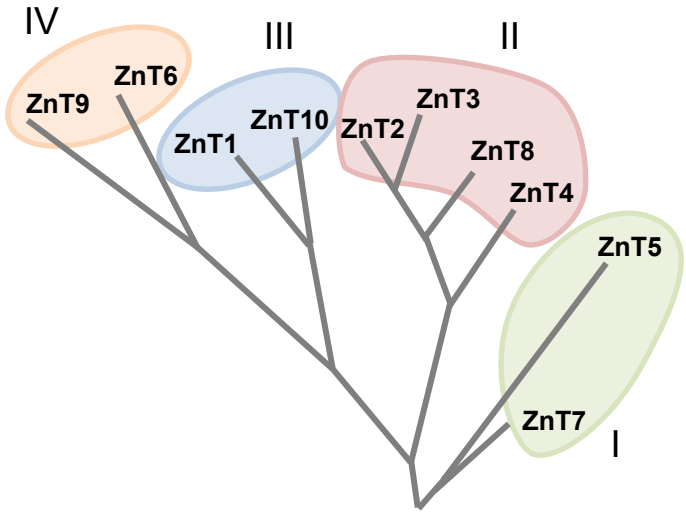


Figure 5-1. Phylogeny and classification of the mammalian ZIP and ZnT zinc transporters. ZIP family can be classified into 4 groups: I (green), II (red), gufA (orange) and LIV-I (blue). ZnT family members can be classified into 4 groups: I (green), II (red), III (blue) and IV (orange). Adapted from Jeong et al (2013) [88] and Huang et al (2013) [89].

The subfamily classifications of both ZIPs and ZnTs do not appear to have any influence of the localization or function of these zinc transporters (Figure 5-2). The purpose of the expressing multiple members of these transporters in male germ cells is unknown. Indeed certain members were more highly expressed than others. However there was no enrichment of any subfamily of ZIPs or ZnTs in either pachytene spermatocytes or round spermatids. The expression of the different members of the ZIP and ZnT members may reflect the localization of different members to specific cellular compartments (figure 5-=2) and be related to specific roles. In support of this idea is the finding that residues that are upstream of the first transmembrane domain in ZnTs usually contain subcellular targeting signals and vary greatly between family members [89].

Knock out models of different ZIP and ZnT family members have failed to show any fertility phenotypes (reviewed in [88, 89]), suggesting functional redundancy for many of these transporters. To gain further insight into the role zinc transporters in male germ cells, investigation into the subcellular localization of ZIPs and ZnTs are needed as well as proper characterization of the consequences of ZIP and ZnT knockout on germ cell development.

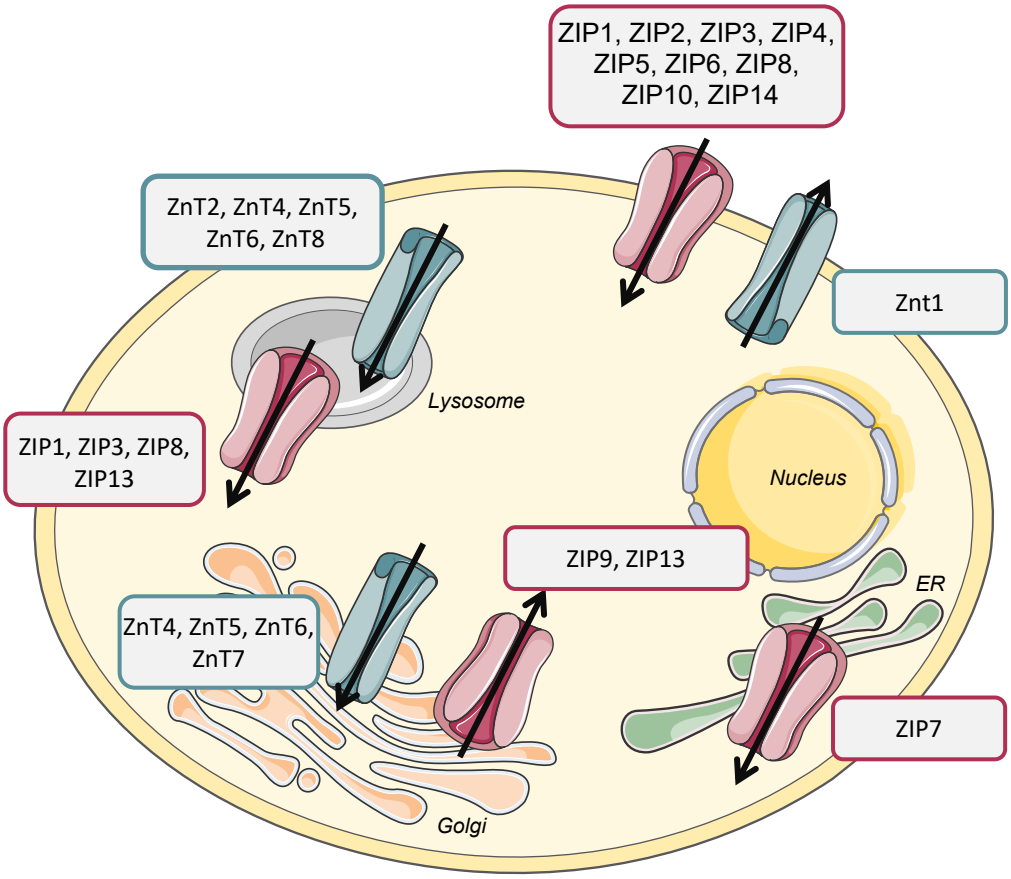


Figure 5-2. Cellular localization of members of the mammalian ZIP and ZnT zinc transporters. ZIP family members (red transporters) increase intracellular zinc levels by importing zinc from the extracellular space or releasing it from organelles. ZnT family members (blue transporters) decrease intracellular zinc by exporting zinc out of the cell or sequestering it into organelles. Members of both the ZIP and ZnT families have been localized to the plasma membrane, lysosomes, Golgi and endoplasmic reticulum (ER). Figure was created in part using images from Servier Medical Art (www.servier.com) , licensed under a Creative Commons Attribution 3.0 Unported License and adapted from Jeong et al (2013) [88] and Huang et al (2013) [89].

We found that chronic CPA treatment altered the expression of four members of the ZIP family of zinc transporters in pachytene spermatocytes: ZIP5, ZIP6, ZIP 13 and ZIP14. This is the first evidence that zinc transporter expression and zinc transport can be influenced by a drug and involved in stress response. Previously, the expression of some ZIP transporters, including ZIP6 and ZIP10 in the testis [91], has been shown to be influenced by zinc status (reviewed in [88, 105]). In yeast, this is mainly through binding of ZAP1, a transcriptional activator, to the zinc responsive element (ZRE) in the promoter of zinc related genes [106, 107]. Additionally, aging [108], hormones (testosterone, prolactin and thyroid hormone) [109-111], cadmium [112], cigarette smoke [113] , glucose and inflammation [114] have been shown to alter ZIP transporter expression.

The expression of metallothioneins, another group of important regulators of cellular zinc concentration, were not altered in either pachytene spermatocytes or round spermatids after CPA treatment. These cysteine rich proteins have the ability to bind and release zinc via the thiol groups,and can thus act as a sink or a source of intracellular zinc.

We hypothesised that male germs cells take up more zinc in order to protect themselves from CPA treatment by acting as an antioxidant and a cofactor for DNA repair proteins. An additional and perhaps alternative mechanism could be through signalling pathways. In support of this theory, ZIP14 has been shown to control G-protein coupled receptor-mediated signaling in the growth plate, pituitary gland and liver [115]. Zinc has been shown to play important roles in intracellular signaling by acting as a second messenger [116], inhibiting the activity of enzymes, such as caspases [117,

118] and MAPK [119], and modulating signaling pathways including NF-κB [120, 121]. Thus increased zinc uptake in male germ cells after CPA treatment might be affecting germ cell survival through intracellular signaling. To investigate this possibility, the activity of caspases and MAPK and NF-κB signaling pathways could be assessed in male germ cells after CPA treatment with and without the zinc chelator TPEN.

An important question is by which mechanism CPA is regulating zinc transporter expression. Since both ZIP5 and ZIP14 are zinc responsive [88], it is possible that CPA treatment led to a reduction in zinc concentration resulting in increased ZIP expression. However, we did not see a significant decrease in testicular zinc concentrations after CPA treatment. Since we observed altered ZIP expression specifically in pachytene spermatocytes, it is possible that CPA treatment reduced zinc concentrations in a cell specific manner. Therefore analyzing the zinc concentration in different germ cell types instead of the whole testis could be useful. This could be accomplished in testis sections using laser ablation-inductively coupled plasma mass spectrometry (LA-ICP-MS) or X-ray fluorescence microscopy (XRFM) which allows mapping of the distribution of zinc ions in tissues with sub-cellular spatial resolution (reviewed in [122]).

Interestingly, ZIP14 is a predicted target of miR-497, a miRNA that we observed was down-regulated in pachytene spermatocytes after CPA treatment. ZIP5 has been shown to be targeted by miR-328 and miR-193a and predicted to be a target of miR-30b-3p [123]. After CPA treatment, none of these miRNAs were altered. However miR-328a*, which is predicted in-silico to target ZIP5 but to a lesser extent [123], was significantly up-regulated after CPA treatment in pachytene spermatocytes. Additionally, closely related miR-30d and miR-30a* were altered after CPA treatment. Thus miRNAs

altered by CPA treatment may be involved in modulating changes in ZIP transporter expression. The luciferase reporter gene assays and the use of miRNA mimics and antagonists as outlined in the previous section could be used to examine this relationship.

Changes in zinc transporter expression and zinc transport as a protective and adaptive response in male germ cells to CPA treatment merit more investigation. Future studies could include the investigation of whether the loss of the up-regulated transporters in male germ cells abolishes the CPA increase in zinc uptake and intensifies the toxic effects of CPA treatment, including DNA damage, oxidative stress and cell death. Whether other ZIP transporters might be dysregulated to compensate for this loss could also be assessed. The effect of over expression of ZIP transporters on germ cell response to CPA treatment could also be examined. Similar to what was described in the previous section, these studies could be accomplished using in-vivo gene transfer and RNAi based techniques. Intra-testicular injection of DNA constructs has been shown to result in successful integration into the germ cell genome [124] and robust expression of transgenes [125]. Alternatively, germ cell specific K.O. and transgenic animals could be generated. With the arrival of CRISPR technology, the generation of animals, including rats, with genetically modified male germ cells is now possible [126].

5.5 Oncofertility: on the quest to protect male germ cells during chemotherapy

Human exposures to environmental and therapeutic toxicants, especially chemotherapeutic agents, during germ cell development are contributing factors to male subfertility [127-129]. Classic chemotherapeutic drugs were developed on the basis that the vast majority of normal cells are quiescent and that rapidly dividing and metabolically active cells, such as cancerous cells, are particularly sensitive to perturbations in DNA synthesis [130]. Unfortunately, this selectivity (or lack thereof) means that frequently dividing normal cells, such as hair follicles, myelopoietic bone marrow cells, intestinal epithelial cells and male germ cells are also targeted by chemotherapeutic agents. Additionally, the chromatin remodelling and transcriptional quiescence that occur in the later steps of spermatogenesis make the male germ cells especially vulnerable to DNA damaging agents [131].

Oncofertility merges the fields of oncology and reproductive research in order to provide and develop options for the reproductive future of cancer patients. As outlined previously in Chapter 1, methods to preserve fertility in male cancer patients are currently limited to sperm cryopreservation [132]. Thus simple methods that could help mitigate some of the toxic effects of chemotherapy on male germ cells are needed.

The unexpected findings that CPA treatment altered 1) the expression of miRNAs with predicted targets involved in zinc binding; 2) the expression of mRNAs that code for zinc binding proteins and transporters; and 3) zinc transport in male germ cells led us to investigate whether zinc supplementation could protect male germ cells from CPA. The results from Chapter 4 revealed that zinc supplementation reduced levels of oxidative stress and DNA damage induced by chronic low-dose CPA treatment. The specific mechanisms by which zinc supplementation reduces CPA induced oxidative stress and

DNA damage were not investigated. However there is sufficient evidence in the literature about the cellular and molecular roles of zinc to speculate on how it may protect male germ cells against CPA treatment. As described in chapter 1, zinc can act as an antioxidant through several different mechanisms [133-137]. Moreover, zinc deficiency causes elevated levels of oxidative stress and oxidative damage in the testis [100].

Another important regulator of intracellular zinc levels and oxidative stress is the family of metallothieniens. These cysteine rich proteins have the ability to bind and release zinc via the thiol groups, and can thus act as a sink or a source of intracellular zinc [138]. Additionally, metallothioneins are thought to have important ROS scavenging abilities [139, 140]. The expression of transcripts for metallothioneins was not altered after CPA treatment. However, it is possible that the protective effect of zinc supplementation on CPA induced ROS is due, at least in part, to metallothioneins, as zinc in an important regulator of these proteins. This possibility merits further investigation.

In addition to its role as an antioxidant, zinc plays a structural role in many proteins, most notably zinc finger proteins (reviewed in [141]). Zinc finger proteins are most often DNA binding and include not only a large number of transcription factors but also components of the DNA repair machinery. For example, the DNA damage repair proteins XPA and RPA of the NER pathway [142], OGG1 [143] and PARP1 [144] of the BER pathway and BRCA1 of the homologous repair pathway [145] all contain zinc binding motifs. The importance of zinc for DNA repair has been shown by the increase in DNA damage [146, 147] and decrease in the activity of some of these enzymes, such

as PARP [148, 149], in zinc deficient models. Importantly, zinc repletion is rapidly able to reverse these effects [147].

The tumour suppressor protein p53 plays an important role in regulating the events that lead to DNA repair (reviewed in [150]) and also male germ cell death [151-153] by binding specific DNA sequences to transcriptionally activate downstream targets. The DNA-binding domain of p53 is stabilized by the tetrahedral coordination of a zinc ion [154, 155]. Zinc depletion has been shown to reduce p53 DNA binding activity by altering the stability and the DNA-binding domain conformation [147]. Additionally, the ability of zinc to restore p53 conformation and activity of mutant p53 in cancer cells in-vitro and in-vivo [156, 157] has led to the development of a new class of drugs termed zinc metallochaperones, to re-establish chemo sensitivity in cancers with p53 mutations [158, 159]. These drugs act by binding extracellular zinc, diffusing across the plasma membrane and releasing it intracellularly thereby increasing intracellular zinc [160].

Despite finding a protective effect of zinc supplementation, we did not observe a decrease in zinc concentration in the testes of CPA treated rats. It is possible that rather than deplete zinc levels, zinc availability in germ cells is decreased due to an increased demand in response to CPA insult. More studies are needed to tease apart specific mechanisms of the protective role of zinc. These studies would include investigating the anti-oxidant activity of zinc by measuring malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) levels, bi-products of lipid peroxidation, 8-oxodG and 8-oxoG, markers of DNA and RNA oxidative damage, as well as Cu/ZnSOD activity in male germ cells.

Our immunofluorescent studies indicated increased DNA damaged after CPA treatment, especially in spermatogonia and early spermatocytes, and that the level of damage was reduced with the addition of zinc. However, changes in zinc transporters and transport were observed in isolated pachytene spermatocytes and the protective effects of zinc in oxidative stress were observed in both pachytene spermatocytes and round spermatids. Thus it would be of importance that DNA damage levels be directly assessed in these cell types. The single cell gel electrophoresis (COMET) assay can be used to evaluate DNA damage at the level of the individual cells [161]. We have previously employed the COMET assay to evaluate mature sperm integrity [50], but the use of this assay on the developing germ cells will provide additional information on the susceptibility of germ cell types to CPA damage and further support a role for zinc and zinc transport in protecting male germ cells from DNA damage. The ability of zinc to decrease DNA damage by improving DNA repair activity could be addressed by measuring the activity of DNA repair enzymes OGG1 and PARP1 and DNA binding capacity of p53 in germ cells treated with CPA and supplemented with zinc.

In addition to mechanistic studies, more extensive studies are required to evaluate the full potential of zinc in protecting male germ cells from CPA induced damage and preventing the negative progeny outcomes that have previously been described. A longer treatment period would need to be done to evaluate the effect of CPA treatment and zinc supplementation on a full cycle of spermatogenesis in rats. A similar study to the original studies done over 30 years ago to evaluate chronic CPA treatment on the male reproductive system and pregnancy outcome is necessary [48, 49]. In this proposed study, male reproductive organ weights (testis, epididymis, prostate, seminal

vesicles), reproductive hormones (testosterone, LH, FSH), testicular sperm counts, sperm motility and morphology and testicular histology would be assessed at the end of a 9 week treatment period. Throughout the treatment period, mating studies would be undertaken at different time points to evaluate effects of zinc supplementation in different cell types as done previously. This would include evaluation of mating activity, pre-implantation loss, post-implantation loss and evaluation of live born pups.

An important addition to these mating studies will be the evaluation of the sperm chromatin quality after zinc supplementation. The importance of zinc in sperm chromatin structure and the detrimental effect of CPA treatment on chromatin condensation and integrity was described in Chapter 1. Several available assays could be used to examine the potential of zinc supplementation in improving sperm chromatin quality after CPA treatment. The sperm chromatin susceptibility assay (SCSA) evaluates sperm chromatin integrity using FACS by measuring the susceptibility of sperm DNA to acid denaturation using the DNA stain acridine orange that fluoresces green when associated with double stranded DNA and red when associated with single stranded DNA [162]. This technique has previously been used to demonstrate the detrimental effects of zinc deficiency and CPA on sperm chromatin integrity [163, 164]. The level of compaction of sperm DNA can be assessed using the fluorescent chromomycin A3 staining (CMA3) and monobromobinane thiol labelling (mBBr) assays which indicate the amount protamine present and the extent to which the protamines are cross-linked by di-sulfide bonds [165, 166]. These have previously been used to assess the effects of CPA on mature sperm [164]. The COMET assay mentioned above and used previously will also be useful to directly evaluate the ability of zinc supplementation to

prevent CPA treatment to induced DNA strand breaks in mature sperm [161]. Additionally, since zinc supplementation was able to decrease CPA induced oxidative stress in germ cells, the level of oxidative DNA damage can be assessed by immunofluorescent staining for 8-oxodG in mature sperm.

A limitation in our zinc supplementation study was the use of a single concentration of zinc. A dose response pilot study would be necessary to determine the optimal concentration of zinc that will protect male germ cells from CPA toxicity without causing any systemic toxicity. Zinc is considered to be relatively non-toxic. Zinc toxicity occurs at high doses (above 100-300 mg/day in adults) and can lead to gastrointestinal upset, headaches, copper deficiency and impaired immune function [167]. Interestingly, zinc toxicity in the testis of mice results in similar effects as zinc deficiency: germ cell death, arrest of spermatogenesis and decreased sperm count [168]. The dose given in our study is well below the maximum tolerated dose in rats (over 250 mg/kg of diet) [169] and also below the concentrations which are gonadotoxic [168]. Finally, different types of zinc supplements or delivery methods are available. Zinc sulfate ($ZnSO_4$), zinc acetate ($Zn(OAc)_2$) and zinc picolinate (ZnPic) have also been used in animal models to protect against cisplatin toxicity [170, 171] and inflammatory conditions [172, 173]. Another interesting option would be to examine the effectiveness of the new zinc metallochaperone drugs that help transport zinc across membranes [158, 160].

5.6 Integrating zinc supplementation into clinical practice

The use of complementary or alternative medicines (CAM), including supplements, is widespread. It is estimated that as much as 20-50% of cancer patients use some form of CAM depending on the country [174-176]. Canada and the United States have some of the highest rates of CAM use [174]. Patients resort to CAM as an approach to exhaust all possibilities in search of a successful treatment or in the hopes of reducing some of the toxic side effects associated with chemotherapy [177]. An important question to consider is whether supplements used with chemotherapy can antagonize the anti-cancer effects of these drugs. Currently, the use of supplements, predominantly antioxidants, with chemotherapy is controversial. Although there are no specific guidelines, both the American and Canadian Cancer societies and the National Cancer Institute advise patients to discuss the use of any supplements with their physicians [178-180].

Many clinical trials have been carried out assessing the use of different antioxidant supplements with chemo- and radiotherapy (reviewed in [181]). Many of these supplements show potential in reducing chemo- and radio-therapy induced toxicities. For example, several studies have indicated that coenzyme Q10 (CoQ10) is capable of reducing cardiotoxicity in patients receiving different combinations of chemotherapy, including fluorouracil, doxorubicin, daunorubicin, cyclophosphamide and vincristine, and radiation therapy [182-184]. A few trials have examined the use of selenium supplementation. They showed a decrease in nephrotoxicity and bone marrow suppression in patients treated with cisplatin [185] and a decrease in gastrointestinal upset, loss of appetite, weakness and hair loss in patients that received combination chemotherapy (doxorubicin, CPA, vincristine and prednisolone) [186] or radiation

therapy [187, 188]. With the exception of one clinical trial that showed an increased rate of cancer reoccurrence and mortality in patients who were smokers while undergoing radiotherapy and being supplemented with α-tocopherol and β-carotene [189], the vast majority of trials have not reported any adverse effects associated with antioxidant supplementation. Clinical trials that are designed to examine the effect of antioxidant supplementation on clinical outcome and survival are rare. The trials that have looked at clinical outcomes and survival in patients receiving supplements (vitamin A, vitamin E and ellagic acid) saw no significant difference between the supplemented and control groups [190-193]. The exception is melatonin, a potent antioxidant that plays various roles in regulating circadian rhythms, sleep, aging and tumour growth [194]. Clinical trials in patients receiving various chemotherapeutic agents (including cisplatin, etoposide, mitoxantrone, fluorouracil, doxorubicin, CPA, vincristine, gemcitabine, oxaliplatin and paclitaxel) have shown that melatonin not only decreases myelosuppression and neurotoxicity but also improves the clinical response to chemotherapy, increases tumour regression and increases overall patient survival [195-200]. Thus, although there is very little information available to date, it is possible that antioxidant supplementation could protect patients from the toxic side effects of cancer treatment without altering their efficacy. At minimum, there doesn't seem to be any danger in adding antioxidant supplementation to cancer treatment plans.

The use of zinc in cancer patients has been less well studied. A handful of clinical trials have been done; they show that zinc supplementation decreased mucositis [201], dysphagia [202] and the incidence of opportunistic bacterial and fungal infections [203] in patients receiving radiation therapy. Importantly, in patients with head and neck

cancer receiving radiotherapy and patients receiving concomitant radiotherapy and chemotherapy (fluorouracil and cisplatin), zinc supplementation improved the three year local tumour reoccurrence rate and the 5 year local tumour reoccurrence rate and overall survival respectively [204, 205].

Considering the importance of zinc for the immune system [206], zinc may improve patient survival by enhancing immune function. Zinc deficiency has been associated with decreased interleukin-2 (IL-2) production [207], a key event in the activation and proliferation of T-lymphocytes, and zinc supplementation stimulates IL-2 production in-vivo and in-vitro [208-210]. Thus zinc could increase the production of anti-tumour T-cells. In support of this, IL-2 administration is capable of mediating tumour regression in humans [211] and was the first immunotherapy approved for cancer treatment (reviewed in [212]).

In addition to its immunomodulatory role, zinc supplementation may improve patient survival rate via cytotoxic effects on cancer cells. Zinc sulfate ($ZnSO_4$) had a cytotoxic effect on cultured human leukemia myelogenous leukemia cell lines (K562) without causing damage to normal human lymphocytes [213]. In addition, the cancerous cells showed increased DNA damage compared to normal human lymphocytes when exposed to H_2O_2 in the presence of zinc sulfate ($ZnSO_4$). These in-vitro results suggest that zinc can protect normal cells while enhancing the cytotoxicity of damaging agents such as H_2O_2 in cancerous cells. Moreover, zinc ionophores have been shown to inhibit proliferation and decrease survival of A549 human lung and PC3 human prostate cancer cell lines [214]. Interestingly, these ionophores were also able to limit tumour growth of A549 and PC3 cells in xenograft models with no observable toxicity[215].

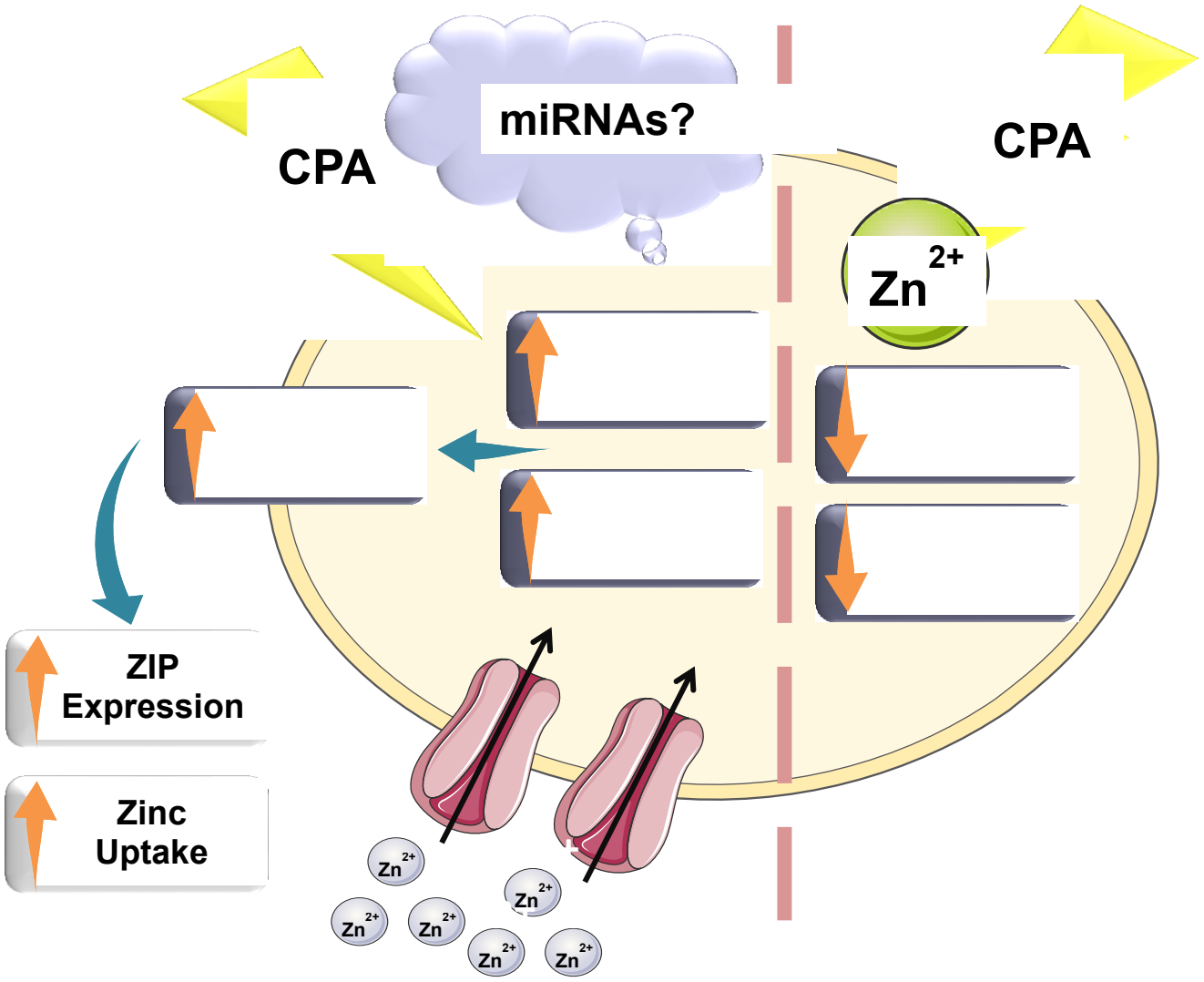
Our study was not designed to assess the effect of zinc supplementation on the efficacy of CPA treatment. Animal models for the diseases that are commonly treated with CPA could be used to assess both the germ cell protective effect of zinc supplementation and potential for either interfering or even improving CPA efficacy. Two leukemia rat models are available that are commonly used in pre-clinical testing [216, 217]. The APL model generated in Brown Norway rats in particular may be useful as it has previously been used to test the efficacy of CPA treatment combined with radiotherapy in treating acute myelocytic leukemia [217]. The effect of zinc supplementation on CPA efficacy in these animal models could be evaluated haematologically and by assessing the overall survival of the animals. An alternative method would involve using luciferase or GFP transfected human cancer cell lines xenografted into immune deficient mice. Using this method, the effect of zinc supplementation on the efficacy of CPA treatment for leukemia [218] and other diseases such as neuroblastoma [219], retinoblastoma [220] and lung cancer [221] could be evaluated by fluorescence activated cell sorting (FACS) of blood and bone marrow samples [218] and in-vivo imaging of tumour formation and metastasis [219-221]. These types of studies would need to be integrated into zinc supplementation dose optimization studies.

Thus, although zinc shows promise in protecting male germ cells from the toxic effects of CPA, additional studies are still needed before chemotherapy supplemented with zinc can be implemented in clinical practice.

5.7 Conclusion

Concerns over paternal exposure to toxic agents, such as cyclophosphamide, and the effects on fertility and progeny have provided the basis for the studies in this thesis. In this thesis we used transcriptomics to gain further knowledge about the effects of CPA treatment on the developing male germ cells. We found that CPA treatment alters the expression of miRNAs and mRNAs in a cell specific manner in both pachytene spermatocytes and round spermatids. Most unexpectedly, we found a role for zinc and zinc transporters in the germ cell response to toxic effect of CPA treatment. Together, these studies suggest that CPA treatment alters zinc requirements in male germ cells as a consequence of increased oxidative stress and DNA damage. The reduced availability of zinc is reflected by changes in expression of transcripts for and miRNAs that target zinc binding proteins. In response, germ cells increase zinc import via the up-regulation of zinc transporters. Finally, zinc supplementation can protect the male germ cells from CPA induced oxidative stress and DNA damage (Figure 5.3).

This thesis provides important information and novel insights into the germ cell response to CPA insult. Furthermore, it provides preliminary evidence for the potential use of zinc supplementation in reducing the germ cell toxic effects of chronic CPA treatment.


miRNAs?
CPA
CPA
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Expression
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Figure 5-3. Summary of thesis findings. Cyclophosphamide treatment causes increased oxidative stress and DNA damage in male germ cells leading to an increased demand for zinc. In response, the expression of members of the ZIP family of zinc transporters and intracellular zinc transport are increased. MicroRNAs may play a role in the effect of CPA treatment by post-transcriptional control of the expression of genes involved with zinc binding, response to oxidative stress and DNA damage and spermatogenesis. Zinc supplementation decreased CPA induced oxidative stress and DNA damage in male germ cells. Thus zinc plays an important role in the germ cell response to CPA treatment and has the potential to protect germ cells against toxic exposures. Figure was created in part using images from Servier Medical Art (www.servier.com) , licensed under a Creative Commons Attribution 3.0 Unported License.

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5.9 Original Contributions

1) Profiled miRNA expression in isolated pachytene spermatocytes and round spermatids from male Sprague-Dawley Rats. Other studies have only examined miRNA expression in whole testis or isolated germ cells from mice

2) Profiled miRNA expression after chronic CPA treatment in isolated pachytene spermatocytes and round spermatids. This is the first study to show that a therapeutic agent can alter miRNA expression in male germ cells.

3) Examined the expression profiles of all 14 members of the ZIP and all 10 members of the ZnT families of zinc transporters in isolated pachytene spermatocytes and round spermatids. Other studies have only shown the expression of select zinc transporters in male germ cells

4) Determined differences in the expression of ZIP and ZnT transporter expression in isolated pachytene spermatocytes and round spermatids.

4) Developed a live cell imaging assay using the zinc fluorescent probe Fluozin3-AM and hight-content screening to measure zinc uptake in male germ cells.

5) Determined differences in zinc uptake over time between isolated pachytene spermatocytes and round spermatids.

6) Examined whole genome gene expression after chronic CPA treatment in isolated pachytene spermatocytes and round spermatids. Previously, only the expression of stress response genes was examined after CPA treatment in these cells.

8) Reported that CPA treatment has an apparent repressive effect on gene expression in pachytene spermatocytes.

7) Associated chronic CPA treatment with changes in the expression of miRNAs and genes involved with zinc in pachytene spermatocytes and round spermatids.

9) Reported increased expression of members of the ZIP family of zinc transporters (ZIP5, ZIP6, ZIP13, ZIP14) in pachytene spermatocytes after chronic CPA treatment.

10) Determined that zinc uptake is increased in pachytene spermatocytes after CPA treatment in comparison to control.

11) Showed that CPA treatment increases levels of reactive oxygen species using the fluorescent probe CellROX and live imaging of male germ cells. Although increased oxidative stress has been previously shown, this is the first study to show increased ROS levels visually in isolated cultured pachytene spermatocytes and round spermatids after CPA treatment.

12) Demonstrated that zinc supplementation can decrease CPA induced increased levels of ROS in isolated pachytene spermatocytes and round spermatids.

13) Determined a distinct distribution pattern of γH2Ax foci volume in germ cells in the testis cross-sections from CPA treated animals compared to controls.

14) Determined that CPA treatment results in γH2Ax foci with larger volume in testis sections compared to controls.

15) Demonstrated that zinc supplementation can reduce levels of DNA damage caused by CPA treatment in male germs.